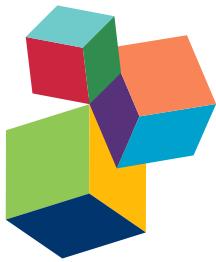


INTERACTION BETWEEN HYALURONIC ACID AND ITS RECEPTORS (CD44, RHAMM) REGULATES THE ACTIVITY OF INFLAMMATION AND CANCER

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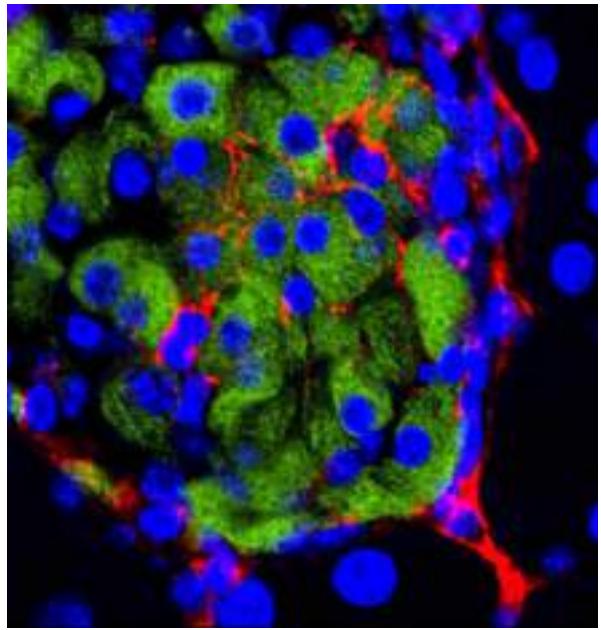
INTERACTION BETWEEN HYALURONIC ACID AND ITS RECEPTORS (CD44, RHAMM) REGULATES THE ACTIVITY OF INFLAMMATION AND CANCER

Topic Editor:

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The biological outcome of Hyaluronan (also hyaluronic acid, abbreviated HA) interaction with its CD44 or RHAMM receptors recently attracted much attention within the scientific community owing to a *Nature* article by Tian X et al. (*Nature* 2013; 499:346-9). The article described a life span exceeding 30 years in naked mole rats, whereas the maximal lifespan of mice, to which the naked mole rat is related, is only 4 years. This observation is accompanied by the finding that the naked mole rat, in contrast to the mouse, does not develop spontaneous tumors during this exceptional longevity. The article provides evidence that interaction of long tissue HA (6000-12,000 kDa) of the naked mole rat with cell surface CD44, in contrast to the interaction of short tissue HA (less than 3000 kDa) with the mouse CD44, makes the difference. More specifically, this communication shows that the interaction of short HA with fibroblasts' CD44 imposes on them susceptibility for malignant transformation, whereas the corresponding interaction with long HA imposes on the fibroblasts a resistance to malignant transformation.

The article does not explain the mechanism that underlines these findings. However, the articles, that will be published in the proposed Research Topic in the Inflammation section of *Frontiers in Immunology*, can bridge not only this gap, but also may explain why interaction between short HA and cell surface CD44 (or RHAMM, an additional HA receptor) enhances the development of inflammatory and malignant diseases. Furthermore, the articles included in the proposed *Frontiers* Research Topic will show that cancer cells and inflammatory cells share several properties related to the interaction between short HA and cell surface CD44 and/or RHAMM. These shared properties include: 1. Support of cell migration, which allows tumor metastasis and accumulation of inflammatory cells at the inflammation site; 2. Delivery of intracellular signaling, which leads to cell survival of either cancer cells or inflammatory cells; 3. Delivery of intracellular signaling, which activates cell replication and population expansion of either cancer cells or inflammatory cells; and 4. Binding of growth factors to cell surface CD44



Hyaluronic acid localization on pancreatic β cells. Section from pancreatic islets derived from diabetic NOD mice were subjected to double fluorescence staining with anti-insulin (green) and biotinylated hyaluronic acid binding protein (HABP; red). DAPI staining was used to detect cell nuclei. Analysis by confocal microscopy revealed that hyaluronic acid (HA; red) are localized on β cell membrane (green). It was suggested by Nathalie Assayag-Asherie from Naor's laboratory that the interaction of the cell bound HA with the β cell surface CD44 imposes apoptosis on these cells resulting in type 1 diabetes (Nathalie Assayag-Asherie et al., Can CD44 be a mediator of cell destruction? the challenge of type 1 diabetes. PLoS One. 2015; 10(12): e0143589)

of cancer cells or inflammatory cells (i.e., the growth factors) and their presentation to cells with cognate receptors (endothelial cells, fibroblasts), leading to pro-malignant or pro-inflammatory activities.

Going back to the naked mole rat story, we may conclude from the proposed articles of this Frontiers Research Topic that the long HA, which displays anti-malignant effect, interferes with the above described pro-malignant potential of the short HA (perhaps by competing on the same CD44 receptor). Extrapolating this concept to Inflammation, the same mechanism (competition?) may be valid for inflammatory (and autoimmune) activities. If this is the case, long HA may be used for therapy of both malignant and inflammatory diseases. Moreover, targeting the interaction between short HA and CD44 (e.g. by anti-CD44 blocking antibodies) may display also a therapeutic effect on both malignant and inflammatory diseases, an issue that encourages not only fruitful exchange of views, but also practical experimental collaboration.

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Editorial: Interaction Between Hyaluronic Acid and Its Receptors (CD44, RHAMM) Regulates the Activity of Inflammation and Cancer

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The Editorial on the Research Topic

Interaction Between Hyaluronic Acid and Its Receptors (CD44, RHAMM) Regulates the Activity of Inflammation and Cancer

An old Indian legend tells a story about six blind men who touched an elephant. The first man who touched his leg said: “it is a pillar.” The second man who touched the tail said: “it is a rope.” The third man who touched the trunk of the elephant said: “it is a thick branch of a tree.” The fourth man who touched the ear said: “It is a big hand fan.” The fifth man who touched the belly said: “It is a huge wall.” The sixth man who touched the tusk of the elephant said: “It is a solid pipe.” Each one of them loudly insisted that his claim is the right one. Then wise old man arrived to the place of scene, listened to their arguments and said “all of you are right, but all together you identified an elephant.”

Similarly, each one of the 18 chapters of this e-book tells a different, fascinating story about the “biological polygamy” of hyaluronan and its receptors. Yet, this story is focused on the author’s specific field of interest or discipline. On the other hand, when the 18 chapters are collected in one e-book, each of them fosters the others and collectively a complete scene is created.

Hyaluronan or hyaluronic acid (HA), which resides in the interstitial collagenous matrices, increases viscosity and hydration and binds to a “link module motif” of HA-binding proteoglycans (e.g., CD44) and link proteins. HA is a non-sulfated, linear glycosaminoglycan (GAG) composed of repeating disaccharides of (β , 1–4)-glucuronic acid (GlcUA) and (β , 1-3)-N-acetyl glucosamine (GlcNAc). In most tissues, native HA has a high molecular mass of 1000–10,000 kDa, with extended molecular lengths of 2–20 μ m. HA plays crucial roles in structuring tissue architecture, in cell motility, in cell adhesion, and in proliferation processes (1, 2).

Hyaluronic acid is synthesized by three HA synthase (HAS) proteins. These generate predominantly high molecular weight-HA (HMW-HA) of between 200 and 2000 kDa. HA catabolism is mediated by hyaluronidases, mechanical forces, and oxidative stress (reactive oxygen and nitrogen species). The degradation generates different-sized HA polymers (or fragments), abbreviated low molecular weight-HA (LMW-HA; <200 kDa) and HA oligomers (1).

In general (exceptions do exist), LMW-HA is pro-inflammatory and pro-cancerous, whereas HMW-HA is anti-inflammatory and anti-cancerous. In this context, a vicious cycle is generated. Inflammatory conditions activate the production of HAS, which synthesizes HA. Subsequently, the HA is degraded by hyaluronidases and reactive oxygen species, the generation of which is also induced by the inflammation. The resulting cleaved HA fragments further propagate the inflammation. This perpetuating cycle can be blocked by competition with excess HMW-HA. To this end, Tian et al. found that naked mole-rat fibroblasts secrete HMW-HA, which is over five times larger (6000–12000 kDa), than human or mouse HA (500–2000 kDa). The HMW-HA accumulates in naked

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mole-rat tissues. This rodent has a lifespan exceeding 30 years and is resistant to cancer. Interestingly, once HMW-HA is removed by knocking down HAS-2, or by overexpressing hyaluronidase 2, which cleaves HMW-HA, the naked mole-rat cells become susceptible to malignant transformation and form tumors (3). Notably, the pro-inflammatory role of LMW-HA, including HA fragments and oligo-HA, displays not only pathological effects but eventually also physiological activities, such as expression of β -defensins to combat microbial infections (4) or induction of inflammation to accelerate wound healing (5).

CD44 glycoprotein is expressed on the surface of many mammalian cells, including leukocytes, endothelial cells, epithelial cells, fibroblasts, and keratinocytes. Extensive alternative splicing of nine variable exons and distinct post-translational modifications generate many CD44 isoforms. Standard CD44 is the smallest and most abundant isoform, whereas the other variants are expressed in a cell-specific manner (e.g., on epithelial cells or keratinocytes), as well as in multiple pathologies, including rheumatoid arthritis, diabetes, multiple sclerosis, and cancer (2, 6). The physiological activities of CD44 stem from its multiple functions, including mediating cell–cell and cell–matrix interactions, cell proliferation, cell adhesion, cell migration, hematopoiesis, lymphocyte activation, cell homing, cell extravasation, cell survival, and apoptosis, as well as epithelial–mesenchymal transition (EMT) (7). However, these functions can be converted to pathological activities when exaggerated or they escape out of control, like in cancer or chronic inflammation. Most of the CD44 studies are limited to preclinical models. However, the use of anti-CD44 antibodies in a few clinical trials resulted in life-threatening toxicity (8). Therefore, the risks vs. the benefits must be carefully evaluated before CD44-targeting strategies are translated to the clinic.

The receptor for HA-mediated motility (RHAMM or CD168), such as CD44, is also alternatively spliced, albeit at a much lower intensity. Variant forms of RHAMM are found on both cell surfaces and inside the cells (9). However, unlike CD44, RHAMM isoforms do not have the link module domain. Instead, they have a BX7B motif that binds HA, where “B” represents arginine or lysine, and “X” represents any non-acidic amino acid (10). RHAMM supports both malignancy and wound healing processes.

As CD44 supports both chronic inflammation and cancer progression in many (but not all) experimental models and human diseases, CD44 targeting, e.g., by antibody, was successfully documented in many preclinical studies, such as collagen-induced arthritis (CIA) (11). Surprisingly, we found that CD44 targeting by CD44 gene deletion in the embryo aggravates CIA, rather than ameliorating it. It appears that a CD44 redundancy process in the CD44 deleted embryo allows up-regulation of RHAMM, which replaces CD44 also during adulthood. The substituting RHAMM supports CIA joint inflammation more effectively than CD44 (11), because it is a better supporter of cell migration. It is not surprising that CD44 targeting in the adult is not redundant, like in the embryo, as CD44 in the embryo displays a survival-supporting function that generates pressure for ultimate RHAMM replacement. Such a developmental pressure does not exist in the

adult, so that CD44 targeting is not compensated by functional RHAMM at this phase of life, and the therapeutic effect by CD44 targeting, can be documented.

TLR-4, a principle innate receptor of bacterial LPS, is also an important receptor of HA (1). TLR-4 activates nuclear factor (NF- κ B protein via two major routes: a myeloid differentiation factor (MyD) 88-dependent pathway that acts via NF- κ B to induce pro-inflammatory cytokines and a MyD88-independent pathway that acts via type I interferons to increase the expression of interferon-inducible pro-inflammatory genes.

Siiskonen et al. describe the mysterious and unexpected functions of hyaluronan synthase 1 (HAS-1), which is less known and less explored than its two HAS-2 and HAS-3 enzyme “step brothers,” which also engage in HA synthesis. As HAS-1 embryonic gene deletion does not influence the normal phenotype, this raises the questions: are its functions compensated (by redundancy) by the two other HAS enzymes, and if so, why has HAS-1 has been preserved in the course of evolution?

Receptor (e.g., CD44) sensitivity to hyaluronan quantity and size provides a biosensor of the state of the microenvironment (inflammation, cancer stroma, or wound healing) surrounding the cell. Hence, to learn more on the chemical profile of HA in the context of these parameters or on technologies associated with its quantification, specification, isolation, and size determination in both fluids and tissues, it would be highly beneficial to read Cowman et al. communication.

Readers interested in the structural alternations associated with the HA-binding domain (HABD) of CD44 after HA binding, cannot miss Guvench’s chapter. The authors (Guvench et al.) performed extensive all-atom explicit-solvent molecular dynamics (MD) simulations of HABD and the conclusions are presented in this communication. However, the HABD was analyzed independently of the rest of the CD44 molecule, while the transmembrane domain and especially the cytoplasmic tail influence the binding affinity as well (2, 6). Furthermore, it should be recalled that in this study, the conclusions are limited to HABD interaction with HA oligomers, whereas larger HA molecules were not evaluated.

Monslow et al. comprehensively reviewed the role of HA in health and disease, especially in relation to HA size. Their size definition for HA is formulated as follows: HMW-HA: >1000 kDa; intermediate (medium) molecular weight-HA (MMW-HA): 250–1000 kDa; LMW-HA: >10–250 kDa; and oligo-HA (<10 kDa). However, there is no consensus on these definitions and standardization of these values by an international workshop is necessary. In general, there is a consensus that HMW-HA controls normal homeostasis and displays anti-inflammatory and anti-cancerous effects, with a few exceptions. Many researchers consider LMW-HA and oligo-HA pro-inflammatory and pro-cancerous GAGs, as well as stimulators of pro-inflammatory cytokines. Yet, there are many contradictory findings. This confusion is related to the lack of consensus on size definition, polydispersity of HA commercial products (different HA sizes in the same product), the use of HA from different animal sources or from different tissues, and, finally, the impurity of commercial products. These reservations must be taken into account whenever a new study on HA is undertaken.

Four-methylumbellifero (4-MU) is an HA-antagonizing product, described by Nagy et al. The product inhibits HAS synthesis by reducing the availability of UDP-GlcUA to the enzyme, thus, interfering with HA synthesis and consequently with HA-related pathologies, such as cancer and autoimmunity. As 4-MU is an already approved drug called "hymecromone" for biliary spasm, the road to 4-MU therapy of inflammatory diseases and malignancy has been largely paved.

Hyaluronan and CD44 reside in the lipid rafts, cholesterol- and glycosphingolipid-enriched membrane microdomains that regulate the membrane receptors as well as signal delivery from the cell surface into the cell. Murai et al. examines in particular lipid raft regulation of HA binding to the CD44 of T lymphocytes and malignant cells, binding, which leads to rolling interactions on vascular endothelial cells, an important phase in inflammation and cancer development.

If the reader centers his/her interest on the inter-relationship between the tumor and its inflammatory microenvironment in context to HA, he/she can be referred to the article by Nikitovic et al. The authors focus their discussion on the influence of the cancer inflammatory environment on tumor growth, with specific emphasis on stromal HA.

The interplay between the tumor and its stromal microenvironment is also documented by Schwertfeger et al., using breast cancer as an example. The generation of a pro-tumorigenic inflammatory environment during breast cancer development requires LMW-HA-induced recruitment and activation of inflammatory macrophages. The macrophages release NF κ B-regulated pro-inflammatory factors (IL-1 β , IL-12, reactive oxygen species), normally involved in tissue repair. Hence, the cancer cells "stole" the inflammation supportive machinery from the wound healing process.

Such inter-relationships between the tumor and its microenvironment are described also in hematological tumors. Gutjahr et al. call our attention to the pro-cancerous survival (or anti-apoptotic) signals delivered by the tumor inflammatory environment, focusing on acute lymphocytic leukemia (CLL). Long-term survival and proliferation of CLL cells requires their dynamic interaction with stromal and immune cells in lymphoid organs. Interactions of HA with cell surface CD44 or RHAMM contribute to CLL cell localization, and hence to CLL pathophysiology. Deep mining of these complex interactions may reveal links more susceptible to therapeutic targeting, such as CD44v6, RHAMM, VLA-4, ZAP-70, or HAS (for details, see this communication).

Lee-Sayer et al. focus their attention on the inter-relationships between HA and CD44 in cells involved in the innate and adoptive immune system in the context of inflammation. Under innate inflammatory conditions, dendritic cells express HA on their membrane and T cells upregulate CD44. In the adoptive phase, interactions between the HA of the antigen-presenting dendritic cells and the activated CD44 of T lymphocytes may allow intimate contact between the co-stimulating molecules of the former and accessory molecules of the latter, leading to activation of the lymphocyte's T cell receptor. Going one step further, the HA and the CD44 molecules may also be considered co-stimulating and accessory molecules.

If the reader wants to know how HMW-HA and LMW-HA are involved in allergic inflammation, he/she should focus on the communication by Kim et al. The reader can surmise, following extrapolation from the inflammation data, that HMW-HA is anti-allergic, whereas LMW-HA is pro-allergic. The mechanisms underlying these effects, including the role of microRNAs, are reported in detail.

McDonald and Kubes describe the cell trafficking roles on endothelial cells in the liver, which are different than those in other tissues. Recent evidence implicates serum-derived hyaluronan-associated protein (SHAP) as an important co-factor that strengthens the binding of HA to CD44 under shear stress, resulting in improved cell extravasation. Finally, the authors indicate that HA-CD44 interaction supports not only destructive chronic inflammation but also the trafficking of stem cells that resolve the inflammation, the balance between the two determining the tissue's fate.

Bourguignon and Bikle suggest that the interaction of large HA (>1000 kDa) with cell surface CD44 leads to Rac-signaling and normal keratinocyte differentiation, DNA repair, and survival function. On the other hand, the interaction of small/fragmented (10–100 kDa) HA (generated by UV irradiation) with cell surface CD44 stimulates RhoA/ROCK activation, NF κ B/Stat-3 signaling, and microRNA-21 production, resulting in proliferation and inflammation, as well as in the progression of squamous cell carcinomas (SCC). A balance that favors the "good" Rac-signaling over the "bad" RhoA signaling can be generated by Y27623, a ROK inhibitor, vitamin D, or by triggering HAS-2, which activates the production of large HA. These therapeutic approaches may be used for therapy of patients with UV irradiation-skin diseases (for more details, see the article).

Misra et al. comprehensively describe technologies that can be used to modulate the signals of HA-HA receptor interactions in favor of the patient. A sophisticated approach is Misra's technology relating to transferrin-coated nanoparticles, which include CD44v6 shRNA, to silence the CD44v6 gene in tumor cells expressing transferrin receptor. Readers, who seek information on this fascinating approach, or to other therapeutic strategies based on disrupting HA-CD44 interactions and subsequent signaling, are invited to read this chapter.

The use of a CD44-targeting peptide, Ac-KPSSPSEE-NH₂, is another therapeutic strategy, documented by Finlayson, to combat CD44-associated pathological activities in experimental vascularized eye, tumor xenografts, or in clinical trials. If the reader wishes to know more on the peptide's mechanism of action, it is recommended to read this chapter.

Jordan et al. focuses our attention on normal and aberrant cellular signaling generated after interaction of HA with its receptor (mainly CD44), under different physiological and pathological settings. These include bacterial infection, viral infection, interstitial lung disease, wound healing, chronic inflammation (autoimmunity), and cancer. The outcome of such aberrant signaling is uncontrolled cell migration, cell proliferation, cell survival (e.g., of cancer cells), apoptosis [e.g., of β cells in type 1 diabetes; (12)], angiogenesis, and EMT, leading to different pathologies.

Both hematopoietic stem cells (HSCs) and leukemia stem cells (LSCs), also known as leukemia-initiating cells (LICs) seek

a “shelter” called a bone marrow “niche.” The niche maintains the “stemness” of the host cells, i.e., supports their survival and homing as well as regulates the balance between their quiescence and growth. Once HSCs are transplanted into a leukemic patients, they eventually compete with LICs for lodging in the niche, engaging their cell surface CD44 in interaction with the HA of the niche. In this communication, Zöller raises the question: how can an advantage be imparted to the transplanted HSCs over the patient’s LICs in the context of HA–CD44 interaction, in view of their largely identical biological nature, when they compete for “shelter” in the same niche. The answer to the question may be found in this communication.

Orian-Rousseau’s communication is focused on the role of CD44 isoforms as co-receptors, especially for receptor tyrosine kinases (RTK). She further calls our attention to the involvement of CD44 in Wnt signaling, both as a regulator of the Wnt receptor (via interaction with LRP6) or as a Wnt target gene, e.g., for CD44v6 or Met-RTK expression. Involvement of CD44 in Wnt signaling, leading to EMT, is also discussed. Finally,

Dr Orian-Rousseau speculates on the function of CD44 in cancer stem cells (CSCs), which has so far has been studied as a biomarker for these cells, but its role in CSCs remains elusive. Integration of the CD44v6 co-receptor (activated by HA ?) and Met-RTK (activated by hepatocyte growth factor) with Wnt signaling may explain what could be the role of CSC CD44 in colorectal cancer and perhaps other malignancies, i.e., by promotion of cell migration and metastasis.

In conclusion, the elephant unveiled in this e-book reveals a fascinating story about the HA–CD44 interaction, which not only exposes the underlying mechanism of this interaction but also allows identification of weak links, which can be targeted by various therapeutic approaches in both cancer and inflammatory diseases.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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Hyaluronan synthase 1: a mysterious enzyme with unexpected functions

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Hyaluronan synthase 1 (HAS1) is one of three isoenzymes responsible for cellular hyaluronan synthesis. Interest in HAS1 has been limited because its role in hyaluronan production seems to be insignificant compared to the two other isoenzymes, HAS2 and HAS3, which have higher enzymatic activity. Furthermore, in most cell types studied so far, the expression of its gene is low and the enzyme requires high concentrations of sugar precursors for hyaluronan synthesis, even when overexpressed in cell cultures. Both expression and activity of HAS1 are induced by pro-inflammatory factors like interleukins and cytokines, suggesting its involvement in inflammatory conditions. Has1 is upregulated in states associated with inflammation, like atherosclerosis, osteoarthritis, and infectious lung disease. In addition, both full length and splice variants of HAS1 are expressed in malignancies like bladder and prostate cancers, multiple myeloma, and malignant mesothelioma. Interestingly, immunostainings of tissue sections have demonstrated the role of HAS1 as a poor predictor in breast cancer, and is correlated with high relapse rate and short overall survival. Utilization of fluorescently tagged proteins has revealed the intracellular distribution pattern of HAS1, distinct from other isoenzymes. In all cell types studied so far, a high proportion of HAS1 is accumulated intracellularly, with a faint signal detected on the plasma membrane and its protrusions. Furthermore, the pericellular hyaluronan coat produced by HAS1 is usually thin without induction by inflammatory agents or glycemic stress and depends on CD44–HA interactions. These specific interactions regulate the organization of hyaluronan into a leukocyte recruiting matrix during inflammatory responses. Despite the apparently minor enzymatic activity of HAS1 under normal conditions, it may be an important factor under conditions associated with glycemic stress like metabolic syndrome, inflammation, and cancer.

Keywords: hyaluronan, hyaluronan synthase, CD44, inflammation, cytokines, cancer

INTRODUCTION

Hyaluronan is the most abundant matrix polysaccharide, which maintains tissue homeostasis, gives compressive strength for tissues, acts as an ideal lubricant in body fluids and accelerates growth and healing. In addition, excess hyaluronan promotes cancer progression and mediates inflammation. Therefore, membrane-bound hyaluronan synthases (HAS1–3), special enzymes responsible for hyaluronan production, have a key role in regulation of these conditions. Despite highly homologous amino acid sequences, HAS's differ in subcellular localization, enzymatic activity, and regulation (1).

Despite almost 20 years of active research to sequence hyaluronan synthase genes, it is not known why vertebrates have three different isoforms of these enzymes, which are coded by separate genes on different chromosomes, to synthesize a single sugar polymer. Most research has focused on HAS2 and HAS3, while HAS1 has received the least attention and remains the most enigmatic, with only a few published reports of its biological effects on cellular behavior or association with disease states.

Knocking out the activity of hyaluronan synthase genes has provided a better understanding about normal HAS function.

Knockout of *Has2* results in embryonic lethality with severe cardiac and vascular malformations (2), while the knockout of *Has1* or *Has3* does not have any apparent phenotype under non-stressed conditions (3, 4). However, double knockout of *Has1* and *Has3* leads to enhanced inflammation and accelerated wound closure of mouse skin (5), suggesting that they are necessary for the regulation of acute inflammation following injury.

A number of recent studies have highlighted the role of HAS1 in health and disease. Interestingly, *Has1* was the most upregulated gene in aneuploid mouse embryonic fibroblasts (MEFs) with malignant properties (6) and splice variants of *HAS1* are suggested to contribute to genetic instability (7), suggesting that it is susceptible to genetic alterations during oncogenic transformation. Surprisingly, HAS1 immunostainings of breast carcinoma cells correlated with hyaluronan staining, estrogen receptor negativity, HER2 positivity, high relapse rate, and short overall survival. In stromal cells of tumors from the same patients, the staining level of HAS1 was related to obesity and large tumor size (8). Human mesenchymal stem cells from different donors express *HAS1* in variable but significant levels (9), suggesting its contribution to formation of a hyaluronan niche that maintains stemness of the

cells. *HAS1* is upregulated during human keratinocyte differentiation (10) and its expression correlates with levels of HA synthesis, indicating that HAS1 is an important regulator of skin homeostasis. Furthermore, as compared to other isoforms, differences in HAS1 substrate requirements (11–13), subcellular localization, and the structure of the hyaluronan coat (7, 13, 14) have been reported, suggesting an independent role of HAS1 in the regulation of cell and tissue homeostasis. However, a comprehensive review of HAS1 has not been published. Therefore, the purpose of this review is to summarize and discuss the current knowledge of this mysterious enzyme. In this review, the abbreviations *Has1* and Has1 are used for non-human gene and protein, and *HAS1* and HAS1 for human gene and protein, respectively.

GENETICS AND FUNCTION OF *Has1* GENES AND PROTEINS

Hyaluronan is synthesized by HAS enzymes found in vertebrates, some bacteria, and a virus (15). The first *Has* was cloned in Group A *Streptococcus pyogenes* and it was predicted to be an integral membrane protein (16). The first human *HAS* gene was isolated by two research groups almost simultaneously. Shyjan and co-workers used functional expression cloning in Chinese hamster ovary (CHO)-cells (17) and Itano and Kimata screened cDNA libraries of human fetal brain (18).

Mammalian cells have three distinct synthase genes, *Has1-3* (the human genes are abbreviated here as *HAS1-3*). They are well-conserved with highly homologous amino acid sequences, but located on separate chromosomes. In humans, *HAS1* resides in chromosome 19 at q13.3–13.4, *HAS2* is located in chromosome 8 at q24.12 and *HAS3* is in chromosome 16 at q22.1 (19). *HAS1* gene has five exons, whereas *HAS2* and *HAS3* both have four (20). Several alternative splice variants of *HAS1* have been reported in Waldenström's macroglobulinemia (21), multiple myeloma (22), and bladder cancer (23). *In silico*, the *HAS1* gene has 46 possible transcription-factor binding sites 500 bp upstream of the transcription start site (20).

Has1 is not essential for embryogenesis. *Has2* knockout mice die at embryonic day 9.5 due to cardiovascular defects (2), but mice deficient in *Has1* (3) or *Has3* (4) are viable and fertile. Furthermore, double knockout *Has1* and *Has3* mice have been developed and are phenotypically normal (5).

The three hyaluronan synthase proteins in humans are designated as HAS1, HAS2, and HAS3. Mammalian hyaluronan synthases are integral membrane proteins with 4–6 transmembrane domains in addition to 1–2 membrane-associated domains (15, 24). The synthase enzymes need Mg²⁺ or Mn²⁺ to produce hyaluronan, in addition to the uridine diphosphate (UDP) sugar precursors, UDP-glucuronic acid (UDP-GlcUA), and UDP-N-acetylglucosamine (UDP-GlcNAc) (15, 25). The synthesis takes place at the inner surface of the plasma membrane utilizing cytoplasmic precursors (26). Human and mouse enzymes add the precursor sugars to the reducing end of the growing polymer (27–29), while amphibian *Xenopus laevis* Has utilizes the non-reducing end (30), like the *Pasteurella multocida* hyaluronan synthase (31).

It has been suggested that the HAS enzymes do not require any primers for the synthesis of hyaluronan (32). The adenosine triphosphate-binding cassette (ABC) transporters have been proposed to be important for hyaluronan translocation on the plasma

membrane of fibroblasts (33), requiring a concurrent efflux of K⁺ ions (34). However, ABC transporters do not seem to contribute to the translocation of hyaluronan in breast cancer cells (35). The Has protein has been shown to produce hyaluronan in a combined process of synthesis and membrane translocation, as demonstrated by Has reconstituted into proteoliposomes in *Streptococcus equisimilis* (Se) (36). In addition, there is an intraprotein pore in Has and the synthase itself is able to translocate hyaluronan in liposomes containing purified Se-Has (37).

REGULATION OF *HAS1* EXPRESSION AND ACTIVITY

The three *HAS* genes are often regulated in parallel (38, 39) and the synthesis of hyaluronan reflects changes at the mRNA level (40–44). *HAS1* expression is transcriptionally regulated by transforming growth factor-β (TGF-β) in synoviocytes (45, 46) and by the pro-inflammatory cytokine interleukin-1β (IL-1β) in fibroblasts (44, 47, 48), while these factors may have similar or opposite effects on other *HAS*s, depending on cell type. The nuclear factor kappa B (NF-κB) (49) and tyrosine kinases (50) have been shown to be important for IL-1β-induced *HAS1* activation, while induction of *HAS1* by TGF-β seems to act through the p38 MAPK pathway (51). There is evidence that some of the effects are mediated by transcription-factors sp1 (52) and sp3 (53). **Table 1** summarizes the growth factors and cytokines that regulate *Has1/HAS1* expression. In addition to these factors, ultraviolet B radiation induces a fast up-regulation of *Has1* expression in rat epidermal

Table 1 | Transcriptional regulation of *Has1/HAS1* by different growth factors and cytokines (↑ increased, ↓ decreased).

Agent	Cell/tissue	HAS1	Reference
EGF	Human fibroblast	↑	(44)
EGF	Human oral mucosal cell	↑	(44)
FGF2	Human dental pulp	↑	(58)
FGF2	Human periodontal ligament	↑	(59)
FGF	Human fibroblast	↑	(60)
Forskolin	Human orbital fibroblast	↑	(48)
IGF	Human fibroblast	↑	(60)
IL-1β	Human fibroblast	↑	(44)
IL-1β	Human fibroblast	↑	(61)
IL-1β	Murine uterine fibroblast	↑	(47)
IL-1β	Human orbital fibroblast	↑	(48)
IL-1β	Human dermal fibroblast	↑	(53)
PDGF	Human fibroblast	↑	(62)
Progesterone	Murine uterine fibroblast	↓	(47)
Prostaglandin D2	Human orbital fibroblast	↑	(63)
Prostaglandin E2	Human synoviocyte	↑	(64)
TGF-β	Human fibroblast	↑	(65)
TGF-β	Human keratinocyte	↑	(65)
TGF-β	Human synoviocyte	↑	(46)
TGF-β	Human synoviocyte	↑	(45)
TGF-β	Human dermal fibroblast	↑	(53)
Estradiol	Human vascular smooth muscle cell	↓	(66)
4-MU	Human aortic smooth muscle cell	↓	(67)
TGF-β1	Human synoviocyte	↓	(68)
TGF-β	Human mesothelial cell	↑	(40)

keratinocytes (54). Additionally, *Has1* expression levels are raised in renal (55) and pulmonary (56) ischemia and hyperglycemia (57). The synthesis of hyaluronan by HAS1 is also regulated by the substrate concentrations of the precursor sugars (discussed in detail later in this review).

There is evidence that the activities of HAS2 and HAS3 are regulated by posttranslational modifications like phosphorylation (38, 69), ubiquitination (70), or O-GlcNAcylation (71). Whether these modifications are involved in the regulation of HAS1 activity is not completely known. Phosphorylation seems not to regulate HAS1 activation (72), but HAS1 can exist in multimers of full length-HAS1 or its variants, formed by intermolecular disulfide bonds (73).

The reported length of hyaluronan polymers produced by each of the mammalian Has differs, but the obtained results vary depending on the experimental set-up (74–77). For example, in membrane preparations from CHO-cells transfected with recombinant Has isoforms, Has2 produced the largest hyaluronan (over 3.9×10^6 Da), Has3 produced intermediate length hyaluronan ($0.12\text{--}1 \times 10^6$ Da), and HAS1 produced the smallest polymer (0.12×10^6 Da). However, all isoforms produced high molecular weight hyaluronan (3.9×10^6 Da) in live cells (76). The size of the growing hyaluronan chain is increased or decreased by mutation of certain cysteine or serine amino acids in the Has1 protein in *X. laevis*, suggesting that the size of the hyaluronan chain is affected by the ability of the synthase to bind it (74).

SUBCELLULAR LOCALIZATION AND TRAFFIC OF HAS1 AND ITS IMPACT ON FORMATION OF HA-COAT

Our understanding of the localization and traffic of Has proteins has been deepened after recruitment of fluorescent HAS fusion proteins together with live cell imaging (78–80). All studies reported so far suggest that like other Has/HAS isoforms, Has1 follows the normal intracellular route from rER to Golgi (78), and its traffic is regulated similarly to other HAS isoforms (13), as shown

by manipulation of its traffic in live cells by factors like 4-MU and brefeldin A (BFA).

A typical subcellular localization pattern of GFP-HAS1 is presented in **Figure 1**. The GFP-HAS1 signal is mainly cytoplasmic, rather than on the plasma membrane, being distributed either diffusely or in cytoplasmic patches, and partially co-localizing with the Golgi apparatus (13, 14, 73). Only a small proportion of the total cellular pool of HAS1 is located on the plasma membrane, even when activated with glucosamine (12), or inflammatory cytokines like TNF- α or IL-1 β (13). Occasionally, HAS1 signal is seen on or near the plasma membrane, usually as patches or concentrated spots (arrows in **Figure 1**), or on the plasma membrane protrusions (13, 14). The low plasma membrane signal of HAS1 is in parallel with the low activity level of HAS1, because latent HAS enzymes are thought to stay in the ER–Golgi compartment.

In addition to the full-length form, HAS1 has multiple transcript variants resulting from alternative splicing. Transfected HAS1V-GFP constructs localize with cytoskeletal structures like microtubules (7, 73). The reticular localization of the standard form of HAS1 (**Figures 1** and **2**) suggests that all forms of HAS1 studied so far are associated with the cytoskeletal network or endoplasmic reticulum, which is a distribution that is not typical for HAS2 or HAS3, and indicates different regulation and binding partners.

The size of the pericellular hyaluronan coat correlates with activity of hyaluronan synthesis. Interestingly, even high overexpression of HAS1 in cell types with little or no endogenous hyaluronan production is not enough to produce a clearly visible hyaluronan coat (12, 13, 76). Furthermore, like previously published (12–14), the coat produced by HAS1 has a clearly different, more “cloudy” structure (**Figures 1** and **2**), as compared to the tight and concentrated coat around plasma membrane protrusions produced by HAS2, and especially HAS3 (**Figure 2**). However, the size of the coat produced by HAS1 can be induced upon induction by inflammatory agents or glucosamine (12, 13). The effect of

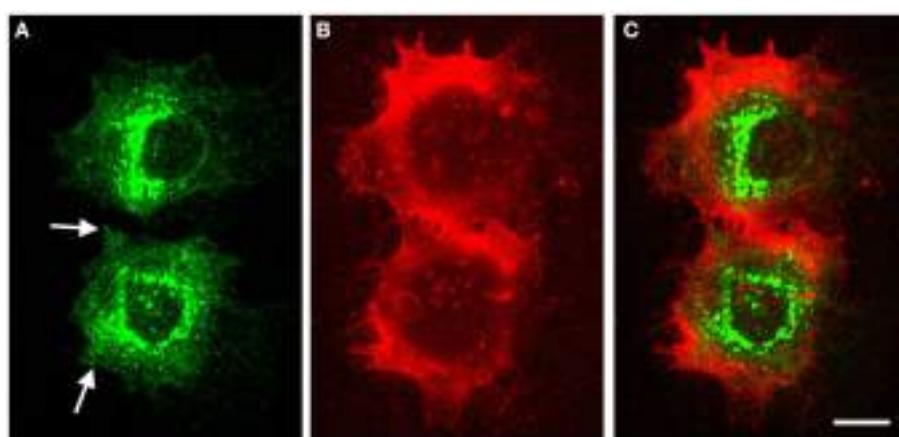


FIGURE 1 | Intracellular localization of GFP-HAS1 and structure of pericellular hyaluronan coat induced by GFP-HAS1 overexpression. Confocal optical sections of live MCF-7 breast cancer cells transfected with EGFP-HAS1 (green) and stained with fHABC to visualize the

hyaluronan coat (red). Localization of EGFP-HAS1 is shown in **(A)**, fHABC in **(B)**, and merged images in **(C)**. Arrows in **(A)** point patches of signal near the plasma membrane. Scale bar 10 μ m. Original data published in Ref. (13).

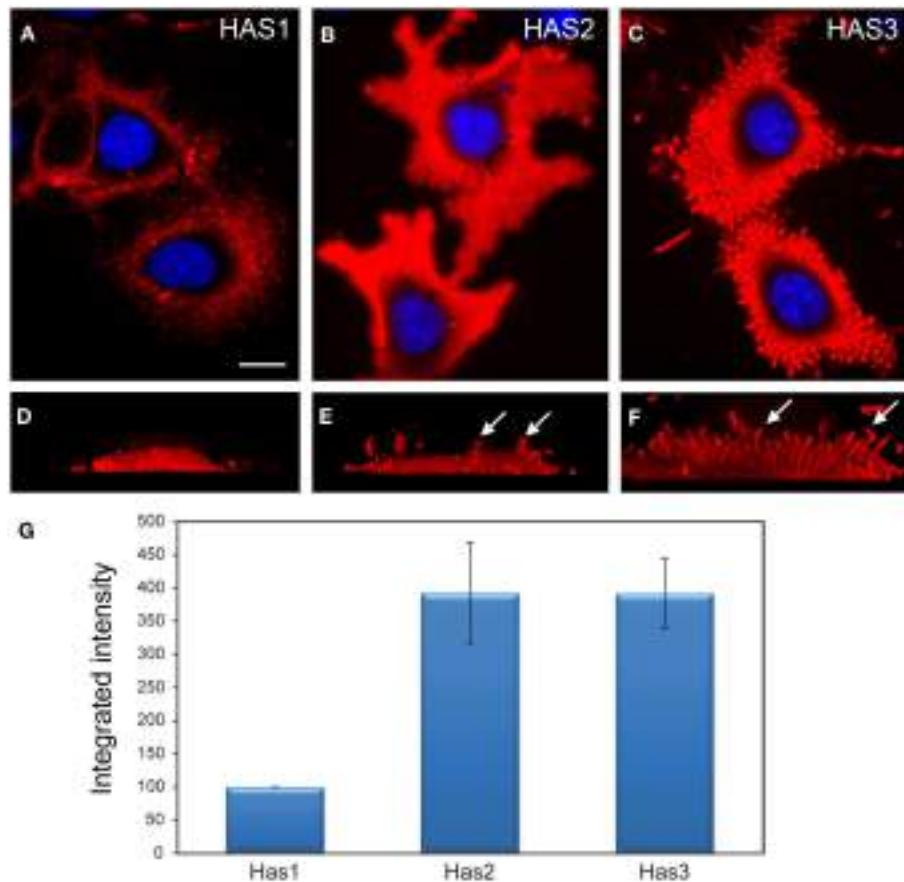


FIGURE 2 | Comparison of the structure and intensity of the pericellular hyaluronan coat in MCF-7 cells overexpressing the three HAS isoforms. Structure of the hyaluronan coat of live MCF-7 cells transfected with fusion proteins Dendra2-HAS1 (**A,D**), Dendra2-HAS2 (**B,E**), and Dendra2-HAS3 (**C,F**) and labeled with fHABC (red). Single confocal sections obtained from the middle level of nucleus (blue) are shown in (**A–C**). Vertical views created from compressed image stacks of

horizontal optical sections are shown in (**D–F**) to show the dorsal protrusions (arrows). The integrated intensity (mean intensity \times area) of hyaluronan coat probed with fHABC in the three HAS transfectants was measured in thresholded area of optical sections through the center of nucleus (**G**). Mean of three independent experiments is represented (total number of measured cells in each group = 92). Magnification bar in (**F**), 10 μ m. Original data published in Ref. (12, 81).

glucosamine is presented in **Figure 3**. Additionally, the hyaluronan coat synthesized by HAS1 is largely dependent on hyaluronan interactions with CD44 (13).

Detailed studies on tissue distribution and subcellular localization of endogenous HAS's have been challenging due to the lack of reliable antibodies and apparently low expression level of HAS's in many cell types. Subcellular localization of endogenous HAS1 detected with affinity purified polyclonal antibodies shows a similar pattern to exogenously expressed HAS1 fusion proteins (14). HAS1 immunostainings have shown notable levels of HAS1 in mesothelial cells, fibroblasts (14), and human chondrosarcoma cells (9). Furthermore, MEFs have prominent Has1 staining (6). Examples of HAS1 immunostainings in cultured cells are summarized in **Figure 4**. These results are in line with the notable mRNA levels of *Has1/HAS1* observed in these cell types (6, 12, 13).

Staining patterns of HAS1 in tissue sections is in line with cell culture studies. Immunostainings of Has1 in developing tissues (14) and HAS1 in tumor tissues (8, 82–85), endometrium (86),

and oral mucosa (87) have been published recently. In tumor tissues, HAS1 is typically expressed in tumor cells (8, 83–85), as well as in stromal fibroblasts (**Figure 5**). The localization of HAS1 is mainly intracellular, corresponding to the staining observed in cell cultures. Typical staining patterns vary from diffuse to granular with deposits next to the nucleus, which suggests HAS1 accumulation in the Golgi area (arrowheads in **Figure 5**), similar to that seen in cell cultures.

HAS1 REQUIRES HIGH CELLULAR CONTENT OF UDP-SUGARS FOR ACTIVATION

An important factor affecting activity of all HAS enzymes is the cytoplasmic availability of substrates, namely, UDP-GlcUA and UDP-GlcNAc. Many studies have shown that treatments influencing either UDP-GlcUA or UDP-GlcNAc levels regulate hyaluronan production [reviewed by Vigetti et al. (88)]. This role of substrates is particularly interesting in regulation of HAS1 as its activity of hyaluronan production in many cell models is low or absent unless stimulated.

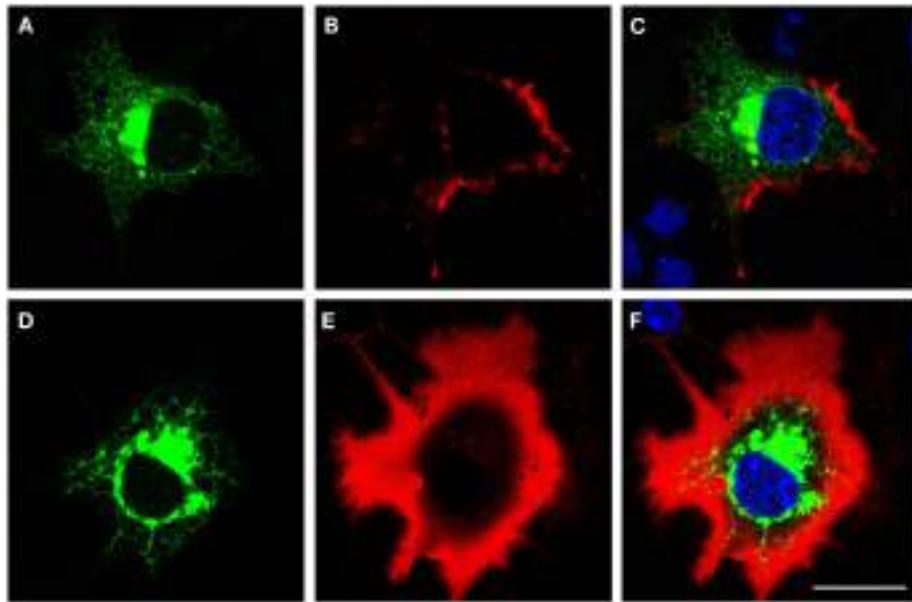


FIGURE 3 | Glucosamine induces the growth of hyaluronan coat produced by HAS1. Confocal optical sections of pericellular hyaluronan coats on COS-1 cells expressing Dendra2-HAS1 without glucosamine (**A–C**) and

after 6 h incubation with 1 mM glucosamine (**D–F**). Green, Dendra2-HAS1; red, hyaluronan coat; blue, nuclei. Magnification bars 20 μ m. Original data published in Ref. (12, 13).

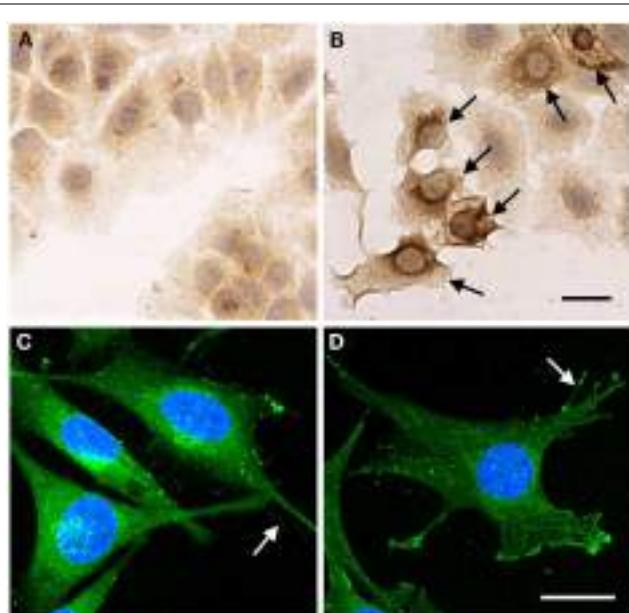


FIGURE 4 | Subcellular localization of endogenous HAS1 detected by immunostainings. MCF-7 cells transiently transfected with empty vector (**A**) and HAS1 expressing plasmid (**B**), followed by immunostaining with polyclonal HAS1 antibodies (brown color). Arrows in (**B**) show the HAS1 overexpressing cells. A 3D confocal projection of human chondrosarcoma cell (HCS) (**C**) and transformed mouse embryonic fibroblast (MEF) (**D**) stained with HAS1 immunofluorescence (green). Arrows in (**C,D**) point plasma membrane protrusions. Blue, nuclei. Magnification bars in (**B,D**) = 20 μ m. Original data published in Ref. (6, 9, 14).

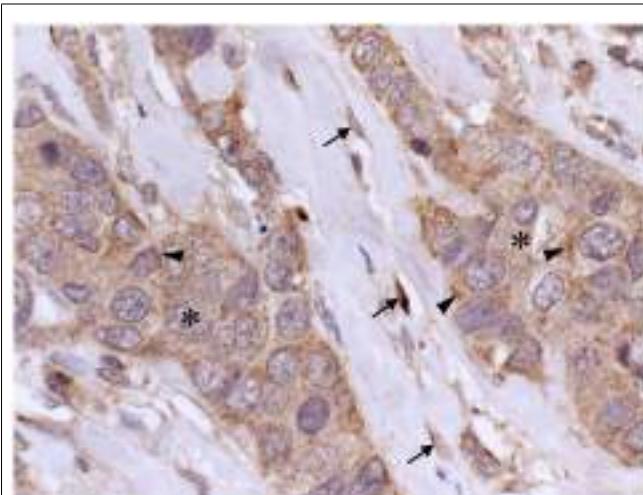


FIGURE 5 | Localization of HAS1 in breast cancer tissue. A paraffin section of breast carcinoma immunostained with HAS1 polyclonal antibody (brown). Nuclei are labeled blue. A mainly cytoplasmic localization of HAS1 is detected in carcinoma cells (asterisks) and in stromal fibroblasts (arrows). Special accumulation of staining is seen intracellularly (arrowheads). Magnification bar 50 μ m. Original data published in Ref. (8).

In order to study the effect of UDP-GlcUA on hyaluronan production, 4-methylumbelliferyl (4-MU) and overexpression of enzymes involved in either UDP-Glucose (UDP-glucose pyrophosphorylase) or UDP-GlcUA (UDP-glucose 6-dehydrogenase) production have mainly been used (39, 89, 90).

These reports rely mostly on mRNA data to explain the altered hyaluronan production. The effect of UDP-GlcUA fluctuations on HAS1–3 expression levels vary considerably from one cell line to another and it is often impossible to reveal the exact role of HAS1 during these changes. A recent investigation demonstrated that availability of UDP-GlcUA can have a direct effect on HAS1 activity, as treatment of MCF-7 cells overexpressing HAS1 with an inducing agent and 4-MU significantly decrease hyaluronan coat compared to cells treated with the inducing agent only (13). It has been reported that Has1 has a lower affinity for UDP-GlcUA than other Has's, and the K_m of Has1 is about double that of Has2–3. Interestingly, availability of the other substrate, UDP-GlcNAc, did not considerably influence the K_m of Has1 toward UDP-GlcUA, whereas levels of UDP-GlcUA did have a significant effect of the K_m toward UDP-GlcNAc (11).

The affinity of Has1 for UDP-GlcNAc is lower than the affinity of Has2–3 as with UDP-GlcUA. The K_m toward UDP-GlcNAc of Has1 is about two to three times higher than that of the other Has's. Interestingly, all Has enzymes exhibit lower affinity toward UDP-GlcNAc than for UDP-GlcUA (11). Treatments with compounds like mannose and glucosamine that regulate UDP-GlcNAc content also affect cellular hyaluronan secretion levels (12, 91). Similar to the level of UDP-GlcUA, the availability of UDP-GlcNAc influences both mRNA levels and activity of all HAS's. The differences in substrate affinities are well demonstrated in intact cells using HAS1 overexpressing cell lines. Both COS-1 and MCF-7 cell lines have negligible endogenous hyaluronan production, and even overexpression of HAS1 enzymes does not cause prominent changes in it. Upon treatment with glucose or glucosamine, compounds that increase the amounts of hyaluronan substrates, the HAS1 enzyme is able to produce significant amounts of hyaluronan (12, 13). Furthermore, this effect of substrate availability on HAS1 activity is dose dependent (12).

The above mentioned findings on the regulation of HAS1 activity point out that although HAS1 has a minor role in total cellular hyaluronan production, it may have significant effects when induced by increased substrate availability. Since the affinity of HAS1 for its substrates is lower compared to the two other HAS's, the fluctuations in UDP-GlcNAc and UDP-GlcUA levels can have a more significant effect on HAS1 than on HAS2–3.

HAS1 AS A MEDIATOR IN INFLAMMATION

Many recent results suggest HAS1 may play a pivotal role during cell stress, such as inflammation. Earlier in this frontiers review series, Petrey and de la Motte comprehensively discussed the role of hyaluronan in inflammation (92). Whether hyaluronan acts as a pro- or anti-inflammatory molecule is highly dependent on its molecular size. Generally, low-molecular weight hyaluronan fragments mediate pro-inflammatory responses (93) such as recruitment of macrophages and other leukocytes to the injured or inflamed tissue (94, 95) and stimulate transcription of genes related to inflammation including several cytokines and matrix metalloproteinases (96). Growth factors and pro-inflammatory cytokines (**Table 1**) released during inflammation, like TGF- β , IL-1 β , and TNF- α , which stimulate inflammatory cells also induce expression of *HAS1* (44, 45, 64) and *Has1* (97). Expression of *HAS1* is also upregulated in response to prostaglandins (98, 99).

Therefore, *Has1/HAS1* up-regulation has been noted in many diseases associated with inflammation such as murine atherosclerosis (100), human osteoarthritis (101), murine infectious lung disease (102), and human rheumatoid arthritis (45). *HAS1* expression is also increased, among several other genes, in osteophytic chondrocytes (103). Interestingly, the expression of both *HAS1* and *HAS2* was reduced in the synovium of patients with osteoarthritis or rheumatoid arthritis compared to healthy controls (104). Moreover, elevated HAS1 expression is observed in oral lichen planus, which is a chronic inflammatory disease of the oral mucosa (87). It is worth noting that in oral lichen planus the increased HAS1 expression is detected in the basal layers of the epithelium, which is the most affected, inflamed area in lichen planus.

It is not known whether the product of HAS1 enzyme of certain polymer length, HAS1 enzyme itself or hyaluronan with HAS1 and hyaluronan binding proteins like CD44 mediate the pro-inflammatory responses. One explanation for HAS1 involvement in inflammation might be that HAS1 is associated with production of a special type of pericellular hyaluronan coat, which is pro-inflammatory. Recently, Siiskonen and co-workers showed that inflammatory agents and glycemic stress induce HAS1 to produce an expanded pericellular hyaluronan coat (13). Compared to Has3-induced hyaluronan coat, which is rather tight and formed around microvillus protrusions (105), HAS1 produces a looser, but extensive pericellular hyaluronan coat, which is dependent on CD44. In several cell types, these types of hyaluronan coats have been shown to associate with monocyte binding (106, 107). It has even shown that hyaluronan produced by Has1 binds mononuclear cells more effectively than hyaluronan produced by the two other Has enzymes (77). This could provide an explanation for the central role of HAS1 in inflammation.

In rheumatoid arthritis, the rate of hyaluronan synthesis is altered. Hyaluronan accumulates in joints affected by rheumatoid arthritis, which causes periarticular swelling and morning stiffness (108). In synoviocytes isolated from RA patients, *HAS2* and *HAS3* are constitutively activated, but *HAS1* is the gene that responds readily to pro-inflammatory cytokines like IL-1 β (49) and TGF- β (45). However, IL-1 β is not able to stimulate *Has1* expression in healthy synoviocytes like in type-B synoviocytes isolated from rheumatoid arthritis patients (49, 109). This IL-1 β -induced *HAS1* up-regulation is dependent on the activation of the transcription-factor NF- κ B (49), like many other pro-inflammatory molecules. In type-B synoviocytes, IL-1 β stimulation induces the translocation of NF- κ B into the nucleus, which results in up-regulation of *HAS1* mRNA expression (49). Similarly, in fibroblast-like synoviocytes, viral infection causes NF- κ B activation and increased HA release due to *HAS1* up-regulation. This *HAS1* up-regulation is reversed with mitogen-activated protein kinase p38 and JNK inhibitors indicating that viral RNA activates *HAS1* through these signaling pathways (110). Moreover, *HAS1* activation is blocked with commonly used anti-inflammatory drugs, hydrocortisone, and dexamethasone, in TGF- β stimulated synoviocytes (51). In these cells, glucocorticoids block p38 activation, which results in suppressed *HAS1* expression (51). Interestingly, sodium salicylate inhibits IL-1 β induced *HAS1* activation and HA release in type-B synoviocytes (64). This might explain some of the beneficial effects of sodium salicylate in the treatment of rheumatoid arthritis.

In addition to its role in rheumatoid inflammation, altered *HAS1* levels contribute to other inflammation-related states. In murine models of asthma, *Has1* mRNA is increased at an early stage, but later decreased (111, 112). In thyroid dysfunction associated with activation of the thyrotropin receptor, hyaluronan is accumulated through up-regulation of *HAS1* and *HAS2* (113). Taken together, HAS1 seems to be fundamentally involved in the inflammatory processes. However, many questions are still waiting for an answer.

HAS1 AS A PREDICTOR OF CANCER PROGRESSION

Hyaluronan content is known to be increased in many cancers, which may be altered due to hyaluronan synthase expression. Few studies have shown a direct association of HAS's with cancer progression *in vivo*, but interestingly, HAS1 associates with tumor progression and prognostic factors in many cases. Increased expression of *HAS1* is associated with poor patient survival in ovarian cancer (114, 115), colon cancer (116), Waldenström's macroglobulinemia (21), and multiple myeloma (22). In multiple myeloma and Waldenström's macroglobulinemia, the occurrence of *HAS1* splice variants, rather than the full length *HAS1*, is related to cancer prognosis. *HAS1* expression is also increased in bladder cancer, correlating with increased hyaluronan levels (23), and predicting metastasis (117). In bladder cancer, HAS1 has been shown to modulate HA and CD44 levels, affecting tumor growth and progression (118). Accumulation of hyaluronan is associated with poor patient survival in breast cancer (119, 120). Recently, HAS1 and HA stainings were found to correlate with each other in breast carcinoma cells of these tumors, and HAS1 was associated with estrogen receptor negativity, HER2 positivity, high relapse rate, and short overall survival. In addition, expression levels of stromal HAS1 and HAS2 were related to obesity, large tumor size, lymph node positivity, and estrogen receptor negativity (8).

In serous ovarian tumors, *HAS1* has been shown to be very low or totally absent, whereas the levels of *HAS2* and *HAS3* mRNA or staining levels are not elevated compared to normal ovaries or benign tumors (83). Interestingly, the levels of HAS1 and HAS2 immunostainings are decreased in melanomas, correlating with reduced hyaluronan content and poor overall survival observed in these tumors (85, 121).

CONCLUSION AND FUTURE CHALLENGES

The hyaluronan coat produced by HAS1 differs from that of other isoenzymes, as shown by fluorescent hyaluronan binding probes. The flossy and loose coat is typical for cells with mesenchymal origin, like fibroblasts, mesothelial cells, synovial fibroblasts, and chondrocytes. Furthermore, as Table 1 summarizes, most of the cells that respond to cytokines or growth factors by upregulating *Has1/HAS1* levels, are of the same mesenchymal origin. Additionally, these cell types secrete active proteoglycans and other molecules participating in hyaluronan coat formation, like versican, I α I, and TSG6, which are important players in inflammation (92) and are associated with hyaluronan cables detected in fixed cells. However, other HAS's are active in these cells, and cell types solely expressing *HAS1* are not available, making it challenging to study the specific contribution of HAS1. The most specific method

so far is the artificial overexpression of fluorescently tagged HAS1 in cells with low levels of HAS enzymes (12–14).

Interestingly, HAS1 overexpression in many epithelial cell types has shown a low activity in normal culture conditions, without addition of glucosamine or inflammatory cytokines. This suggests that these cell types may lack factors that are crucial for HAS1 activity. Several studies suggest that HAS1 has a low capacity to retain hyaluronan chains on the plasma membrane, thus other molecules may be required to retain hyaluronan chains on the plasma membrane and assemble the hyaluronan coat. A potential molecule for these interactions is CD44, which seems to play a special role in the formation of the HAS1-induced coat (13).

The complexity of hyaluronan metabolism, existence of three isoenzymes, and the crucial role of HAS2 make it complicated to study the biological effects of HAS1 in animal models. Furthermore, since most human tissues and cells express all HAS isoforms, it is impossible to get comprehensive answers and make conclusions on the role of a single isoenzyme. Furthermore, many cells and tissues express low or negligible levels of *HAS1* mRNA. However, variable sensitivity of the methods used and other limitations may explain the low or absent *HAS1* levels detected in some cases.

Several trials have been done to solve the function and regulation of this puzzling enzyme. Evidently, HAS1 is an important regulator during inflammation and in states with altered sugar metabolism. However, contradictory results raise several new questions, which need to be resolved before we can elucidate the exact role of HAS1.

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The content and size of hyaluronan in biological fluids and tissues

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Hyaluronan is a simple repeating disaccharide polymer, synthesized at the cell surface by integral membrane synthases. The repeating sequence is perfectly homogeneous, and is the same in all vertebrate tissues and fluids. The polymer molecular mass is more variable. Most commonly, hyaluronan is synthesized as a high-molecular mass polymer, with an average molecular mass of approximately 1000–8000 kDa. There are a number of studies showing increased hyaluronan content, but reduced average molecular mass with a broader range of sizes present, in tissues or fluids when inflammatory or tissue-remodeling processes occur. In parallel studies, exogenous hyaluronan fragments of low-molecular mass (generally, <200 kDa) have been shown to affect cell behavior through binding to receptor proteins such as CD44 and RHAMM (gene name HMMR), and to signal either directly or indirectly through toll-like receptors. These data suggest that receptor sensitivity to hyaluronan size provides a biosensor of the state of the microenvironment surrounding the cell. Sensitive methods for isolation and characterization of hyaluronan and its fragments have been developed and continue to improve. This review provides an overview of the methods and our current state of knowledge of hyaluronan content and size distribution in biological fluids and tissues.

Keywords: hyaluronan, quantification, assay, molecular mass, molecular weight

Introduction

Hyaluronan (hyaluronic acid, HA) is found in vertebrate tissues, as a key component of the extracellular matrix. It has a simple covalent structure consisting of alternating β -D-glucuronate and N-acetyl- β -D-glucosamine sugars. The linear anionic polymer has a semi-flexible structure, causing it to adopt an expanded wormlike random coil. The domain of a coiled chain can be conceptually described as a sphere [mostly filled with (unbound) solvent], with a dynamically changing chain configuration. The apparent volume occupied by a single isolated molecule depends strongly on the chain length, and thus the molecular mass, M . The volume increases approximately as M raised to the 1.8 power, as dictated by polymer chain statistics (1). Where HA chains are crowded together, their domains are forced to interpenetrate, and this leads to severe non-ideality in behavior. The non-ideality determines such properties as the large colloid osmotic pressure, viscoelasticity, and effect on partition of other macromolecules (excluded volume) in the biomatrix. Since the molecular mass of HA in normal biological fluids and tissues is normally

very high (ca. 1000–8000 kDa), the non-ideality effects dominate the physicochemical properties of HA in the extracellular matrix (2–4).

In addition to the physicochemical effects due to mutual macromolecular crowding, HA has important binding interactions. At the cell surface, HA provides a protective coat that is tethered to receptors embedded in the cell membrane (5–7). Beyond the cell surface, HA organizes proteoglycans (e.g., versican, aggrecan) and other binding proteins via specific non-covalent interactions, creating a further highly hydrated and charged domain (8–10). In inflammation and other specific tissue-remodeling processes, covalent transfer of the heavy chain domains of I α I to HA can be catalyzed by TSG-6 protein (11–14). The HA-protein assemblies, whether covalently or non-covalently mediated, are integral to maintenance of an expanded pericellular matrix.

Normally, HA has a high turnover rate (15, 16). Thus, the extracellular environment is constantly renewed. The need for renewal may reside in the protective role HA fulfills. Facile degradation of HA by reactive oxygen and nitrogen species (ROS/RNS) (17, 18) during active inflammation can weaken the protective HA coat that usually protects the cell. The HA acts as a scavenger of damaging free radicals and other chemical agents. If the rate of HA synthesis keeps pace with the rate of degradation and turnover, the homeostatic environment is maintained (19).

When the rate of HA degradation is not adequately compensated by its synthesis, fragments of the polymer might be present at significant levels and consequently cells are poorly protected. Changes in the physicochemical control of the pericellular environment take place. HA fragments compete in binding interactions with proteins, altering the integrity of the biomatrix. Fragments can displace high M HA in interactions with cell surface receptors, resulting in changes in receptor clustering and altered signaling (20). Fragments can also signal through alternate receptors (21–23). In these ways, HA may be regarded as a biosensor of damaging processes in the cellular microenvironment. Altering the balance of high and low M HA is a stimulus that sets in motion multiple cellular response mechanisms. These can be purely defensive, such as HA fragment-induced expression of β -defensins to combat microbial infection in the gut (24, 25). But sustained responses can also lead to chronic inflammation via aberrant signaling through receptors and consequently increased expression of inflammation mediators (26).

Tissue-remodeling processes, including wound healing and tumor progression, are associated with changes in HA content and size (27–31). HA synthesis is usually increased during remodeling, but increased expression of hyaluronidases may also occur, and together with macrophage-generated ROS/RNS, degrade HA. The balance of high and low M HA may differ from the homeostatic case, thus altering both the physicochemical and signaling effects of HA. To understand HA biology, we are faced with multiple questions: (1) What is the content of HA present, (2) What is the molecular mass distribution of the HA, and (3) Can we control pathological processes by altering the content, size, and binding interactions of HA?

Isolation of HA

There are a number of methods appropriate for assaying the content and size of HA in biological fluids and tissues. Depending on the method, it may be necessary to purify HA to remove/digest bound proteins and sulfated glycosaminoglycans prior to assay.

The isolation of HA follows protocols (25, 30, 32–35) that are quite similar to those historically employed in the purification of DNA. The requirement for specific steps depends on the nature of the sample: fluid tissue vs. conditioned medium from cell culture vs. solid tissue. For solid tissues, the HA is extracted into soluble form, and liberated from proteins. Protein removal can be accomplished by digestion with a protease, or by denaturing the protein by gentle mixing with chloroform. Lipids are removed with acetone or other organic solvent mixtures. Removal of low-molecular mass contaminants may require dialysis, or precipitation of the HA with ethanol or isopropyl alcohol. DNA and RNA can be enzymatically digested. There are many variations on these steps. A sample protocol for extraction of HA from solid tissue might include the following steps: digestion with a protease such as proteinase K, boiling to denature enzyme, centrifugation, extraction with chloroform, centrifugation, dialysis, precipitation with ethanol, centrifugation, re-dissolution, digestion with Benzonase (or DNase plus RNase), boiling to denature enzyme, and repeat of steps starting with chloroform extraction. Abbreviated protocols can be used for fluid samples, or where the HA needs to be liberated but not purified because specific assay will be employed.

The above purification will not remove other glycosaminoglycans. Sulfated glycosaminoglycans can be removed by anion exchange chromatography. Unsulfated or undersulfated chondroitin, which is rare in normal tissues but may be significant in remodeling tissues, is not removed by this process. At this point in the procedure, specific isolation of HA can be accomplished by affinity methods, such as use of a biotinylated HA-specific binding protein and streptavidin-coated magnetic beads, or other similar medium such as gel beads (36, 37).

It is worth noting that most isolation methods in current use have not been validated with respect to quantitative yield of HA, or preferential extraction/isolation of specific HA sizes. In particular, losses of very low M HA may be significant in some procedures. It is also possible to degrade HA during isolation. Endogenous enzymes may cause some of this degradation. However, most degradation is the result of ROS generation catalyzed by contaminating iron (II) or copper (I), and molecular species that regenerate the active metal ion oxidation states. Thus, use of papain activated with cysteine can lead to HA degradation (38). The presence of EDTA can also enhance the ability of contaminating iron to catalyze formation of hydroxyl radicals. Iron contamination is better inactivated by chelation with deferoxamine (34). Also, of note, EDTA and phosphates can be co-precipitated with HA using ethanol.

Testing for degradation of HA during isolation can be easily accomplished by “spiking” the initial fluid or tissue with a pure HA sample of known M and low polydispersity in M , and then testing its size in the final isolate. Spiking samples with known amounts of HA can also be used to detect losses during isolation,

including losses due to non-specific interactions with surfaces or other macromolecules that HA may not normally contact.

Methods to Analyze Content of HA

The most simple and historical assay for HA is measurement of uronic acid content. The assay involves hydrolysis in concentrated sulfuric acid, so that protein content is not a problem. Other glycosaminoglycans that contain uronic acid will contribute to the result, and should be separated or removed from HA if possible. The uronic acid assay has been widely employed, especially in analysis of fluids (synovial fluid, vitreous) with high HA and low sulfated glycosaminoglycan content.

Hyaluronic acid content can be also determined by analysis of the oligosaccharide products of enzymatic digestion. Quantification is accomplished by methods such as HPLC, capillary electrophoresis (CE), mass spectrometry, or fluorophore-assisted carbohydrate electrophoresis. These methods have primarily been employed to determine relative amounts of different glycosaminoglycans in a sample, rather than absolute quantities.

The most sensitive, specific, and accurate methods for determination of HA content are based on enzyme-linked sorbent assays (ELSA, ELISA-like assays) (39–47). The specific detection of HA is an important step of these methods, because purification of low M HA is difficult, and contaminants interfere with non-specific detection modes. The specificity is based on the use of molecular species such as proteins or proteoglycans that recognize and bind HA but no other biological molecules. For example, the aggrecan proteoglycan binds HA specifically (8, 9). The intact proteoglycan may be used, or a terminal fragment called globular domain 1 – interglobular domain – globular domain 2 (G1-IGD-G2), often referred to as HA-binding protein (HABP) or HA-binding region (HABR). The link protein, also called CRTL1 or HAPLN1, is similar to the G1 domain of aggrecan, and is another suitable protein for specific detection of HA. Isolated HABP, usually a mixture of the aggrecan HABR and the link protein, may also be used. Similarly, versican proteoglycan G1 domain is useful. Hyaluronectin, a HA-specific binding protein isolated from brain, may be used. Recently, a recombinant fusion protein of human TSG-6 and the Fc domain of human IgG, and a second variant of the fusion protein in which the heparin-binding region of TSG-6 was mutated to become inactive, were found to be suitable for development of a specific HA assay (47). Other HA-specific binding molecules could be used.

There are two types of ELSA. The first type, sandwich assays, are sensitive and reproducible, but fail to adequately quantify low M HA (39–42). This is because the plate surface, coated with an HABP, strongly binds HA, but does not allow further probing of short HA chains by the detector protein (43). Longer HA chains have accessibility as a result of looped sections above the surface. (The same problem occurs in HA blotted to positively charged nylon membranes after electrophoresis, from which short HA chains cannot be detected.) The second type of ELSA is competitive assays. In these, HA is usually immobilized on a surface such as the wells of a plastic 96-well plate. Alternative surfaces are suitably modified magnetic beads. Soluble HA samples, either standards or unknowns, are mixed with the specific binding agent,

usually a protein or proteoglycan. The soluble HA competes with immobilized surface HA for the specific binding agent, so that the resulting surface-bound amount of the binding agent is a measure of the amount of soluble HA in the sample being analyzed (41, 44, 45). There are multiple possible detection schemes to quantify the bound agent. For example, if aggrecan proteoglycan is the specific binding agent, it can be quantified with an antibody to the keratan sulfate chains of aggrecan, and a suitably labeled second antibody. When the specific binding agent is a labeled (e.g., biotinylated) HABP, it can be quantified by binding of the label to a specific agent such as streptavidin, which is, in turn, conjugated to an enzyme or other detectable species. Radiolabeled HABP may also be used but is less desirable on the basis of safety and disposal. Because the recognition step in a competitive binding assay occurs in solution, HA chains as short as approximately decasaccharides can be accurately detected, depending on the labeled binding protein used. The results of sandwich and competitive assays have been shown to be in good agreement for high M HA (46).

HA Content in Biological Fluids and Tissues

The content of HA in many normal biological fluids has been determined. Here, we cite a few relevant results. HA is a major component of articular joint synovial fluid, where it provides the viscoelasticity and lubrication necessary for protection of cartilage surfaces. Its concentration in the human knee joint is approximately 2–3 mg/ml, being slightly higher in younger adults than in older adults (48–50). HA is also a major component of the vitreous body of the eye, but at a lower concentration of approximately 200 µg/ml, in the phakic human eye vitreous (51). The concentration in the aqueous humor is lower still, being only about 1 µg/ml (52). Human lymph fluid contains HA at a concentration of about 0.1–18 µg/ml (36). In the blood serum of healthy human adults, the concentration of HA is lower still, being usually between 10 and 100 ng/ml, mostly 20–40 ng/ml, and averaging about 30 ng/ml (36, 40, 44). Normal human urine also contains a low level of HA, around 100–300 ng/ml (44), and human milk similarly contains HA at about 200–800 ng/ml (25).

The HA content of solid tissues varies widely. Bovine nasal cartilage contains approximately 1200 µg HA/g wet tissue weight (44). The HA content of human articular cartilage is similar, being about 500–2500 µg/g (53). Human skin contains approximately 400–500 µg HA/g tissue, mostly in the dermis (54). Fetal skin and young skin have higher HA contents than older skin. Other organs have much less HA. Laurent and Tengblad (44) reported HA contents of approximately 1–100 µg HA/g wet tissue weight for most organs. Rabbit kidney had 103 µg/g, brain had 65 µg/g, muscle had 27 µg/g, liver had 1.5 µg/g, and cornea had 1.3 µg/g. Armstrong and Bell (34) also reported rabbit tissue HA contents of 500 µg/g for skin, 200 µg/g for large intestine and heart, 130 µg/g for small intestine, and 80–90 µg/g for lung and muscle tissues.

Measurement of HA content is of continuing high interest, because there are multiple studies correlating changes in HA content with tissue remodeling and pathological processes. While the normal HA concentration in human serum is usually <40 ng/ml, it is elevated (>46.5 ng/ml) in hepatic cirrhosis (55), in rheumatoid arthritis (56, 57) (highly variable; reports up to nearly 200 µg/ml,

but more generally between 0.07 and 6.4 µg/ml), in ankylosing spondylitis (57) (7–13 µg/ml), and in osteoarthritis (57, 58) (0.04–2.3 µg/ml). The elevated HA concentration in serum of patients with hepatic cirrhosis is utilized as one component of a diagnostic assay. A small but significant elevation (frequently, about twofold) of HA in serum is found in multiple types of untreated cancer (59–61). Radical surgery to remove the tumor causes the HA concentration in serum to return to the normal range. Most interestingly, it was found that the low-molecular mass component of serum HA can be used to differentiate metastatic from non-metastatic breast cancer (62), which may form the basis of a new diagnostic test.

In solid tissues, many but not all cancers progress in a tumor microenvironment of increased HA content (28). Further, some non-aggressive cancer types such as non-malignant fibroadenoma produce elevated HA (63, 64). The presence of HA may therefore not be sufficient by itself to promote tumorigenesis. However, high levels of HA accumulate in lung, colorectal, prostate, bladder, and breast carcinomas and in these cancers are linked to tumor aggression (28). For example, the HA content of human lung tissue increases 4- to 200-fold in lung carcinoma (65), 100-fold in grade 3 ovarian cancer (66) and 7-fold in prostate cancer (67). Increased tumor HA accumulation is also linked to tumor aggression. The HA content of malignant ovarian epithelial tumor correlates with tumor grade and with metastasis. Elevated HA accumulation within the stroma or tumor parenchyma of breast cancer is associated with unfavorable prognosis of the patient. Recent studies have further linked high stromal HA staining to HER2 positive tumors and poor outcome parameters including time to relapse, large tumor size, lymph node positivity, hormone receptor negativity, high body mass, and shortened overall survival (68). Elevated HA in the tumor microenvironment is linked to inflammation (69). Thus, high amounts of both tumor-associated macrophages and HA are concurrent in breast carcinoma. High macrophage numbers correlate with high tumor HA, HAS expression and poor outcome, suggesting that HA facilitates a macrophage tumor supporting function in breast cancer. The link between inflammation and cancer has led to recent interest in HA as a contributor to a pro-tumorigenic inflammatory environment, as detailed in a companion article in this issue (70).

As for cancer, wound healing and fibrosis are associated with inflammation and increased HA content (71). An approximately twofold increase was observed in HA content of rat skin during healing of excisional wounds (30). Similarly, scleroderma patients with early stage disease have an approximately twofold increase in serum HA (72). Many other pathological states characterized by inflammation similarly have elevated HA, as estimated by immunohistochemical analyses (73, 74).

Methods to Analyze HA Molecular Mass Distribution

It has long been appreciated that degradation of HA negatively affects its biomechanical properties. For example, degradation of HA in articular joint synovial fluid can reduce the viscosity and elasticity of the synovial fluid, and has also been shown to reduce its lubricating ability (49, 75). The widespread and successful

uses of solutions of high molecular mass HA as a viscosurgical tool in ophthalmic surgery, and as an analgesic treatment for osteoarthritis, are based on this understanding. More recently, the discovery that exogenous HA fragments can alter cellular behavior by signaling through multiple receptor proteins, and that the existence of such fragments *in vivo* is likely, based on increased hyaluronidase levels and reactive oxygen and nitrogen species in tissue remodeling and pathological processes, has led to increased interest in measuring the size distribution of HA in biological fluids and tissues.

Many current methods for determination of the M distribution of HA from tissues and biological fluids have been optimized for highly purified HA. A commonly employed method used commercially is size exclusion chromatography with multiangle laser light scattering (SEC-MALLS) (76, 77). However, detection of very low M HA by light scattering is inherently insensitive, and the SEC-MALLS method requires a highly purified HA sample. CE (78) is similarly limited to pure HA samples. MALDI-TOF mass spectrometry (79, 80) has high sensitivity, but requires a pure sample and HA with M larger than about 10 kDa becomes difficult to analyze. A new method that has extremely high sensitivity and works best for low M HA is gas-phase electrophoretic mobility molecular analysis (GEMMA), but it still requires pure HA (81).

The most widely used methods, to date, for size distribution analysis of imperfectly pure HA isolated from biological samples are size exclusion chromatography with enzyme-linked sorbent assay (SEC-ELSA) (36, 56, 82), and agarose or polyacrylamide gel electrophoresis (83–86) with staining or with blotting and specific detection. Both methods are capable of detecting a wide range of HA sizes. Gel electrophoresis with staining can analyze samples on the microgram scale, and can tolerate some impurities in the sample, but non-specific staining by those impurities can interfere with size distribution analysis of the HA. Blotting of gels to positively charged nylon and detection of HA using a labeled specific binding protein works only for HA with $M > 100$ kDa, as a result of strong surface binding (43). To address the issues of limited sample amount, purification difficulty, and the importance of analyzing both high and low M HA simultaneously, we recently developed a method using size-dependent fractionation of HA by anion exchange on a spin column, and quantification of HA in the fractions using a competitive ELSA assay (IEX-ELSA) (37). All of these methods require calibration with purified HA samples of known size.

HA Size in Biological Fluids and Tissues

The average M and distribution of M for HA present in biological sources have been studied primarily for fluid tissues such as synovial fluid, vitreous, serum, lymph, and milk. Until recently, the emphasis has been on documenting reduction of the average M , which strongly affects the biomechanical properties of HA solutions (48, 87–89). This has been done using physicochemical methods such as viscometry, light scattering, and sedimentation-diffusion. Interest in the distribution of sizes present, and the possibility that specific sizes have unique biological effects, has led to an increasing number of studies by chromatographic and electrophoretic separation methods.

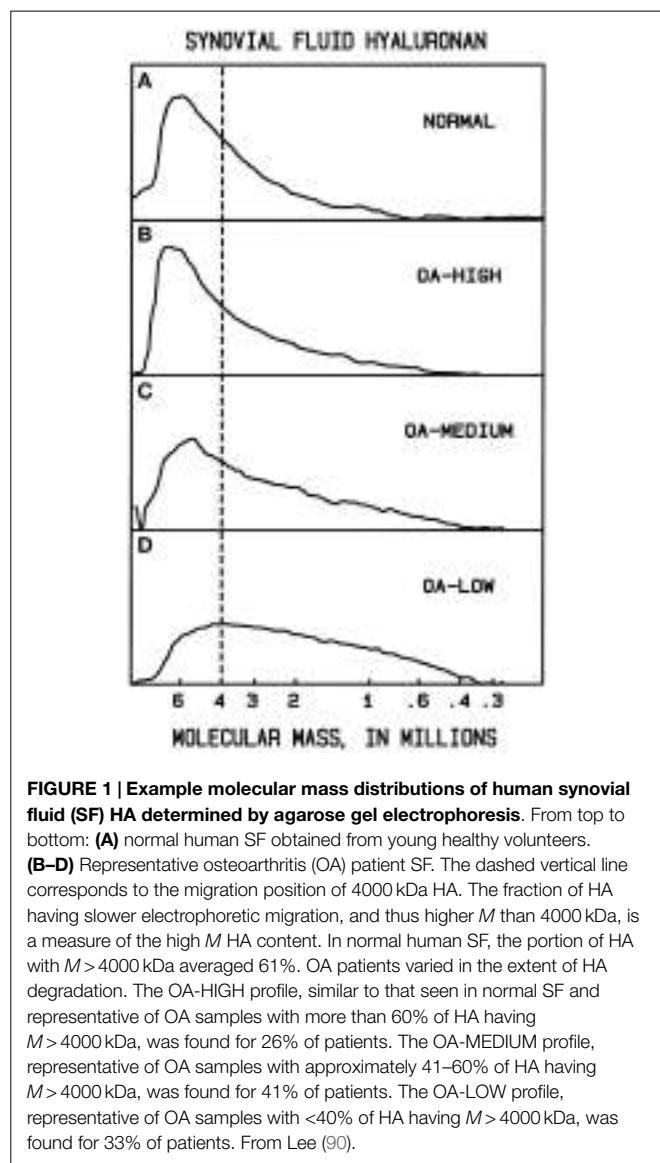


FIGURE 1 | Example molecular mass distributions of human synovial fluid (SF) HA determined by agarose gel electrophoresis. From top to bottom: (A) normal human SF obtained from young healthy volunteers. (B–D) Representative osteoarthritis (OA) patient SF. The dashed vertical line corresponds to the migration position of 4000 kDa HA. The fraction of HA having slower electrophoretic migration, and thus higher M than 4000 kDa, is a measure of the high M HA content. In normal human SF, the portion of HA with $M > 4000$ kDa averaged 61%. OA patients varied in the extent of HA degradation. The OA-HIGH profile, similar to that seen in normal SF and representative of OA samples with more than 60% of HA having $M > 4000$ kDa, was found for 26% of patients. The OA-MEDIUM profile, representative of OA samples with approximately 41–60% of HA having $M > 4000$ kDa, was found for 41% of patients. The OA-LOW profile, representative of OA samples with <40% of HA having $M > 4000$ kDa, was found for 33% of patients. From Lee (90).

In normal human synovial fluid, most of the HA is very high in molecular mass. Gel filtration chromatography with HA-specific detection (50) and agarose gel electrophoresis with staining (84, 90) show the average M to be approximately 6000–7000 kDa, with little if any HA <1000 kDa. In rheumatoid arthritis and in osteoarthritis, HA can be partially degraded, resulting in a broad distribution of sizes, extending perhaps down to a few hundred kilodaltons (90, 91) (Figure 1). Normal rabbit vitreous HA has mostly high M (2000–3000 kDa), but bovine vitreous HA has mostly moderate M (500–800 kDa) (82). Owl monkey vitreous has very high M HA (84).

For fluids containing HA at very low concentrations, determination of the M distribution is correspondingly difficult. Despite this, evidence for the occurrence of HA below 100 kDa (<250 disaccharides) in M is accumulating. Human milk contains mainly HA with an average M of about 440 kDa, and also has been definitively shown to have approximately 5% of HA with $M < 100$ kDa (37). The low M HA is proposed to participate in

stimulating the expression of human β -defensin 2 in the infant intestinal epithelium (24). Human amniotic fluid contains HA with an average M of about 330 kDa at 16 weeks gestation, but the M distribution changes to a mixture of high and very low M HA by 40 weeks gestation (92). HA in lymph fluid is variable in size, and can occur as a mixture of high and lower M components (36), or as a broad distribution of moderate M , ca. 800 kDa average (93). HA in normal blood serum is mainly relatively low M (ca. 100–300 kDa) (36, 56). It is also low in M in saliva and urine (94, 95).

Tumors have been proposed to shed very low M HA into associated body fluids. The quantity of such very low (but undetermined size) M HA in patient serum, obtained by centrifugal filtration, has been reported to be associated with metastatic breast cancer (62). It has also been reported in saliva of patients with head and neck tumors (95), and in the urine of patients with bladder cancers (94). The precise size of all such HA has not yet been determined, but should be accessible using recent improvements in methods. Rarely, high M HA is found in serum, as, for example, associated with Wilm's tumor (96).

For solid tissues, the pattern is a bit simpler. Normal healthy tissues are almost always associated with high M HA. HA with average $M > 2000$ kDa is found in young human cartilage (53). Larger HA averaging closer to 4000–6000 kDa is found in human skin (54), in rabbit skin (34), and in rat skin (30, 97). High M HA is found in rooster combs (32). High M HA is also found in skeletal muscle, lung, heart, ileum, and colon of the rabbit (34). Little if any low M HA is found in these healthy tissues.

Remodeling tissues and tumors show evidence of some lower M HA. Reduction in HA M occurs in older human cartilage (53). Low M HA also occurs in healing rat skin wounds (30), in human skin following irradiation with UVB (74), and in mouse cervix undergoing postpartum remodeling (98). It is found in rat kidney after ischemia–reperfusion injury (99). Human prostate tumor HA has also been reported to contain some low M HA of indeterminate size (67). Many of the above-described studies of reduced M HA should be regarded as indicative but not conclusive proof of the presence of specific low M HA species. Recent improvements in techniques for analysis of very low quantities of polydisperse HA will allow this uncertainty to be addressed. Future studies should also include spiking samples with multiple monodisperse HA species to show that the isolation methods cause no degradation, or preferential isolation of high or low M HA.

It is interesting to consider that all efforts to determine the content and size of HA in biological tissues and fluids have made the tacit assumption that the HA has a constant chemical structure, except for variation in size. Since degradation by ROS/RNS can cause chemical changes including ring opening reactions, it is possible that HA assays and size analyses may be influenced by such changes, if present at significant levels. Further examination of this possibility is warranted.

Conclusion

It is now well established that HA synthesis is significantly increased in remodeling tissues and tumors. The concomitant

presence of hyaluronidases and ROS/RNS makes it likely that fragments of HA can be created by degradation of high *M* polymers. The balance of synthetic and degradative activities, coupled with turnover through outflow or internalization, will determine the steady state *M* distribution of the tissue HA. HA shed into lymph or blood from a tumor may represent only the lowest *M* fraction of that present. Whether HA fragments of particular sizes exist in sufficient amounts within a tissue or tumor environment to trigger specific cellular responses is not yet clear. The fact that exogenous HA fragments can elicit such effects is suggestive but not yet a proof of their role *in vivo*. There is good reason to expect clarity on these issues in the near future.

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Author Contributions

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Revealing the mechanisms of protein disorder and N-glycosylation in CD44-hyaluronan binding using molecular simulation

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The extracellular N-terminal hyaluronan binding domain (HABD) of CD44 is a small globular domain that confers hyaluronan (HA) binding functionality to this large transmembrane glycoprotein. When recombinantly expressed by itself, HABD exists as a globular water-soluble protein that retains the capacity to bind HA. This has enabled atomic-resolution structural biology experiments that have revealed the structure of HABD and its binding mode with oligomeric HA. Such experiments have also pointed to an order-to-disorder transition in HABD that is associated with HA binding. However, it had remained unclear how this structural transition was involved in binding since it occurs in a region of HABD distant from the HA-binding site. Furthermore, HABD is known to be N-glycosylated, and such glycosylation can diminish HA binding when the associated N-glycans are capped with sialic acid residues. The intrinsic flexibility of disordered proteins and of N-glycans makes it difficult to apply experimental structural biology approaches to probe the molecular mechanisms of how the order-to-disorder transition and N-glycosylation can modulate HA binding by HABD. We review recent results from molecular dynamics simulations that provide atomic-resolution mechanistic understanding of such modulation to help bridge gaps between existing experimental binding and structural biology data. Findings from these simulations include: Tyr42 may function as a molecular switch that converts the HA-binding site from a low affinity to a high affinity state; in the partially disordered form of HABD, basic amino acids in the C-terminal region can gain sufficient mobility to form direct contacts with bound HA to further stabilize binding; and terminal sialic acids on covalently attached N-glycans can form charge-paired hydrogen bonding interactions with basic amino acids that could otherwise bind to HA, thereby blocking HA binding to glycosylated CD44 HABD.

Keywords: CD44, hyaluronan, binding, free energy, molecular dynamics, glycosylation, inhibition, unfolding

Introduction

The structure of the cell surface protein CD44, from its N-terminus to its C-terminus, consists of a globular hyaluronan binding domain (HABD), a stalk domain, a single-pass transmembrane domain, and a cytoplasmic domain (1, 2). Amino acids located N-terminal to the transmembrane domain are on the extracellular side of the cell membrane, and amino acids located C-terminal to the

transmembrane domain are on the intracellular side (**Figure 1**). Post-translational modifications to CD44 include glycosylation of the extracellular portion (3–5), palmitoylation of amino acids immediately C-terminal to the transmembrane domain (6–9), and phosphorylation of the cytoplasmic domain (10–12). The already-complex structural biology of CD44 is further complicated by variable splicing of the RNA transcript of the *CD44* gene, which yields a variety of different patterns of amino acid insertion into the stalk domain and which modulates CD44 function (1, 13, 14), and by shedding that produces soluble CD44 (15).

Atomic-resolution structures can lead to substantial insight into the function of a biomolecule. Such high-resolution structural information is typically obtained from X-ray crystallography or NMR spectroscopy experiments, with examples being as small as a single zinc finger domain (16) and as large as ribosomes (17) and virus capsids (18). However, not all biomolecules are equally amenable to having their structures solved by these methods. Glycoproteins and proteoglycans are particularly challenging because of the difficulty in obtaining pure samples and the inherent flexibility of these two types of biomolecules. Sample homogeneity is a challenge for glycoproteins and proteoglycans because the carbohydrate component results from non-template-based enzymatic synthesis, leading to carbohydrate microheterogeneity at a given glycosylation site even though the protein component for a given

sample is identical throughout (19). The carbohydrate component is also flexible (20, 21), especially in comparison to globular proteins with their well-defined tertiary (three-dimensional) structures, and which therefore were crystallized early in the development of the field of structural biology (22) and still compose the majority of publicly available experimental atomic-resolution structures.

In contrast to globular proteins, which exist in an aqueous environment, transmembrane proteins are located in biological lipid bilayers. Therefore, this environment must be suitably reproduced in samples in order to do experimental structural biology, which can be very challenging (23, 24). Additionally, heterologous expression of transmembrane proteins and subsequent purification can be more difficult than for water-soluble globular proteins because of the limited surface area of the cell membrane for expression, the resulting toxicity to the organism being used for expression, and the subsequent need to reconstitute the protein in a lipid environment after extraction and purification (25, 26).

Intrinsically disordered proteins provide another contrast to globular proteins in that the former lack well-defined unique stable three-dimensional structures (27–29). In X-ray experiments, this results in crystallographic disorder, diffuse scattering, and therefore undefined atomic coordinates (30). While NMR has been used extensively to study intrinsically disordered proteins, solution NMR experiments yield data that represent ensemble averages, which can limit understanding of the various discrete conformations that such proteins may assume (31).

CD44, with its multiple domains, poses a number of challenges for characterization by X-ray crystallography or NMR spectroscopy. The N-terminal HABD, which is similar to globular proteins, is in its biologically relevant form a glycoprotein that has numerous glycosylation sites (1). Furthermore, HABD in the presence of hyaluronan (HA) has characteristics of an intrinsically disordered protein (32, 33). The stalk domain that connects the extracellular HABD to the cell membrane has both N- and O-glycosylation sites (34). And, with alternative splicing, the stalk domain can have proteoglycan characteristics, namely a protein core with glycosaminoglycan (GAG) attachments in the form of chondroitin sulfate and heparan sulfate (35). Recalling that CD44 is a transmembrane protein, in addition to the challenge associated with being located in the cell membrane, the transmembrane domain can be post-translationally modified by the attachment of lipids, which further complicates its structural biology since this modification can alter its interactions with the membrane bilayer (9, 36, 37). Finally, the cytoplasmic domain is likely disordered when not non-covalently bound to intracellular adapter proteins (12).

Atomic-resolution experimental structural biology on CD44 has been largely limited to the ~150 amino acid HABD in its non-glycosylated form because of the many specific challenges above. X-ray structures for HABD exist for both human and mouse isoforms. Human HABD has been reproducibly crystallized in its apo form (i.e., not bound to HA, inhibitors, or peptides) (38, 39), as well as with unidentified peptide (39) found to be non-covalently bound to the face of HABD opposite that of the location of the HA-binding site. However, there are no publicly available X-ray structures of human HABD complexed with either HA or with inhibitors of HA binding. In contrast, mouse HABD has been co-crystallized with both oligomeric HA (oHA) (40, 41) and

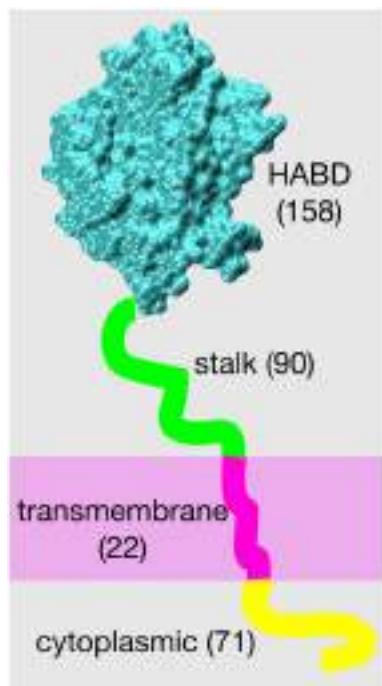


FIGURE 1 | CD44 structure. The four different structural/functional regions are denoted by different colors, and labels include the number of amino acids in each region for the standard splice variant “CD44s.” Results of variation in RNA splicing include additional amino acids in the stalk region and loss of amino acids composing the cytoplasmic region. Amino acid numbering for the human isoform begins with residue 21 because of cleavage of a 20-residue N-terminal signal peptide.

small-molecule inhibitors (40), as well as in its apo form (41). These co-crystals reveal the binding mode of HA with HABD, which is presumably the same for human HABD given the ~85% sequence identity between the two forms, and the 100% sequence identity of the HA-binding site (42). NMR structures exist for human HABD, both in its apo form (38) and bound to oHA (32). However, unlike X-ray structures with HA, NMR structural characterization of the bound form lacks atomic coordinates for HA and therefore does not provide comprehensive information into the non-covalent atomic contacts between HABD and bound HA. All of these previous structural biology examples are of CD44 HABD; the only non-HABD example of experimental CD44 structure is a complex consisting of a nine amino acid long portion of the 72 amino acid cytoplasmic domain complexed with the radixin FERM domain (12).

Molecular dynamics (MD) is a physics-based approach to the modeling and simulation of biomolecules (43). In all-atom explicit-solvent MD simulations, all the atoms of the system, including those for the solvent, are included as interaction sites for computing the forces in the system. The values of the forces as a function of atomic positions are determined by a combination of a mathematical expression and parameters, commonly called a “force field,” that encodes properties such as the energetic cost to stretch a bond or the energetic benefit of a van der Waals or charge-pairing interaction (44). These forces are numerically integrated to propagate the system, and this is done in an iterative manner to generate a trajectory, analogous to a movie, that shows how the positions of the atoms in the system evolve with the passing of time (45). Typical present-day simulations involve tens of thousands to hundreds of thousands of atoms with trajectory lengths of tens to hundreds of nanoseconds, which require tens to hundreds of millions of consecutive integration steps. MD simulations can be used to determine not only the time-evolution (dynamics) of the system but also the relative probabilities, and therefore free energies, associated with different states (thermodynamics) (46). As such, MD is an especially useful tool for studying flexible biomolecules at an atomic level of resolution, which makes it an ideal complement to experimental structural biology techniques like X-ray crystallography and NMR spectroscopy (47).

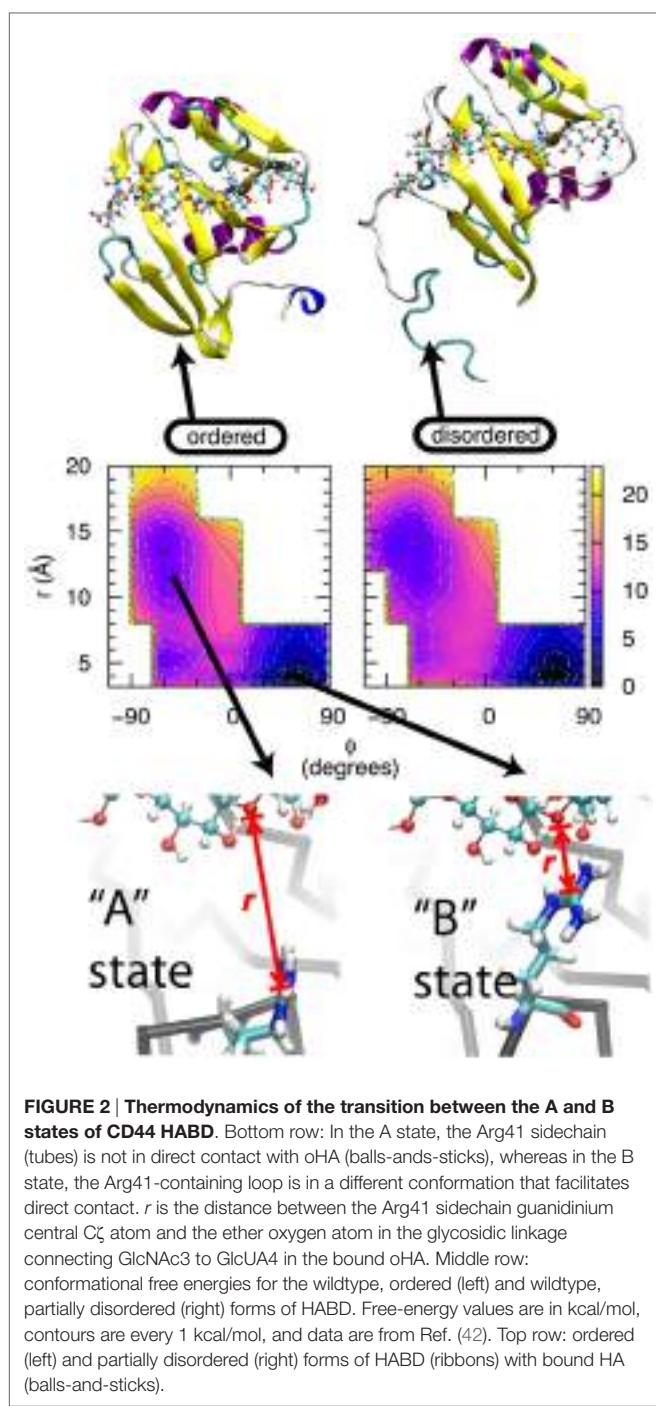
Over the past several years, our research group has applied all-atom explicit-solvent MD simulations to extend the understanding of the function of the CD44 HABD. These efforts have aimed to address the following scientific questions: (1) what is the mechanism and associated thermodynamics of a conformational change in an arginine-containing loop at the HABD binding site that is associated with HA binding? (41); (2) why does HABD transition from a well-ordered (folded) three-dimensional structure to one that is partially disordered when it binds to HA? (32, 33, 48); and (3) why do covalently attached sialylated N-glycans inhibit HA binding while unsialylated ones do not? (49–51) This article reviews the contribution MD simulations have made toward developing answers to these questions. We note that others have also recently applied MD simulation to the study of CD44 HABD, with topics including conformational flexibility and the microscopic structure and dynamics of water surrounding HABD (52, 53), and the mechanism and thermodynamics of the ordered to partially disordered transition (54).

Conformational Switching at the CD44 HABD Binding Site

Two lines of experimental evidence lead to the hypothesis that direct contact between CD44 Arg 41 and HA is a major source of binding affinity (“Arg41” reflects amino acid numbering in the human form of CD44; the equivalent amino acid is Arg45 is the mouse form. For simplicity, the human numbering will be used throughout this text.). From X-ray crystallography, Arg41 and the loop that contains it change conformation depending upon binding of oHA. In the apo form, the loop is in an open conformation that locates this sidechain too far away to contact oHA if it were present, and HABD is said to be in the “A” state (38, 41). In the complexed form, the loop can be either in the open conformation, or in a closed conformation that facilitates direct contact between the positively charged guanidinium group of the Arg41 sidechain and bound oHA, in which case HABD is in the “B” state (40, 41) (**Figure 2**, bottom row). From mutation data, the Arg41Ala single point mutation essentially destroys the ability of HABD to associate with oHA (41, 55, 56). This thermodynamic information demonstrates the critical nature of Arg41 in binding. However, the X-ray crystallographic data do not say anything about the thermodynamics of the A and B states, other than they are both sufficiently stable to be trapped as crystals when oHA is bound. And the mutation data do not provide information about the conformation of Arg41 when oHA is bound. We therefore applied MD simulations in an attempt to tie these two previous findings together.

All-atom explicit-solvent MD simulations can be used to determine true thermodynamic free energies for a variety of biomolecular processes (46, 57–60). This is because of two reasons: (1) a model system under study includes water molecules and allows for full conformational flexibility of the included biomolecules, which means both solvent effects and entropic effects are explicitly included (that is, not as an approximation, but as part of the system under investigation); and (2) there exist exact mathematical expressions to determine thermodynamic quantities directly from simulation data. One approach to determining the free-energy difference between two conformational states x and y of a biomolecule from MD simulation is to compute the reversible work required to transition the system from state x to state y by integrating the measured average force along the transition path (61). Since free energy is a thermodynamic state function, it does not depend on the actual path used to convert the system from state x to state y . However, in practice obtaining good numerical convergence as well as plausible biological insight both depend upon determining a physically reasonable transition path.

For CD44 HABD, the two states are A, having an open loop and the Arg41 sidechain separated from bound oHA, and B, with a closed loop and the Arg41 sidechain in direct contact with oHA. While a simple distance between Arg41 and oHA can be used to discriminate between different conformations of the sidechain, it is not immediately obvious that a similarly simple metric can be used to discriminate between the open vs. closed loop conformations. In an effort to identify such a metric, we first compared the conformations of the backbone dihedral angles ϕ , ψ for Arg41 and three amino acids on either side: -Lys-Asn-Gly-Arg41-Tyr-Ser-Ile-. This



revealed a difference in the Tyr42 φ dihedral where in the A state $\varphi = -60^\circ$ and in the B state $\varphi = +60^\circ$ (62). This was followed by MD simulation where force was applied to this dihedral angle to gradually convert it from one value of φ to the other. In one case, starting from the A state and increasing φ converted the system to the B state, not just with respect to the Tyr42 backbone but also with respect to the separation distance between Arg41 and oHA. In the other case, starting from the B state and decreasing φ converted the system to the A state, including breaking of the

contact between Arg41 and oHA. In neither case was any force applied to directly affect the Arg41 to oHA distance; rather, changes in this distance spontaneously resulted from changes in the loop backbone conformation. Furthermore, a similar effect could not be achieved using the backbones of other amino acids in the loop (62). Thus, the reaction path for interconversion between the A and B states was defined in terms of two progress variables: the value of the Tyr42 backbone dihedral angle φ , and the distance r between the Arg41 sidechain guanidinium central C ζ atom and the ether oxygen atom in the glycosidic linkage connecting GlcNAc3 to GlcUA4 in the bound oHA.

Extensive simulations were subsequently done to compute the free energy of the system as a function of the progress variables φ and r (42). All simulations were of the human HABD (hHABD) complexed with [-4GlcUA β 1-3GlcNAc β 1-]₄ ("HA8"), which was computationally constructed by combining information from mouse and human structures. The four hHABD-HA8 systems considered were: wildtype, ordered; wildtype, partially disordered; Arg41Ala, ordered; and Arg41Ala, partially disordered. **Figure 2**, middle row shows the data for both wildtype systems, and the major free-energy minima, which are the most stable states for each system, correspond to A and B.

Wildtype simulation data demonstrate that the B state of the hHABD-HA8 complex is more stable than the A state by ~8–9 kcal/mol. This is true for both the ordered and partially disordered forms of hHABD (**Figure 2**, top row). Additional simulation data demonstrate that the analogous transition for the Arg41Ala mutant is substantially less favorable at ~6 kcal/mol. From these data, it is possible to calculate the loss in binding affinity associated with the point mutation, with values of 2.2 kcal/mol for the ordered form and 2.3 kcal/mol for the partially disordered form (42). These simulation data are in close agreement with existing experimental data measuring the loss in binding affinity to be 2.5 kcal/mol (41), which helps validate both the force field and the convergence of the simulations. Taken together, these results support the idea that formation of direct contact between HA and the Arg41 sidechain is a substantial source of favorable binding free energy (41).

In contrast to some of these findings, Plazinski and Knys-Dzieciuch, in their simulation studies, found that the φ -related free-energy barrier was not correlated with the Arg41-HA distance (54, 63). The authors also observed a low free-energy barrier associated with separation of Arg41 from HA, with the A and B states reducing to an average dynamic structure (54, 63). They suggested that a possible explanation might be differences in the force fields used in their studies (54), since the previous analogous work found the A and B states to be distinct (62). Differences in force fields, which are the underlying physical models used to represent the bonded and non-bonded interactions in such simulations, can indeed cause such differing results. When such differences are inferred based on differing simulation results and conclusions, one possibility is to review the methodology involved in the force field development (44, 64). Another is to compare the outcomes of the particular simulations with the existing experimental data for inconsistencies. In this particular case, relevant experimental data include the crystal structures of the A and B states of HABD complexed with oHA, and the mutation data showing loss of binding affinity

in the Arg41Ala mutant. Dynamic averaging would manifest as crystallographic disorder with poorly resolved electron density for Arg41, which is in contrast to the existing crystallographic data. Additionally, rapid equilibrium between short and long separation distances between Arg41 and oHA suggests a weak interaction between them, which is in contrast to the mutation data.

Binding and Unfolding in CD44 HABD

Early experimental studies of the Arg41Ala mutation that predated the CD44 structural studies also probed the contribution of other basic amino acids by both point and truncation mutations (55). This was a logical course of action in the absence of HABD three-dimensional structure since HA contains a negatively charged carboxylate group every other monosaccharide and Arg and Lys sidechains are positively charged, suggesting the possibility of charge-charge interaction as a mechanism of binding. From that perspective, it is not surprising that these mutations all decrease the strength of binding of HA oligomers. But, taken in the context of the subsequent structural information, the explanation is less obvious, since these additional amino acids, in contrast to Arg41, are located spatially far from the now-known binding site (41). Further complicating the situation is the observation that the portion of HABD that contains these amino acids 153–169 goes from having well-defined three-dimensional structure to becoming unfolded in conjunction with HA binding (32, 33, 48). That is, the change in the conformation, which correlates with the binding of HA, in this span of amino acids located in the C-terminal most region of HABD is what defines the ordered-to-partially disordered HABD transition.

The above suggests two questions: why do basic amino acids distant from the binding site affect affinity? And why is the affinity greater when the sequence containing these amino acids unfolds and becomes disordered? To answer these two questions, we return to the previous set of free-energy data from MD simulations. For wildtype hHABD, the free-energy data associated with the interconversion between the A and B states are qualitatively the same regardless of whether hHABD is in the ordered or partially disordered form (Figure 2, middle row). The same is also the case for the Arg41Ala mutant, where the free-energy change in going from A to B is independent of whether those distant amino acids are folded or not (42) (data not shown here). Not only are there strong qualitative similarities in free-energy data between the ordered and partially disordered forms, but the quantitative values are also very similar. For the A→B transition of the binding site in the wildtype, ordered form, the associated free-energy change is −8.7 kcal/mol, and for this transition in the wildtype, partially disordered form the value is a very similar −7.8 kcal/mol (42). Likewise for the Arg41Ala mutant, in the ordered form the value is −6.5 kcal/mol and in the partially disordered form it is −5.5 kcal/mol (42). The small difference of ~1 kcal/mol is within the precision that can be expected from these particular computational experiments.

In an allosteric mechanism, a conformational change distant from the site affects the energetics at the binding site. In the case of HABD, the independence of the energetics of the binding site A→B transition from the ordered vs. partially disordered form of HABD contradicts the hypothesis that allostery is at work. That is,

conformational switching at the binding site appears no more or less favorable if the HABD C-terminal region is folded or unfolded.

Analysis of the MD trajectories that were generated as part of the free-energy experiments yielded a result that, in hindsight, is obvious: flexibility from partial unfolding permits favorable electrostatic interactions between HA and the C-terminal HABD amino acids that cannot occur when the domain is fully ordered. In the ordered form of HABD, the amino acids in question are locked into a folded conformation that keeps them far from bound oHA, while in the partially disordered form, this is no longer the case (Figure 2, top row). Because this span of amino acids is no longer in consistent contact with the rest of the HABD domain, it assumes the properties of a random coil peptide, which through random fluctuations can collide with bound oHA. If this collision happens in a way that brings one of the basic sidechains into close proximity with oHA, a favorable contact can be formed. Unlike the Arg41 interaction, which has a well-defined mechanism based on specific interactions of the sidechain with a particular limited section of bound oHA, the flexibility of the disordered amino acids and the repeating nature of the HA polymer permit the possibility of a wide range of basic sidechain interactions with bound HA (42), consistent with the long-standing finding that a 13-amino acid CD44 peptide spanning Arg150–Arg162 itself will bind HA (65). This wide range may conceivably include interaction with HA bound to an adjacent molecule of CD44, though the present simulations had a single copy of the complex and therefore could not directly address the possibility of such *trans* association.

The above computational experiments do suggest a plausible molecular mechanism by which the ordered to partially disordered transition confers increased binding affinity to oHA. However, the simulations involved either the ordered or the partially disordered form of CD44 hHABD, and therefore do not provide any insight into the mechanism of the ordered to partially disordered transition itself. Independent work has been done toward this end, and with the additional aims of estimating the free-energy profile of the transition and clarifying the role of select amino acids in the transition (54). Connected with the proposed transition mechanism was a free-energy change of +25 kcal/mol, implying the partially disordered form in the absence of HA is very unstable relative to the ordered form. While the sign of the free-energy change, associated with loss of a single beta strand at the edge of a beta sheet, agrees with experiment (32), the magnitude is substantially larger than the folding free-energy values for entire small single-domain proteins, which are typically <10 kcal/mol, including for proteins consisting exclusively of beta strands (66). A further difficulty is that the simulation free-energy data for the analogous transition in the Tyr161Ala mutant are identical to those for the wild type. This is in contrast to experimental data, where the Tyr161Ala mutant constitutively exhibits the partially disordered conformation (33, 48). One possible explanation for these apparent inconsistencies is that the study represents the initial steps of the transition (54), such that extending simulations further along the selected reaction coordinate may result in subsequent decreases in free energy. Another possibility is lack of convergence of simulations given the large scale of the transition (54). This was the case for the free energy for the A→B transition, which involves a much smaller conformational change than the ordered to partially disordered

transition. The first study suggested that the A and the B states were essentially equally stable (62), while subsequent work that extended the timescale of the simulations by 40-fold showed the B state to be substantially more stable than the A (42). Importantly, in the case of the ordered to partially disordered transition, the partially disordered form is not a single, well-defined conformation. Rather, the disordered C-terminal HABD amino acids are free to take on a multitude of conformations. Therefore the partially disordered form is actually an ensemble of diverse conformations, and this further complicates computational experiments toward understanding the transition mechanism.

Inhibition by Glycosylation

N-glycosylation of CD44 HABD is known to have variable effects on CD44 function depending on the nature of the N-glycans. One effect is to block HABD binding to HA (49–51). Another is to make CD44 itself a ligand that binds to lectins (67, 68). The biochemistry behind both of these contrasting functions is related and is modulated by *N*-acetylneurameric acid (Neu5Ac) monosaccharide, which is commonly called “sialic acid.” In one case, HABD N-glycans are capped with sialic acids, and this both blocks HA binding and makes CD44 a selectin ligand. In the other case, sialidase activity removes these terminal sialic acids, leaving the bulk of the N-glycan structures intact, and this change restores both HA-binding and removes selectin ligand functionality. While sialidase treatment removes only the terminal monosaccharide from the attached N-glycan, the functional result is the same as removal of the entire N-glycan. For example, Asn point mutation precludes N-glycan attachment and heterologous expression yields non-glycosylated protein. In both of these cases, the functional outcome is the same as sialidase treatment, which implies that inhibition of HA-binding cannot be explained as a consequence of steric blockage of the HABD binding site, since the de-sialylated N-glycan has nearly the same bulk as the sialo-glycan. This mechanism of regulation is not unique to CD44, as the related hyaladherin LYVE-1 demonstrates similar behavior (69).

The sialidase data immediately suggest two sets of simulations of glycosylated HABD: one set with sialylated N-glycan and a second set with asialo N-glycan. Asn25 and Asn120 were selected for computational N-glycosylation based on previous mutagenesis studies showing that cells expressing Asn25Ser and Asn120Ser mutants constitutively bind HA (50). Complex-type N-glycans that were selected (70) based on the existing finding that blocking the metabolic pathway for processing complex N-glycans restores HA binding (71, 72). In conjunction with the two different glycosylation sites and the sialo and asialo forms of the N-glycan, both the ordered and partially disordered forms of CD44 HABD were studied in the simulations. A representative starting conformation of ordered HABD with a sialo glycan attached to Asn25 is shown in the left frame of **Figure 3**; the analogous asialo form would be missing only the atoms colored purple.

The key finding from the comprehensive set of simulations covering the ordered and disordered HABD paired with sialo and asialo N-glycans is that only in the sialo form do stable, long-lasting non-covalent contacts form between the protein and glycan components. Furthermore, these contacts involve

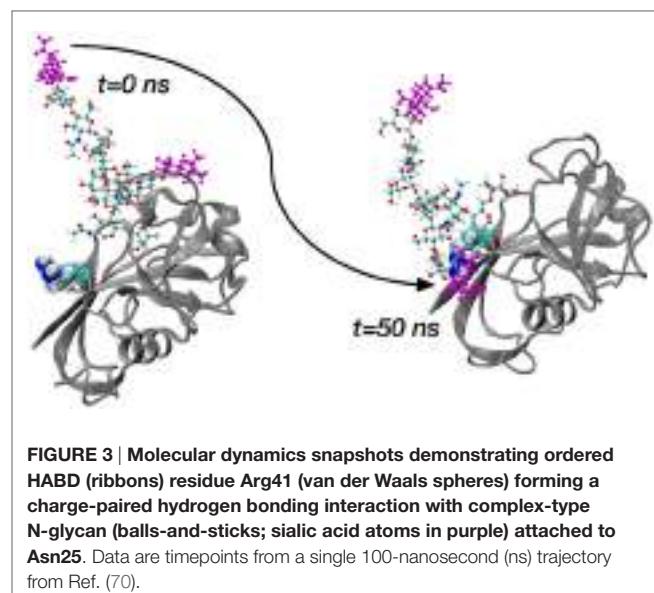


FIGURE 3 | Molecular dynamics snapshots demonstrating ordered HABD (ribbons) residue Arg41 (van der Waals spheres) forming a charge-paired hydrogen bonding interaction with complex-type N-glycan (balls-and-sticks; sialic acid atoms in purple) attached to Asn25. Data are timepoints from a single 100-nanosecond (ns) trajectory from Ref. (70).

the positively charged sidechains of HABD amino acids and the negatively charged carboxylate groups of the terminal sialic acids (**Figure 3**). In contrast, asialo glycans form only brief contacts, which is understandable since they lack the negative charge of the sialo form. Long-lasting contacts in the sialoglycan simulations involve Arg41 and Arg154, and these contacts form spontaneously during the simulation and can last for 40–50% of the simulation length (70). Both of these amino acids can directly associate with HA8 when it is bound, based on findings from the computational experiments on partially disordered HABD, as summarized in the previous section. However, their binding with sialic acid is an interaction that would directly compete with their binding with HA8. Therefore, the view that emerges is that, in the sialo form, the covalently attached N-glycans will form charge-paired hydrogen bonding interactions with Arg sidechains known to be important for HA binding. As further evidence, free-energy simulations, similar to the ones for Arg41–HA8 association described in the previous section, demonstrate that the Arg–sialic acid association is indeed thermodynamically favorable by ~1 kcal/mol (70). We do note that these simulations were limited to only the CD44 HABD, which is present in all splice variants of CD44, and that the simulations did not include HA. Clearly, additional work needs to be done to understand the molecular mechanisms by which glycosylation alters binding, since it has been shown that N-glycosylation of CD44 can also facilitate HA binding (73).

Conclusion

A subset of the computational experiments above suggests the following four conclusions. The first is that the Tyr42 backbone dihedral angle φ can act as a molecular switch to convert the HABD HA-binding site from the open A state to the closed B state, which includes the formation of direct contact between HA and the Arg41 sidechain (62). The second is that the B state is more thermodynamically stable, and this stability is due to direct

Arg41-HA contact (42). The third is that basic amino acids located distant from the HA-binding site in the ordered form of HABD gain sufficient mobility in the partially disordered form to be able to form direct contacts with oHA and further stabilize binding (42). And the fourth is that terminal sialic acids on covalently attached N-glycans can form charge-paired hydrogen bonding interactions with basic amino acids that could otherwise bind to HA, thereby blocking HA binding to glycosylated CD44 HABD (70). In addition to contributing to the mechanistic understanding of CD44-HA binding, these conclusions may be of utility in the future development of small-molecule modulators of CD44 function (40), especially given the potential for CD44 as a therapeutic target (74–76).

However, it should be kept in mind that the role of Tyr42 as a molecular switch, and the discrete nature of the A and B states of the Arg41-containing loop is contradicted by other computational work (54, 63). Furthermore, there do exist experimental data that are in apparent conflict with the above conclusions. As mentioned previously, N-glycosylation of CD44 can facilitate HA binding (73). And, mutation of positively charged amino acids in the disordered region has been found to enhance HA-binding affinity in the context of both purified HABD and cell surface CD44 (41). Given these differences, further investigation is warranted to achieve a comprehensive consistent view. Finally, while outside the scope of this review, there have been substantial efforts using MD simulations to understand the importance of water molecules and of biomolecular conformational entropy changes in HABD binding with HA (52, 53). Findings from these simulations that can inform development of small-molecule modulators of CD44 function include reduced translational and rotational freedom of water molecules in contact with HABD and HA, and loss of HA flexibility associated with binding to HABD.

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The concept of doing computational experiments to address biological questions is appealing, but the technique used here, namely all-atom explicit-solvent MD simulations, requires significant resources. The most obvious resource is computing capacity, since the computing demands are quite large. It is not uncommon for a set of simulations to utilize the equivalent of hundreds to thousands of personal computers running at full speed around the clock for weeks at a time. In practice, this type of computing power tends to be restricted to nationally funded supercomputing centers (77). A second is the development of software capable of making optimal use of modern supercomputers (78–81). And a third is the development of accurate models (i.e., force fields) for the types of molecules that make up the biological systems under study (44, 64, 82). For example, the development of just the carbohydrate component of the force field used in our studies of CD44 HABD involved a collaborative effort spanning over half a decade (83–91). Fortunately, the technique continues to mature, resulting in an increasingly reliable analytical scientific methodology capable of providing accurate and direct insight into questions that could be addressed only indirectly and with great technical difficulty using other approaches.

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Hyaluronan – a functional and structural sweet spot in the tissue microenvironment

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Transition from homeostatic to reactive matrix remodeling is a fundamental adaptive tissue response to injury, inflammatory disease, fibrosis, and cancer. Alterations in architecture, physical properties, and matrix composition result in changes in biomechanical and biochemical cellular signaling. The dynamics of pericellular and extracellular matrices, including matrix protein, proteoglycan, and glycosaminoglycan modification are continually emerging as essential regulatory mechanisms underlying cellular and tissue function. Nevertheless, the impact of matrix organization on inflammation and immunity in particular and the consequent effects on tissue healing and disease outcome are arguably under-studied aspects of adaptive stress responses. Herein, we review how the predominant glycosaminoglycan hyaluronan (HA) contributes to the structure and function of the tissue microenvironment. Specifically, we examine the evidence of HA degradation and the generation of biologically active smaller HA fragments in pathological settings *in vivo*. We discuss how HA fragments versus nascent HA via alternate receptor-mediated signaling influence inflammatory cell recruitment and differentiation, resident cell activation, as well as tumor growth, survival, and metastasis. Finally, we discuss how HA fragmentation impacts restoration of normal tissue function and pathological outcomes in disease.

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Introduction

In the 80 years that have passed since hyaluronan (HA - also known as hyaluronic acid or hyaluronate) was first isolated and purified from the vitreous humor of the eye (1), the perception of this structurally seemingly simple molecule has changed dramatically. From simple beginnings, and being thought of merely as a “space-filler,” our understanding of its role grew slowly at first, steadily gathered steam and has now entered its exponential phase. HA is now recognized as a molecular powerhouse with critical roles in homeostasis, pathological disease onset, progression, and recovery or decline. This is none more so evident than in the number of review articles of which the biological role of HA has been the focus over the last few years alone [2012–2014 nearly 40 reviews, including an entire edition dedicated to its role in cancer (2)]. It is well established that native HA matrix found in homeostasis plays important biomechanical and biophysical roles as a hydrated cushioning agent and/or molecular filter in connective tissue, joints, and skin (3, 4). Furthermore, increased HA accumulation is a hallmark of almost all diseases in which inflammation and/or fibrosis occur, especially tumor growth and metastasis (2, 4–10). Importantly, HA polymer length (and thus its molecular weight, MW) plays a significant part in the nature of its interactions with the extracellular matrix (ECM), cell surface receptors (including its major receptor, CD44) on both

resident and recruited cells, and influences how cells in tissue respond to extracellular cues under these conditions (4, 10–15). The existing literature clearly pinpoints that the MW characteristics of HA are important determinants of its biological activity, through *in vitro* and *in vivo* studies describing how exogenously added HA of different MW affects cellular signaling and function (4, 8, 11, 12, 16–19). There, however, has been limited work to elucidate the distribution of varying sizes of endogenous HA in the tissue in question, the alterations to HA MW that occur during disease progression and how these HA fragments change the biomechanical and biophysical properties of the tissue *in vivo*. Nor have many of the reports where exogenous HA was added, elucidated how this effected the size distribution of endogenous HA, and over time, its effects on tissue architecture and cellular signaling that translate to either recovery of homeostasis or progression of disease. This is especially important in the context of cancer progression, as the effects of altering HA MW may have varying and opposing effects depending on the origin of the cancer, the tissue in which it resides, and the stage of the disease (20). The recent findings in the naked mole rat that suggest a link between the animals' resistance to cancer and the extraordinarily high MW (HWA) HA in its tissues have brought this subject into the limelight (21). For the above reasons, we have confined this review to focus on (i) a summary of the existing knowledge about HA MW distribution *in vivo* under homeostasis and disease, (ii) mechanisms responsible for alterations in HA MW and the occurrence of these mechanisms in pathological settings, and (iii) the opposing effects of HMW-HA versus HA fragments on ECM function, receptor-mediated cellular signaling and disease outcome.

HA Molecular Weight Distribution in Homeostasis and Disease

HA Molecular Weight – Why Do We Care?

Hyaluronan is a polysaccharide of repeating units of D-glucuronic acid and N-acetyl-glucosamine. This highly charged, hydrophilic molecule is among the largest polysaccharides in nature, and in mammals one of the simplest with regards to structure. It is the major, non-proteinaceous component of the ECM, structurally distinct from other glycosaminoglycans (GAGs) in that it is unmodified (i.e., non-sulfated) and linear [non-branching (22)]. In its most common, homeostatic, and native form, HA polymer chain length exists as a HMW molecule, with sizes commonly above 1000 kDa. In this form, HMW-HA possesses biophysical properties that serve as a lubricant to hydrate tissue and create a matrix that sequesters growth factors and cytokines (23). It is uniquely synthesized at the plasma membrane with the completed polymer extruded to the extracellular space by the hyaluronan synthase enzymes (HASs). Increased HAS synthesis and HA accumulation are hallmarks of many pathological conditions (24). HMW-HA is degraded *in vivo* by hyaluronidases (Hyals), a family of enzymes that hydrolyze HA chains into intermediate (medium MW, MMW) or short (low MW, LMW) fragments (18). Changes in HA synthesis and degradation in part mediate the biochemical and rheological alterations to reactive matrices that occur during disease progression. Under certain pathological conditions,

the extent of HA fragmentation is greatly enhanced, causing significant changes in the distribution and size of biologically active HA products, including the accumulation of HA oligomers [<10 kDa or <20 monomers – oligo-HA (25, 26)]. Collectively, these bioactive HA fragments serve to interact with cells and influence behavior in different ways to HMW-HA (27–31).

HA MW Distribution in Health Versus Disease – It is the Small (HA) Things that Matter

A correlation of increased HA levels in the pathological setting is now par for the course. However, understanding the MW distribution of HA *in vivo*, how it varies between different tissues, and how the ratio of HMW-, MMW-, LMW-, and oligo-HA changes during disease progression is also paramount when developing treatment regimens that target HA. Surprisingly, measurements of HA MW distribution *in vivo* have only occasionally been investigated; these are summarized in Table S1 in Supplementary Material.

Upon review of the literature, it became clear that there was no consensus for what was termed HMW- versus MMW-, LMW- and oligo-HA. To better understand and compare the roles of HA of different sizes under various biological settings going forward, we, for the purpose of this review, categorize the various MW forms of HA as follows; HMW-HA (>1000 kDa), MMW-HA (250–1000 kDa), LMW-HA (10–250 kDa), and oligo-HA (<10 kDa). These groups are by no means distinctly distributed; in many settings, HA MW is polydisperse, encompassing more than one size category. In contrast, specific properties of HA are in certain instances associated with a defined and narrow spectrum of its MW (12).

A total of 65 studies reported analysis of HA MW in an array of tissues including skin, brain, eye, prostate, blood, circulating leukocytes, synovial tissue and fluid, cartilage, amniotic fluid, lymphatics, kidney, aorta, gums, lung and lung fluid, heart, larynx, liver, cervix, skeletal muscle, and urine across a variety of species (see Table S1 in Supplementary Material for references). Nineteen of the studies analyzed HA MW under homeostatic conditions exclusively. Surprisingly, we only found eight studies that analyzed HA in the context of cancer. The remaining studies reported HA size in a number of pathological settings, including cardiovascular disease (atherosclerosis and vascular injury), arthritis (rheumatoid and osteoarthritis), liver disease (septic shock and chronic liver fibrosis), vanishing white matter disease, skeletal ischemia, lung disease (asphyxia, cigarette smoke exposure, asthma, fibrosis, ischemia, and hypertension), skin wounding/healing, kidney disease, development, pregnancy, inflammation, and aging. HA exists in a HMW form under homeostatic conditions in almost all of the tissues where it was analyzed, with subtle yet possibly significant differences depending on the tissue and species (1000–7000 kDa). Notably, increased HA fragmentation was evident under pathological conditions, occurring in both inflammatory and fibrotic diseases. HA MW analysis in lung and skin pathologies had been more extensively analyzed compared to other tissues. A small amount of HA was detected in lungs under homeostatic conditions, found predominantly in the HMW form. Following insult or injury, a dramatic increase in total HA as well as fragmentation yielding LMW-HA species was observed.

Comparatively, under homeostatic conditions, skin contained a greater amount of HA, though still present in a HMW form. Following injury (by wounding or exposure to UVB radiation), HA was detected in either a LMW or MMW form (ranging from 100 to 350 kDa). As wounds healed, HA MW gradually transitioned back to its native HMW form after 28 weeks. Two reports analyzed the effects of aging in the context of wound healing and found decreased Hyal activity and delayed wound repair and restoration of HMW-HA in aged animals (mouse and rat). HA, HAS, and Hyal enzymes have been implicated in a variety of cancers. HA size has been reported in prostate, lung, brain, larynx, liver, colon, and urine (bladder) cancer. Increased HA fragmentation was observed in the prostate (1 of 2 reports), urine, larynx, brain tumor cyst fluid, and colon cancer compared to their normal counterparts, with no observed/reported change in MW distribution in lung or liver cancer. Interestingly, the presence of oligo-HA *in vivo* was only reported in five studies, two of which were under homeostatic conditions in the aorta and urine, one in the interstitial fluid of patients with colon cancer, and the remaining two studies in vascular tissue following injury. Collectively, such limited data, therefore, make it challenging to generalize and suggest how to manipulate HA with the goal of altering disease progression. Furthermore, changing HA MW in the milieu in one specific tissue may then not be translatable to the treatment of carcinogenesis and other pathological settings in different tissues.

Physiologic and Pathophysiologic Mechanisms of HA MW Modification *In vivo*

Is Synthesis Important?

Accurate measurements of HA MW profiles may be in short supply, but there is a substantial body of work focusing on the molecular processes that govern HA MW and the methods by which HA fragments accumulate. Under normal homeostatic conditions HA metabolism is carefully controlled to maintain physiological concentration in tissues. Furthermore, changes in HA synthesis and/or degradation are hallmarks of an ongoing pathological process (32). The three mammalian HAS enzymes (HAS1–3) synthesize and secrete HA polymers of different length; HA secreted into the culture media by stable transflectants revealed that HAS1 makes MMW-to-HMW-HA (200–2000 kDa), whereas HAS2 is responsible for only HMW polymers (>2000 kDa). HAS3 produces HA in the LMW-to-MMW range [100–1000 kDa (33)]. Stimuli such as cytokines [IL-1 β , TNF α (34–36), and IL-15 (37)] and growth factors [TGF- β (36, 38, 39), PDGF (39, 40), HB-EGF (41), and EGF (42)] can regulate HAS expression at the transcriptional level. Furthermore, HAS activity has more recently been shown to be controlled by direct phosphorylation (43, 44), O-GlcNAcylation (45), and ubiquitination (46). The availability of UDP-sugar precursors (constituents of the HA disaccharide subunit) is also a rate-limiting step for HA production (47). HAS2 also has a natural antisense transcript at its gene locus (HAS-AS1) that can stabilize HAS2 mRNA (48).

Collectively, there are, therefore, many cellular mechanisms that can regulate HA levels in tissue at the stage of synthesis. However, there is currently no evidence suggesting that any of these factors, or others, alter the ability of the HAS enzymes to modify

the length of HA polymer they produce. HA MW distribution, and specifically the accumulation of smaller polymers, appears to lie solely in mechanisms specifically controlling its degradation, with HAS enzymes only able to replenish existing extracellular HA reservoirs.

HA Turnover, Catabolism, and the Control of HA Fragmentation

Extracellular HA can exist in a number of distinct pools that may have implications for HA turnover in homeostasis. Furthermore, any changes in the processes involved in the transition of HA from one pool to another could potentially affect its biological roles in the surrounding tissue. First, HA is synthesized as a HMW polymer that is retained as a pericellular coat, via retention by the HAS enzymes or receptor-mediated binding of HA following release of nascent chains from the synthases. Alternatively, HA can be released from the pericellular matrix and incorporated as an integral component of the ECM. Some may also be released as a soluble form into interstitial fluids or the circulation. In each case, HA is subject to subsequent degradation and either internalized and recycled by the resident cells or removed via the lymph. Under homeostatic conditions, HA has a high turnover rate, with as much as one third degraded to LMW fragments and replaced, each day. Lymphatic vessels drain considerable amounts of HA via receptor-facilitated uptake (utilizing receptors such as HARE, LYVE-1, and layilin), after which it is predominantly cleared in the liver. A small proportion is cleared by the kidneys (~10%) and only 1–2% is excreted in the urine (3, 49, 50). In tissues containing high amounts of native HA (skin, cartilage, and joints), a significant amount is degraded locally, by processes involving HYAL-mediated cleavage and receptor-aided internalization, via CD44 and RHAMM (49, 51, 52).

Hyaluronan fragmentation can occur via enzymatic or non-enzymatic processes. Enzymatic cleavage of HA by Hyal involves the hydrolysis of β -1-4 linkages in the HA chain (13). Six genes for Hyals have been identified in the human genome (Hyal-1–4, PH-20, and HYALP1), although only five of these encode protein products (not HYALP1) and of which only four can catabolize HA [not Hyal-3 (49)]. Hyal-1 cleaves HA over a wide MW range, down to oligo-HA fragments of only four or six saccharides in length (53). In contrast, Hyal-2 appears to cleave polydisperse HMW-HA to 20 kDa fragments (54), although the lower limit of HA size that Hyal-2 can digest is still unclear. Both intracellular (by Hyal-1) and extracellular (by Hyal-2) degradations of HMW-HA are CD44-dependent (55). PH-20 degrades polydisperse HA to oligo-HA (including HA disaccharides), but its expression is almost exclusively limited to sperm, where it degrades HA in the cumulus layer of oocytes to facilitate fertilization (56). Reactive oxygen/nitrogen species (ROS/RNS) are also capable of non-enzymatic HA depolymerization and fragmentation. This is a non-selective process, resulting in HA fragments of various lengths (57–60).

Under homeostatic conditions Hyal-1 is expressed in the major parenchymal organs, such as the liver, kidney, spleen, and heart, at low levels in lung, skeletal muscle, and placenta, and is also detectable in plasma and urine. In comparison, Hyal-2 is highly expressed in most tissues. Interestingly, neither isoform has been detected in the brain (13). Hyal-1 exists as a 56 kDa glycoprotein

present in tissues and plasma and as a proteolytically processed 45 kDa form that is only found in plasma (12). Whether these two forms cleave HA to dissimilar fragments or have distinct preferences for HA polymers of different MWs remains to be elucidated. Hyal-1 is active at an acidic pH (53), suggesting that at a cellular level it may reside in lysosomal compartments. Hyal-2 shares many of the characteristics associated with Hyal-1; it has a MW of 55 kDa, also exists as a proteolytically processed smaller form and is optimally active at an acidic pH. In contrast, Hyal-2 contains a glycosylphosphatidylinositol (GPI) linkage, thus tethering it to plasma membrane surfaces. There is a consensus that Hyal-2 may also localize to lysosomes, although there is some conflicting evidence that suggests this may not be the case (12). It is also unclear if membrane-tethered Hyal-2 is involved in releasing cell-associated HA from the pericellular environment in order that it can be integrated into the ECM. Hyal-3 is somewhat of an anomaly; strong transcriptional expression has been detected in bone marrow, testes, and kidney, although no changes in HA accumulation were observed in Hyal-3-deficient mice (61). To date, no activity has been detected *in vivo*, although Hyal-3 may contribute to HA metabolism and fragmentation by altering the activity of Hyal-1 (62).

Evidence for Hyal Expression, Activity, and Hyal- or ROS-Dependent HA Fragmentation During Disease Progression *In Vivo*

Any augmentation to Hyal expression, activity, or receptor-mediated lymphatic drainage has the potential to result in the accumulation of smaller bioactive HA fragments in tissue, and this has proven to be a hallmark of a variety of pathological conditions *in vivo*. Indeed, the genetic disorder mucopolysaccharidosis IX arises due to a mutation of Hyal-1. This mutation attenuates the ability of the enzyme to degrade HA, resulting in increased levels of HA in plasma and elevated storage of mucopolysaccharide in lysosomes (63). Table S1 in Supplementary Material documents changes in HA MW distribution in a variety of biological settings, which in some instances have been correlated with, or arise as a result of alterations in Hyal content. This is certainly true in inflammatory disease; platelet-derived Hyal-2 increases the accumulation of HA fragments that in turn stimulate monocytic IL-6 and IL-8 production and downstream inflammatory responses in the local milieu (64). Furthermore, human CD14⁺ monocytes from normal as well as myelomonocytic lineages from leukemia patients express Hyal on their cell surface, thereby possessing the potential to degrade HA in the circulation as well as upon their recruitment to sites of disease (65). Increased Hyal activity, together with increased levels of LMW-HA has been reported in highly inflammatory atherosclerotic plaques during cardiovascular disease (66). Diabetes also correlated with increased Hyal expression in vascular tissue, with increased HA fragmentation (67). Conversely, increased deposition of MMW–HMW-HA led to severe cardiac dysfunction in Hyal-2-deficient mice (68). A variety of lung disorders have been examined for correlations between Hyal levels and HA fragmentation. Hyal-1 expression is increased in a model of pulmonary hypertension leading to accumulation of HA fragments (69). Airway smooth muscle cells from asthmatic or chronic obstructive pulmonary disease (COPD) patients have a reduction in average HA MW (250 kDa) versus healthy controls

(>700 kDa) that correlates with increased expression of Hyal-1 (70). Furthermore, increased Hyal-2 expression, together with decreased HAS2 expression has been reported in patients with COPD (71). Generation of ROS, combined with increased Hyal-2 activity also increases HA fragmentation (72). ROS-dependent HA fragmentation was also supported in two other separate studies, where exposure to cigarette smoke and subsequent ROS generation reduced pulmonary native HA (>500 kDa) to LMW-HA [70 kDa (73)], whereas pulmonary ischemia was associated with increased accumulation of LMW- and MMW-HA fragments [30–495 kDa (74)]. Interestingly, this latter study showed that HA fragmentation resulted solely from ROS activity, and not via Hyal degradation. In a model of skin injury, UVB irradiation of organotypic epidermal cultures induced Hyal, HAS, and CD44 expression, leading to an accumulation of LMW-HA fragments (75). HA fragmentation has also been recognized as a biological marker for rheumatoid arthritis (76). Indeed, TNF α -stimulated synovial fibroblasts from arthritic mice show increased levels of LMW-HA (77).

Alterations in Hyal expression, activity, and HA fragmentation have also been reported in some oncogenic settings. Increased expression and activity of Hyal-1 and Hyal-2 were observed in a study of patients with colorectal cancer, with the highest activity found in the advanced stages of the disease (78). Overexpression of Hyal-1 also promoted mammary tumor growth and increased tumor angiogenesis (79). Increased Hyal activity together with the accumulation of HA fragments promoted pancreatic tumor cell motility (30), and the tumor cell line H460M (derived from human lung cancer) was also reported to produce high levels of Hyal, although not its own HA (80). Hyal expression has been found at elevated levels in other malignancies, including head and neck (81), prostate (82), brain (83), and urinary tract (84). Further reports suggest that increased Hyal levels might serve as a diagnostic marker for the onset and progression of bladder and epithelial ovarian cancer (58, 85, 86). However, evidence exists that contradict these findings. A study of patients with endometrial cancer indicated that tumor tissues had elevated HA levels and correlated with lower expression of Hyal-1 and Hyal-2 compared to healthy controls (87). Decreased Hyal expression has also been reported in squamous cell head and neck carcinoma (88) as well as lung cancer (89). Furthermore, increased Hyal activity by genetic manipulation or intravenous administration suppressed tumor growth in models of colon and breast carcinoma, respectively (90, 91). In a separate study, ablation of HA in the tumor stroma by intravenous injection of Hyal decreased intratumoral fluid pressure and consequentially increased drug penetration in a model of pancreatic ductal carcinoma (92).

Collectively, current evidence suggests that HA synthesis and degradation are delicately balanced. The likelihood exists that alterations in either HA synthesis or degradation can have profound consequences on the other, which may account for opposing outcomes in disease progression. Indeed there is some evidence that supports this notion. Overexpression of HAS1 in prostate cancer was shown to be anti-tumorigenic; however, overexpression of both HAS1 and Hyal-1 increased HA fragmentation that in turn promoted tumor cell proliferation and metastasis (93). Furthermore, Hyal-1 expression in cancer cells themselves functioned as both a tumor promoter and tumor suppressor in

prostate carcinoma (82). In a separate study in breast cancer, expression of antisense HAS2 (ASHAS2) increased accumulation of HMW-HA, while simultaneously causing the downregulation of Hyal-2. Combined, this inhibited the initiation and progression of primary and metastatic tumor progression (94).

The Opposing Effects of Native HA versus HA Fragments on ECM Function, Receptor-Mediated Signaling, and Disease Outcome

HMW-HA - Keeping Tissues in Check?

As discussed, HA in its native state is found in a HMW form (>1000 kDa) that influences normal homeostatic functions in a variety of ways. HMW-HA has the ability to trap large amounts of water, thus possessing biophysical properties that serve to lubricate, hydrate, or space-fill tissues such as joints and connective tissue (13, 95). Its hydrophilic attributes also allow it to act as a molecular sieve and affect fluid absorption rates to and from tissue through changes in its concentration (96). HMW-HA possesses biomechanical properties, and none more so is this evident than during development. The maturing embryo is surrounded by a soft, hydrated matrix, rich in HMW-HA. Soft matrices are commonly considered to inhibit cellular adhesion and proliferation. Uniquely, the HA-rich microenvironment during development facilitates growth and development of tissues, including neuronal growth (97), limbs (98), blood vessels, and the heart (99). The presence of HMW-HA is critical to normal embryogenesis, as targeted deletion of HAS2 results in embryonic lethality, due to abnormal heart development (99). These distinct biomechanical properties of HA in soft tissue have recently been confirmed *in vitro*, where HA-rich soft substrates, as opposed to collagen-rich substrates of the same degree of stiffness but lacking HA, promoted cell spreading, focal adhesion and stress-fiber formation, and normal cell function (100). This might explain how embryonic cells are able to adhere, proliferate, and differentiate under soft conditions during development. It is interesting to speculate whether one may extend this hypothesis to wound healing; one of the first events in the formation of granulation tissue (for example in skin wounds) is an accumulation of HA in a soft fibrin matrix. The HA may serve to facilitate cell migration in the early stages of wound healing, prior to the onset of fibroproliferation, and the generation of a stiffer substratum as a result of increased matrix deposition. A HA-rich fibrin clot may enable swift tissue remodeling and healing in the face of initially soft conditions.

High molecular weight-HA-specific signaling has been shown to result in favorable outcome on cell and tissue function in adult tissues in response to environmental cues. In *in vitro* wound healing models, the incorporation of HMW-HA into collagen gels enhanced gel contraction, vascular smooth muscle cell (VSMC) cell spreading, filopodia formation, and pericellular accumulation of collagen fibers via the HA receptor CD44 (101). HMW-HA also promoted actin stress-fiber arrangement, lamellipodia formation, and cell migration (but not proliferation) in VSMCs (102). Inhibition of CD44 blocked HA-CD44-RhoA-mediated events, with the exception of migration, whereas inhibition of RHAMM (another HA receptor) and downstream Rac signaling only inhibited

HA-mediated migration (102). HMW-HA also enhanced myocardial repair when transplanted simultaneously with bone marrow mononuclear cells in the heart following myocardial infarction. The HMW-HA provided a favorable microenvironment for transplanted cell adhesion and proliferation, leading to reduced inflammation and cardiomyocyte apoptosis, as well as increased angiogenesis and cardiac performance (103). HMW-HA also increased CD44- and NF- κ B-dependent SNAI2 expression leading to increased fibroblast invasion (104). Indeed, the role of HMW-HA in response to injury has been studied quite extensively. In the skin, HMW-HA improved permeability barrier function in aged epidermis via CD44-dependent mechanisms (105). This may in part be attributed to HMW-HA-CD44-dependent mechanisms that control keratinocyte differentiation (106). HMW-HA also enhanced excisional wound contraction compared with saline-treated controls (107). In a diabetic wound-healing model, this was associated with enhanced angiogenesis, TGF- β , and transglutaminase II expression, restoration of cyclin B1/Cdc2 complex and increased mechanical strength (108). Independently, HMW-HA was shown to mitigate astrocyte activation *in vitro* and *in vivo* leading to a reduction in scarring (109). Furthermore, daily subcutaneous administration of a HMW-HA formulation (HYAL-BV 5200) inhibited neointimal formation and macrophage recruitment following balloon catheter-induced vascular injury in cholesterol-fed rabbits (110). Our group reported that the tissue response to vascular injury was CD44-dependent. HMW-HA inhibited mesenchymal cell cyclin D1 expression and subsequent cell proliferation via a CD44-dependent and Skp2-dependent mechanism (111). HMW-HA may also control the response to injury by reducing VSMC apoptosis mediated via TLR4, CD44, and downstream PI3K signaling (112).

Many of the protective/healing effects associated with HMW-HA in response to injury can be attributed to its suppressive effects on the inflammatory response. HMW-HA (1600 kDa) completely blocked monocyte and neutrophil infiltration and MIP-2 and TNF α induction in a model of sepsis-induced lung injury, thus attenuating the injury response (113). T-cell-mediated liver injury, as well as the release of pro-inflammatory cytokines TNF α , IFN γ , MIP2, and IL-4, was also inhibited by administration of HMW-HA (114). HMW-HA increased SDF1b-induced CXCR4 signaling and cell motility, increased vessel sprouting, and angiogenesis. This process was again HA-CD44 dependent, with CD44 physically interacting with CXCR4 in the presence of the CXCL12 ligand (115).

High molecular weight-HA is also able to enhance immunosuppression via binding to the surface receptor TLR4 leading to an increase in the release of the immunosuppressive cytokine IL-10 (116). Interestingly, this immunosuppressive effect has recently been suggested as a mechanism in infectious disease; HMW-HA impaired virus phagocytosis by macrophages and thus increased viral survival within the blood (117).

HMW-HA and Cancer Progression

The protective effect of HMW-HA is also evident in tumorigenesis. Treatment with HMW-HA inhibited post-chemotherapy tumor growth in a human colon carcinoma xenograft model in NSG mice (118). HMW-HA antagonized the pro-inflammatory

effects of IL-1 β -treated chondrosarcoma cells, decreasing COX2, MMP-1, and MMP-13; promoting Akt; and suppressing MAPKs and NF- κ B signaling, via PPAR γ -dependent signaling (119). HMW-HA also inhibited migration of fibrosarcoma cells. Interestingly, this effect was fibrosarcoma cell line-specific and did not occur in any of the other cancer cell lines that were tested (120). Conversely, overexpression of HAS2 (presumably leading to increased accumulation of HMW-HA) facilitated the reversion of cancer cells to a stem cell-like state via Twist and TGF- β signaling and thus promoted tumor cell survival (121). It is, however, unclear if this survival was due directly to HMW-HA signaling, or due to further degradation of the accumulated HMW-HA and pro-oncogenic signaling as a result of an accumulation of HA fragments.

Oligo-HA - the Gloves are Off

Whereas evidence points to HMW-HA as protective and facilitating in the restoration of homeostasis in pathological settings, the effects of oligo-HA could not be more further removed. Many of the effects exerted by oligo-HA in pathological conditions occur via receptor-mediated signaling in immune cells leading to the promotion or protraction of inflammation. Oligo-HA induces the phenotypic maturation of human monocyte-derived dendritic cells (DCs) and the production of inflammatory cytokines IL-1 β , TNF α , and IL-12. Interestingly this was not mediated via the HA receptors CD44 or RHAMM and was partly mediated indirectly via TNFR (122). Subsequently, DC maturation by oligo-HA was found to be mediated by its binding to TLR4 (and downstream p38/p42/44 MAP-kinase pathways). This was confirmed *in vivo*, as oligo-HA induced DC emigration from skin, as well as their phenotypic and functional maturation in the spleen (123). Oligo-HA administration to resting monocytes increases the expression of the scavenger receptor (CD36), uptake of oxidized LDL and their transendothelial migration. This particular response was CD44-dependent and mediated in part via the PKC pathway (124). Together, these data suggest that CD44 is directly implicated in prolonged inflammatory responses in many auto-inflammatory conditions such as atherosclerosis. Furthermore, it suggests that CD44 may promote the conversion of macrophages to foam cells within lesions, leading to increased lesional lipid accumulation and immune cell content, conditions that favor lesion rupture, a risk factor for heart attack and stroke.

Oligo-HA receptor-mediated cell responses are not limited to the immune system. Endothelial cells (ECs) in particular are impacted by oligo-HA. Oligomers of 6, 8, and 10 disaccharides (but not 4 subunits) promoted EC proliferation and VEGF secretion (125). Increased EC proliferation in response to oligo-HA also increased tube formation, upregulation of the adhesion proteins ICAM and VCAM as well as the release of pro-inflammatory cytokines (126). *In vivo*, oligo-HA has been reported as one of the predominant mechanisms by which ECs respond to injury, with these responses mediated via oligo-HA-TLR4-dependent mechanisms (127). However, ECs also express ICAM and CD44, both of which can bind oligo-HA and potentially mediate cellular function (128, 129). Indeed, oligo-HA was shown to induce rapid upregulation of immediate-early genes c-fos, c-jun, jun-B Krox-20, and Krox-24, responsible for angiogenesis, in a

CD44-dependent manner. Additionally, immediate-early gene signaling was not sufficient to induce EC proliferation and was only induced upon long-term treatment with oligo-HA (129). Oligo-HA is also capable of inducing pro-inflammatory signals in chondrocytes. IL-1 β treatment induced inflammatory signaling pathways that were mediated via oligo-HA-CD44 activation, leading to an increase in NF- κ B, TNF α , IL-6, MMP-13, and iNOS, as well as the CD44 receptor itself (130).

A certain degree of inflammation and, therefore, the generation of some oligo-HA is a normal part of the body's response to insult. That being said, excess oligo-HA has often been shown to be detrimental to healing, causing protracted inflammation thus favoring disease progression. In contrast, the effects of oligo-HA on resident mesenchymal cells suggest that it may facilitate tissue recovery and healing. Sustained delivery of oligo-HA by nano-particles increased elastin synthesis and lysyl oxidase expression in rat SMCs to facilitate aortic remodeling following injury (131). Topical application of oligo-HA promoted keratinocyte proliferation and increased skin thickness and barrier function, in a CD44-dependent manner (105). Wound healing models have also revealed that administration of oligo-HA accelerates wound healing by promoting wound closure, the accumulation of M1 and M2 macrophages, release of TGF- β (132), enhanced angiogenesis, lymphogenesis, and ECM deposition (133). Oligo-HA-accelerated wound closure was both CD44- and RHAMM-dependent. Interestingly, although fibroblast proliferation was increased, myofibroblast differentiation within the granulation tissue did not change (132). Protective roles for oligo-HA have also been reported in cardiovascular disease. Together with TGF- β , oligo-HA, but not larger HA polymers (20, 200, or 2000 kDa), cooperatively enhanced elastin matrix regeneration in VSMCs (134), whereas oligo-HA administration protected against neointima formation in the aorta following balloon catheter injury *in vivo* (135). Oligo-HA can also upregulate hsp72 expression by enhancing the activation of HSF1 in response to hyperthermia in synovial cells, which acts as a protective mechanism by suppressing cell death (136). A separate recent study suggests a separate biochemical mechanism by which oligo-HA may facilitate healing. The covalent transfer of heavy chains (HCs) from inter- α -inhibitor ($I\alpha I$) to HMW-HA via the protein product of tumor necrosis factor-stimulated gene-6 (TSG-6) forms a HC-HMW-HA complex, a pathological form of HA that promotes the adhesion and retention of leukocytes to HA matrices (137). The transfer of HCs to HMW-HA is a reversible event mediated by TSG-6, whereas HC transfer to oligo-HA is irreversible. Treatment of HA-HC-rich synovial fluid from arthritic mice with oligo-HA and TSG-6 irreversibly shuttled HCs from pathological, HMW HC-HA to the oligo-HA. This suggests that oligo-HA could thereby facilitate the restoration of HA matrices in the inflamed joint to its normal, unmodified state, by removing HCs from HMW-HA, through more efficient clearance of HCs from tissue (138).

Oligo-HA and Cancer Progression

The current literature is divided upon whether oligo-HA (as well as LMW-MMW-HA as discussed later) promotes or suppresses tumor growth and metastasis. Complexities arise due to

the wide and more varied effects that oligo-HA appears to have on tumors, compared to the effects it has on non-transformed cell types in other pathological conditions. This no doubt stems from the simple fact that tumors can develop in almost all tissues, from different cellular origins, and as a result of different mutations/environmental cues, to the point where you are in effect, looking at the role of one molecule (oligo-HA) on an ever increasing number of functionally and pathologically distinct, tumors. A number of these opposing effects are discussed below.

Oligo-HA induces CXCR7 expression in a TLR4-dependent manner, leading to the proliferation of W3 papillary thyroid carcinoma cells *in vivo* (28). In another independent study, oligo-HA preferentially stimulated a physical association between CD44 with TLR2, TLR4 and the recruitment of MyD88 and actin filament-associated protein 110 (AFAP-110), leading to NF- κ B translocation and downstream expression of IL-1 β and IL-8 in MDA-MB231 breast cancer cells. Combined, this promoted tumor cell invasion (139). Oligo-HA also upregulated the expression and acute phosphorylation of c-met, leading to proliferation, differentiation, and invasion of human chondrosarcoma cells. This effect was dependent on oligo-HA-CD44 interaction and signaling, and not observed with any other HA MW size (29). Similarly, oligo-HA, but not LMW- or HMW-HA, induced MMP-9, -13, and uPAR in Lewis lung carcinoma (LLC) tumor cells, thus facilitating matrix remodeling and tumor cell migration. Interestingly, this response was not dependent on the interaction of HA with CD44, RHAMM, or TLR4 (140). More complex roles of oligo-HA with CD44 have also been reported in pancreatic carcinoma. Oligo-HA, generated by Hyal degradation, enhanced the cleavage of CD44 and its release into the ECM, which in turn enhanced tumor cell motility. This phenomenon was abrogated upon inhibition of HA-CD44 binding. This suggests that tumor cells enhance their own CD44 cleavage via Hyal activity and oligo-HA generation to promote their own motility, tumor invasion, and metastasis (30). In contrast, oligo-HA has the ability to kill many types of tumor cells by triggering apoptosis, while leaving normal cells unaffected. In an interesting extension of these findings, chemo-resistant tumor cells became drug-sensitive when treated in combination with oligo-HA (141). Indeed, this may be one mechanism by which oligo-HA inhibits the growth of B16F10 melanoma growth *in vivo* (142). This phenomenon has been observed in other tumor models and appears to be a response that is dependent on oligo-HA and CD44 interaction. Oligo-HA, competitively blocked binding of endogenous HMW-HA to CD44, consequently attenuating downstream signaling to the PI3K/Akt cell survival pathway, leading to inhibited tumor growth and apoptosis (143, 144). Furthermore, oligo-HA suppressed glioma growth *in vivo*, in part through inhibiting recruitment of progenitor BRCP+ stem cells (145). There is also evidence that oligo-HA abrogated cell-associated matrices and HA retention via CD44 in osteosarcoma, resulting in apoptosis and importantly, suppression of the formation of lung metastases (31).

MMW-HA and LMW-HA - Caught in the Middle?

Low molecular weight-HA and MMW-HA fragments are frequently detected as polydisperse fractions with overlapping MW distributions. They could be considered intermediate fractions, and arise due to partial fragmentation due to varying

concentrations of Hyal, ROS/RNS, as well as the availability of cleavage sites depending on HA interactions with its receptors and other ECM proteins. There is also the possibility that these fractions contain nascent HA that has not yet reached its full length. Taking this into account, MMW-HA and LMW-HA have roles that overlap with either HMW-HA or oligo-HA.

Low molecular weight-MMW-HA has been reported to facilitate the differentiation of many mesenchymal cells that are activated as a normal response following injury, including chondrocytes (146), fibroblasts [together with their expression of growth factors FGF-2 and KGF (147)], keratinocytes (148), and VSMCs. VSMC differentiation was associated with increased collagen deposition (149). LMW-HA improved dermal excisional wound repair, associated with increased expression of CD44 and RHAMM and deposition of type-III collagen in aged mice (150). A separate study also showed improved age-related skin function, when HA was administered to patients with skin atrophy in a CD44-dependent manner (151). Topical administration of LMW-HA also acts as a scavenging agent following xenobiotic treatment (and ROS generation), promoting wound healing in excisional and incisional wound models (152). In the lung, LMW-HA protected against porcine pancreatic elastase-induced bronchoconstriction (153). Its protective effect against elastase was confirmed in a second model where aerosolized LMW-HA blocked experimental emphysema induced by intra-tracheal administration of elastase (154). Conversely, administration of LMW-HA exacerbated ozone-induced airway hyper-reactivity in a CD44-dependent manner, also in the lung, whereas treatment with HMW-HA protected against ozone injury (155). LMW-HA, via TLR4-mediated receptor binding, induced neutrophil apoptosis via an IFN β , TRAIL/TRAILR-dependent mechanism, thus protecting against prolonged inflammation following injury (156). LMW-HA has also been reported to induce apoptosis of myeloid cells via CD44-dependent, tyrosine kinase signaling (157). Its protective effects have additionally been reported in the liver and intestine, by preventing hepatocellular apoptosis via NF- κ B (158) and the expression of murine β -defensin 3 (an ortholog of human β -defensin 2) via TLR4, respectively (159).

Low molecular weight-HA and MMW-HA promote inflammation through direct and indirect signaling mechanisms. Directly, polydisperse LMW-MMW-HA increases inflammatory gene expression and decreases anti-inflammatory signaling in macrophages by downregulating surface expression of A2aR, via CD44 and PKC (160). LMW-MMW-HA binding to HARE has also been shown to activate pro-inflammatory NF- κ B signaling (161). Conversely, LMW-MMW-HA activates the innate immune response via TLR2 and MyD88 (162). LMW-HA can mobilize leukocytes but not hematopoietic progenitor cells to the circulation (163) and increase NO production in primary macrophages (164). Importantly, the activation of elicited, versus resident peritoneal macrophages by LMW-HA have distinct requirements. Both cell types produce pro-inflammatory cytokines (including IL-12) in response to LMW-HA via LMW-HA-CD44 signaling; however, resident macrophages require adhesion-dependent priming to respond to LMW-HA (165). Other pro-inflammatory cytokines released following LMW-HA stimulation include MIP1- α , MCP-1, RANTES, and Crg-2 (166). LMW-MMW-HA is also a potent stimulator of eicosanoids

(including induction of COX2 and PGE2 production via ERK1/2 p38 and JNK signaling) in primary human monocytes and murine wild-type bone marrow-derived monocytes. This activation was also dependent on a HA-TLR4/MyD88 pathway (167). LMW-HA can also influence macrophage polarity. M0 (undifferentiated) and M2 (pro-fibrotic) macrophages can be switched to an M1 (pro-inflammatory) phenotype after a short period of stimulation with LMW-HA (167). However, prolonged exposure to LMW-HA can induce the M2 phenotype (168).

Indirectly, LMW-MMW-HA can stimulate the production of pro-inflammatory stimuli from other cell types. Lung epithelial cells, chondrocytes, liver ECs, and VSMCs release a number of pro-inflammatory cytokines in response to LMW-HA via its binding to CD44 or TLR4, including TNF α , IL-1 β , MMP-13, and iNOS (169–174). In VSMCs, LMW-HA also stimulated cell proliferation and migration via CD44 through ERK1/2 and RhoA signaling (173, 175). One study also found that TLR4 interacts with CD44 in response to LMW-HA and together act as a brake in LMW-HA-induced lung inflammation (176). *In vivo*, LMW-HA promoted splenocyte proliferation, macrophage activation, while suppressing angiogenesis in chicken embryos (177). LMW-HA is critical in the induced fetal growth response to uterine ischemia/reperfusion via TLR4 (178). LMW-HA also decreases the rate of early wound contraction in skin (107), but increased total number of recruited macrophages in the granulation tissue (132).

LMW-MMW-HA and Cancer Progression

In the tumor microenvironment (TME), LMW-MMW-HA has the potential to influence cancer cells, stromal cells, ECs, and infiltrating inflammatory cells. Specifically, LMW-HA facilitates tumor cell adhesion and migration. In fibrosarcoma, this was shown to be dependent on LMW-HA-RHAMM binding, which influenced downstream FAK and ERK1/2 signaling (179). LMW-HA enhanced proliferation (through MAPK and c-fos signaling) and adhesion of LM8 murine osteosarcoma cells with increased MMP-2 secretion. Adhesion in these cells was shown to be dependent on CD44 (180). In fact, LMW-HA-CD44-dependent signaling has been reported in a number of carcinoma cells to activate NF- κ B signaling via a Ras-PKC ζ -I κ B cascade (181), and thus may be one of the many ways in which LMW-HA promotes tumor progression by the activation of cell proliferation. However, much like what has been found for oligo-HA in the TME, LMW-MMW-HA can also inhibit growth of some tumors. MMW-HA stimulated iNOS and subsequent NO production and apoptosis in DCs *in vivo* in glioma. This process was dependent on HA-CD44 interaction and suggests that HA in gliomas may contribute to immunosuppression by promoting apoptosis of infiltrating immune cells (182). In contrasting findings to those above, LMW-HA inhibited colorectal carcinoma growth *in vivo*, by inhibiting tumor cell proliferation via Akt signaling (183). The TME was also affected; LMW-HA induced immunity against the carcinoma by stimulating DC migration, proliferation, and the release of IL-12 and IFN γ , while simultaneously decreasing their release of immunosuppressive IL-10. Interestingly, these responses occurred in a CD44 and TLR4-independent manner (184).

CD44 - An Important Regulator of HA-Mediated Signaling in Cancer?

Among the receptors involved in HA signaling, CD44 is the most abundant, expressed in almost all tissues and across nearly every cell type. Additionally, this type I transmembrane protein is able to bind almost all HA MW species, with the exception of oligo-HA fragments smaller than six saccharides in length (16). Combined, these two contributing factors can arguably account for the variety of different functions of HA-CD44 interactions *in vivo*, any of which could have important outcomes on tumorigenesis.

As well as being bound to the plasma membrane, CD44 can exist as a cleaved, matrix-associated fragment and as a soluble protein and can independently affect cellular function (185, 186). CD44 also exists as a number of splice variants, which are commonly expressed in tumor cells (6). These variants encode additional segments in the membrane proximal region of the extracellular domain that can be differentially glycosylated (187, 188). The CD44 cytoplasmic domain is known to be required for HA binding, the formation and retention of pericellular matrix, and CD44-mediated endocytosis of HA (189, 190). Furthermore, cell adhesion via HA binding can be regulated in part by variable glycosylation of its CD44 extracellular domain, as increased glycosylation inhibits HA recognition (191). These post-translational modifications (N and O glycosylation) of CD44 also affect its ability to signal and shed from the cell surface; however, CD44 cleavage can also occur via a glycosylation-independent mechanism via MMP cleavage (192). Interestingly, murine M0 macrophages when stimulated to induce pro-inflammatory M1 polarization (with LPS/IFN γ), upregulate their CD44 expression and ability to bind HA. Conversely, M0 macrophages polarized to the anti-inflammatory M2 phenotype (with IL-4) also upregulate CD44 expression but with no increase in HA binding. This difference was a consequence of the loss of chondroitin sulfation on CD44 in M1s and conversely an upregulation of chondroitin sulfation on CD44 in M2s (193). It is as-yet unclear if this dynamic physiological regulation of hyaluronan binding also influences the phenotypic differences between the two cell types and the inflammatory state of the TME. Nevertheless, the ability to alter macrophage polarization via CD44 offers a potentially new mechanism to target the inflammatory response *in vivo*, in the context of tumor progression as well as other inflammatory conditions, such as wound healing, lung injury, and atherosclerosis.

CD44 knockout mice are viable and fertile with a modest phenotype; progenitor cell egress from the bone marrow is slightly impaired (194). However, under pathological conditions, deletion of CD44 has profound effects on tissue architecture, signaling, and disease outcome. In a model of non-infectious lung injury, CD44-null mice succumb to unremitting inflammation with impaired clearance of neutrophils, persistent accumulation of LMW-HA, and impaired activation of TGF- β . This phenotype was partially restored by reconstitution with CD44 $^+$ bone marrow-derived cells (195). In contrast, we previously reported that CD44 promotes auto-inflammatory disease progression in a mouse model of atherosclerosis. CD44 expression correlated with increased lesional macrophage and HA content and VSMC activation (196). Furthermore, CD44 on both bone marrow-derived and non-bone marrow-derived cells was important; CD44 on leukocytes

in part promoted the disease via enhancing macrophage and T-cell recruitment to lesions *in vivo*. Leukocyte to EC adhesion and transmigration was also CD44-dependent as was macrophage activation. CD44 on VSMCs also promoted their migration and proliferation (197). Interestingly, we also reported that CD44 is selectively upregulated in athero-prone regions, and CD44 signaling impacts gene expression profiles in the vasculature, including those genes involved with focal adhesion formation, ECM deposition, inflammation, and angiogenesis (198).

CD44 is known to facilitate the rolling and adhesion of circulating leukocytes on the endothelium, and subsequent transendothelial migration (14, 199–201). Targeted inhibition of HA–CD44 binding using a synthetic peptide inhibits leukocyte adhesion and trafficking *in vivo* (202). HMW-HA–CD44 binding in calveolin-enriched microdomains (CEMs) in ECs also promotes barrier function, via the recruitment of c-met, Tiam1, Rac1, dynamin 2, and cortactin to CEMs and their redistribution to areas of cell–cell contact (203, 204). Any alterations to HA–CD44 interaction could, therefore, potentially alter the recruitment of immune cells or the intravasation of tumor cells to the blood stream or extravasation to tissue during metastasis. Administration of LMW-HA suppresses A2aR (a negative regulator of inflammation) via CD44 binding and downstream PKC signaling following lung injury *in vivo* (160). The HA receptor TLR4 has been shown to interact directly with CD44 in order to limit LMW-HA-induced lung inflammation *in vivo* (176). Antisense CD44 inhibited HA binding, tumor growth, and metastasis of colorectal carcinoma cells to the liver (205). Furthermore, peptide inhibition of CD44–HA binding significantly reduced seeding and tumor growth of intravenously introduced B16-F10 melanoma cells in lungs in a model of metastasis (206). Indeed, HA–CD44 interaction has been implicated in the growth of a number of cancers (6). The growth-inhibitory and tumor-suppressive effects of p53 act in part via its ability to bind to a non-canonical sequence in the CD44 promoter, thus inhibiting CD44 expression and downstream tumor-promoting signaling in breast cancer cells (207). Furthermore, a separate study found that human miRNAs miR-373, and miR-520c suppressed CD44 expression, leading to the promotion of breast tumor cell migration and invasion *in vitro* and *in vivo* (208). The standard form of CD44 (CD44s) and CD44v6 are involved in breast cancer cell adhesion and motility via interactions with HA (209). This increased cell motility perhaps occurs as a result of modulation of CD44 into clusters by HMW-HA on the plasma membrane (210). CD44 clusters facilitate cell binding and internalization of HA thus enabling invasion from tumor masses into the surrounding ECM (211). Observation of neuroblastoma cells revealed that these CD44 clusters localized to filopodia and focal bleb-like protrusions in neuroblastoma cells that enabled migration and invasive growth into brain tissue (212). In another study, chondroitin sulfate E fragments enhanced CD44 cleavage and tumor cell motility upon degradation. Much like LMW-HA, these degradation products modulated tumor cell adhesion and migration by binding to CD44 (213). In a separate finding, the non-coding 3'UTR of CD44 was found to act as a binding site for miRNAs that targeted the genes for Col1α1 and fibronectin. Overexpression of the CD44 3'UTR in MDA-MB231 cells antagonized the effects of the

miRNAs on their specific targets and upregulated collagen and fibronectin expression that in turn enhanced tumor cell migration and metastasis *in vivo* (214).

Stromal expression of ECM proteins and GAGs is increased in activated fibroblasts in the TME and is thought to promote tumor cell migration. Thus, targeting CD44 signaling in stromal cells in the TME may also provide a separate avenue to target tumorigenesis. Indeed, CD44 has been implicated in stromal cell function in a number of pathological settings. CD44 facilitates the healing response, by promoting fibroblast infiltration, proliferation, myofibroblast differentiation, and ECM deposition and remodeling following myocardial infarction (215, 216). Fibroblast migration is mediated by CD44-dependent TGF-β activation that promotes stress-fiber formation and directional migration (217). The HA receptor RHAMM may also facilitate cell migration via the regulation of CD44–Erk1/2 complexes at the cell surface (218). Cell proliferation via CD44 is dependent upon the MW of the HA ligand. In VSMCs, HMW-HA binding to CD44 selectively inhibits the GTP loading of Rac and Rac-dependent signaling to cyclin D1 (thereby inhibiting proliferation), whereas LMW-HA binding to CD44 selectively stimulates ERK activation and ERK-dependent cyclin D1 expression [thus promoting proliferation (175)]. Interestingly, HA binding to CD44 increases as a function of HA size. Half maximal saturation is reached with a HA MW of only 30 kDa. Reversible binding was confined to oligo-HA fragments (<10 kDa), with interactions essentially irreversible with large polymers [>30 kDa (219)]. The accumulation of oligo-HA in tumors, combined with increased CD44 expression, may, therefore, be a mechanism to activate alternate, pro-tumorigenic signaling pathways as a means to bypass and eventually overcome the non-reversible protective signals stemming from HMW-HA–CD44 binding.

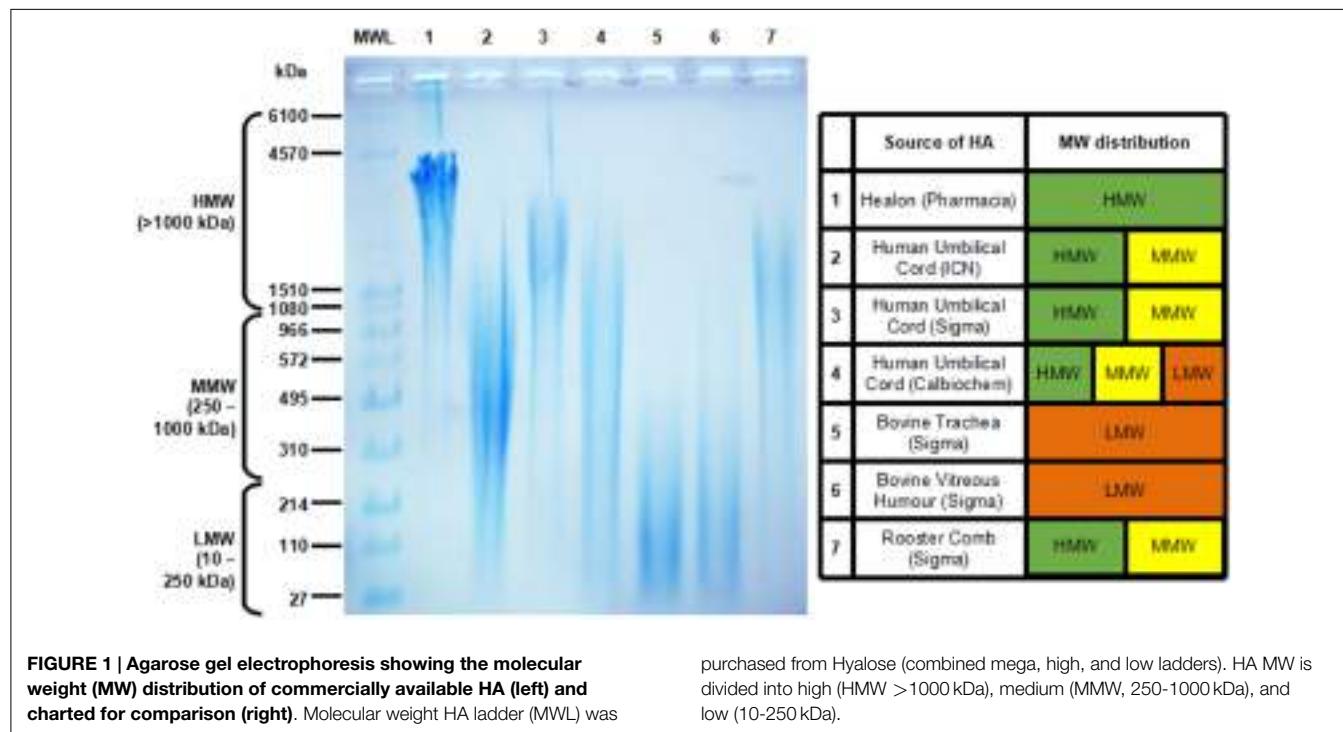
Conclusions, Caveats, and Perspectives

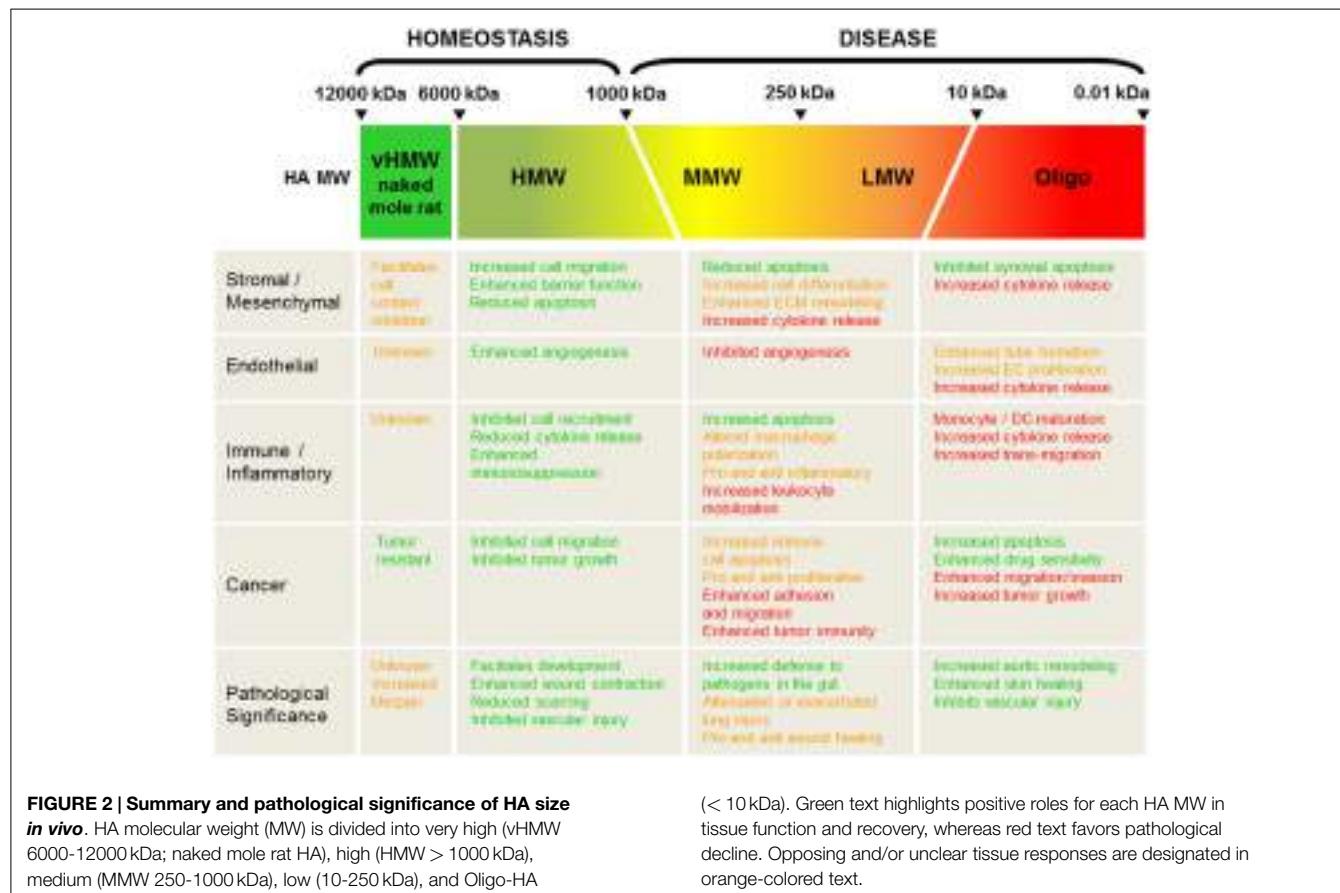
In revisiting the literature, we found that little was known about how HA MW distribution changes *in vivo* during disease, especially when compared with the number of studies that reported changes in HA content in pathological settings. Much of what is known about HA MW in disease *in vivo* has been extrapolated from *in vitro* cell culture experiments and only a handful of early articles where it was analyzed *in vivo*. Furthermore, the limited number of studies, which we found that reported the occurrence of oligo-HA in tissue (five in total) question the pathophysiologic relevance of oligo-HA, and its effects on cell function *in vivo*. Many studies have tested the biological activity of HA (from oligo-HA to HMW-HA) as an exogenously added ligand to cells in culture (with some extending these experiments *in vivo*), and its effects on cellular signaling, gene/protein expression, and cell behavior; some revisited how it changed endogenous HA, including its localization, matrix organization, and cross-linking and turnover, but very few specifically analyzed its effects on endogenous MW distribution. One could argue that HA MW analysis is not a straightforward technology. There are no commercial kits that allow HA MW profiles to be analyzed in a high throughput fashion. Additionally, care has to be taken to first extract HA from its

link proteins, HCs, and other ECM molecules with which it forms higher ordered structures, while simultaneously taking measures to prevent loss of the smaller oligo-HA fragments during purification. The commercially available kits for measuring HA concentration (ELISA and ELISA-like assays) also vary, with some unable to detect HA polymers accurately at the extremes of the MW spectrum (220). On the other hand, thanks to a number of dedicated groups, a variety of reliable methodologies for HA MW analysis do exist, using combinations of size-exclusion chromatography (221), flat-bed polyacrylamide electrophoresis (222, 223), agarose gel electrophoresis (223–227), gas phase electrophoretic mobility molecular analysis [GEMMA (228)], or asymmetrical flow field fractionation with multi-angle light scattering (229, 230), for continuous HA size profiling. Consequently, we anticipate the increased use of these robust methodologies to monitor HA size in various pathological settings *in vivo* in future studies.

High molecular weight HA and oligo-HA exist as distinct pools of HA with unique biological properties at the opposite ends of the HA MW spectrum. On the other hand, LMW-HA and MMW-HA are frequently detected as polydisperse fractions that often overlap. When searching for articles related to LMW-HA and MMW-HA in the literature, we found that the MW range was not always reported, instead being replaced with the average MW for the sample. This was found to be very common in earlier studies, and where LMW-HA or MMW-HA fractions were added exogenously as extracellular cues to examine their effects on cell function. These LMW and MMW fractions were purchased from various companies, with the HA sourced from various tissues from different species. Those most commonly used included umbilical cord (human), rooster comb, trachea and vitreous humor (bovine), and a synthetic polymer of HMW-HA produced by Pharmacia (known as Healon). The polydisperse MW ranges of

HA for these preparations are given in **Figure 1**. Umbilical cord HA was found to be commonly used in the literature as LMW-HA. We compared umbilical cord HA fractions from three separate vendors. Remarkably, the MW distribution of umbilical cord HA was hugely polydisperse and varied depending on the vendor, ranging from 30–1000 kDa for HA from ICN (MP Biomedicals) to 900–3500 kDa from Sigma, and 200–3000 kDa when obtained from Calbiochem. Two of the sources of umbilical cord HA (Sigma and Calbiochem) contained a significant proportion of HMW (>1000 kDa) HA. This high degree of variation in HA MW may account for some of the opposing data we found regarding the function of LMW-HA in the literature where umbilical cord HA had been used. We were only able to obtain single vendor samples for tracheal, vitreous humor, and rooster comb HA. The MW distribution of HA in these fractions in comparison were much narrower, with tracheal and vitreous humor HA in the 30–300 kDa range (LMW) and rooster comb at 600–3000 kDa (MMW-HMW). The synthetic, Healon HA could be considered very high, at 3500–4500 kDa. In order to better understand and compare the roles of HMW-HA versus HA fragments in future studies, non-overlapping HA MW samples with reduced polydispersity for each sized group will help define the often contrasting roles of HA on cell function. To this end, it appears that using umbilical cord HA may not be best suited for this purpose. In any case, HA from this source does not appear to be any longer available, nor is HA from bovine trachea. This leaves Healon, rooster comb HA, and vitreous humor HA as three sources, with non-overlapping MW profiles that can be confidently used to investigate the unique individual roles of different HA MWs on tissue function and disease outcome in future studies. This would be along with the use of LMW-HA, oligo-HA, and narrow-range HA preparations manufactured using unique bacterial fermentation technologies





(spearheaded by Hyalose and now offered by other vendors) that are now readily available (231–233).

Depending on the pathological setting, HA fragmentation may be good or bad, pro- or anti-inflammatory, aid in tissue recovery or promote disease progression, as summarized in **Figure 2**. The complexity in part arises due to the number of different signaling mechanisms that result from HA itself acting as a ligand; it can mediate alternate and often opposing effects via different, yet often the same receptors. Furthermore, HA in tissue is often found as a polydisperse molecule, often covering a MW range from 20 to 2000 kDa with no one, specific length of HA polymer dominating during disease. It is, therefore, interesting that in its polydisperse state, HA can produce such contrasting signaling cues compared to what is exerted by native, HMW-HA in homeostasis. It is possible that changes in HA-mediated cellular signaling occur when a small percent of this polydisperse-fragmented pool passes a threshold that is enough to tip the balance and change the outcome of cellular function. Indeed, we have previously proposed that CD44 may act as a rheostat for cell proliferation through its ability to activate alternative signaling pathways via the binding of HMW-HA versus LMW-HA (22). As small pools of HA fragments have the propensity to do this, it is, therefore, conceivable that therapies to induce even possibly quite modest changes in the ratio of the active HA fragments would be enough to shift the balance of signaling in the favor of tissue repair and recovery. For example, small adjustments in increasing the ratio of HMW-HA to HA fragments may be enough to keep disease in check.

(< 10 kDa). Green text highlights positive roles for each HA MW in tissue function and recovery, whereas red text favors pathological decline. Opposing and/or unclear tissue responses are designated in orange-colored text.

On the other hand, there may be occasions where decreasing this ratio, and increasing the levels of specific HA fragments may also be beneficial. Importantly, the total concentration of HA fragments versus native, HMW-HA also needs to be measured and addressed; simply flooding the system with any size HA might be expected to cause detrimental effects to the tissue. Understanding, therefore, how HA MW distribution changes *in vivo* in different pathological settings, together with the shifts and trends that alter HA signaling, will be crucially important in deciding when and where to intervene to alter the course of disease progression.

It is clear that there is a substantial body of work that has investigated the biological and signaling roles of HMW-HA, MMW-HA, LMW-HA, and oligo-HA on cell function and disease outcome. Collectively, the data suggest that using HMW-HA may be a suitable course of action to aid tissue recovery and a return to homeostasis (**Figure 2**). This has certainly been the case for the treatment of arthritis (234). Other important avenues of research have pinpointed a pivotal role for HA in the regenerative properties of fetal tissues, which unlike their adult counterparts, heal without scarring (235), although the mechanisms by which this occurs remains to be elucidated. A less well-studied area is how MMW-HA, LMW-HA, and oligo-HA impact the biophysical and rheological properties of the tissue microenvironment or the biomechanical properties of HA in the pericellular and ECM. Going forward, these also offer important and exciting areas of investigation that may offer new therapeutic perspectives.

on targeting HA biology in tumor and disease progression and restoration of homeostasis.

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Supplementary Material

The Supplementary Material for this article can be found online at <http://journal.frontiersin.org/article/10.3389/fimmu.2015.00231>

Table S1 | HA size in tissue *in vivo*. HA molecular weight (MW) is divided into High (HMW >1000 kDa), Medium (MMW 250–1000 kDa), Low (10–250 kDa), and Oligo-HA (<10 kDa).

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4-Methylumbelliferone treatment and hyaluronan inhibition as a therapeutic strategy in inflammation, autoimmunity, and cancer

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There have been an increasing number of studies utilizing 4-methylumbelliferone (4-MU) to inhibit hyaluronan (HA) for either experimental or pre-clinical purposes. These studies are notable because of the central role HA plays in many disease processes, including inflammation and cancer progression, and because of the potential utility in repurposing 4-MU, a drug already used in humans for other indications, to treat these diseases.

This review will first briefly summarize the known contributions of HA to inflammation and cancer progression. Then, it will describe the chemistry and pharmacokinetics of 4-MU, particularly in regards to its inhibition of HA production. Finally, it will examine the available clinical data on the use of 4-MU treatment in humans and summarize the available data on safety and efficacy in animal models.

HYALURONAN

HA is an extracellular matrix (ECM) glycosaminoglycan (GAG). It has many roles in normal tissue function and development, including providing support and anchorage for cells, facilitating cell-cell signaling, and facilitating cell movement and migration (1–4).

HA is synthesized by three, independently regulated HA synthase (HAS) proteins. These generate predominantly high molecular weight-HA (HMW-HA) of between 2×10^5 and 2×10^6 Da (5). These enzymes lengthen HA by repeatedly adding glucuronic

Hyaluronan (HA) is a prominent component of the extracellular matrix at many sites of chronic inflammation, including type 1 diabetes (T1D), multiple sclerosis, and numerous malignancies. Recent publications have demonstrated that when HA synthesis is inhibited using 4-methylumbelliferone (4-MU), beneficial effects are observed in several animal models of these diseases. Notably, 4-MU is an already approved drug in Europe and Asia called "hymecromone" where it is used to treat biliary spasm. However, there is uncertainty regarding how 4-MU treatment provides benefit in these animal models and the potential long-term consequences of HA inhibition. Here, we review what is known about how HA contributes to immune dysregulation and tumor progression. Then, we review what is known about 4-MU and hymecromone in terms of mechanism of action, pharmacokinetics, and safety. Finally, we review recent studies detailing the use of 4-MU to treat animal models of cancer and autoimmunity.

Keywords: hyaluronan, 4-methylumbelliferone, hymecromone, immune diseases, cancer

acid and N-acetyl-glucosamine to the nascent polysaccharide as it is extruded through the cell membrane into the extracellular space (4). HA in circulation is rapidly degraded while HA bound to proteins and incorporated into tissues such as joints, basement membranes, and the vitreous of the eye is longer lived (5–8).

HA catabolism is mediated by endogenous hyaluronidases, by bacterial hyaluronidases, by mechanical forces, and by oxidative stress (9). This catabolism results in a continuum of different-sized HA polymers, including low molecular weight-HA (LMW-HA; <120 kDa) and, ultimately, in HA oligomers. One important factor in determining the longevity and size of HA are its interactions with HA-binding proteins, called hyaladherins that protect HA from catabolism and turnover. These include TNF-stimulated gene-6 (TSG-6) and inter-α-inhibitor (IαI) (10, 11). Hyaladherins are thought to interact with HA in such a way as to promote the formation of macromolecular complexes that modulate leukocyte adhesion and activation, thus influencing the inflammatory response (3, 4, 10).

The main receptors for HA are CD44 and RHAMM. Upon binding to HA, intracellular signaling pathways are activated; consequently, the receptors participate in a variety of cellular functions including lymphocyte activation and tumor metastasis.

HA levels are greatly elevated in injured tissues, with production increasing by as much as 80-fold (4). Because HA is highly hygroscopic (12), this increased HA production is likely to drive edema at sites of injury. Consistent with this, HA has

been implicated in vascular permeability changes (13), leukocyte adhesion and egress (14), and migration (15). HA can be organized into a variety of molecular architectures by forming cross-linked complexes with the above mentioned proteins, and can serve as ligands for leukocytes. Such interactions may trap the leukocytes and prevent eventual destruction of the tissue, as well as trap pro-inflammatory mediators (10). These ECM molecules may initiate a cascade of events that promote inflammation by attracting inflammatory cells and promoting their activation (16).

Along with the amount of HA, the size distribution of local HA polymers varies between healthy and inflamed tissues. Longer polymers of HMW-HA typically predominate in most tissues under steady-state conditions while, shorter, LMW-HA polymers predominate at sites of active inflammation (2, 17, 18). In light of these associations, HA size has been called a natural biosensor for the state of tissue integrity (19).

These changes in the size of HA have functional consequences because of the differential impacts of HA polymers of different sizes on injury responses and homeostasis. HMW-HA, which predominates in healthy tissues, typically inhibits inflammation (20–22). Consistent with this, administration of HMW-HA is anti-inflammatory in lung injury models (23), collagen-induced arthritis (24), and a variety of other *in vivo* model systems (25–29). The generally anti-inflammatory properties of HMW-HA may be mediated, in part, through interactions with the HA receptor CD44 [reviewed in Ref. (18)] and/or through hyaladherins known to bind HA, including TSG-6 and I α I (11, 30, 31).

LMW-HA, conversely, is thought to drive local inflammatory responses by acting as a pro-inflammatory “danger signal” or damage-associated molecular pattern (DAMP) through effects on Toll-like receptor (TLR) signaling (3, 32, 33). LMW-HA promotes the activation and maturation of dendritic cells (DCs) (34), drives the release of pro-inflammatory cytokines such as IL-1 β , TNF- α , IL-6, and IL-12 by multiple cell types (35–39), drives chemokine expression and cell trafficking (40, 41), and promotes proliferation (42, 43) and angiogenesis (44). In light of these and other data (45), it seems likely that LMW-HA and HA catabolism contribute to the perpetuation of inflammation in multiple tissues.

HA IN CHRONIC INFLAMMATION

Many chronic disease processes associated with unremitting inflammation are associated with prolonged increases in HA, including type 2 diabetes (T2D) (46, 47), liver cirrhosis (48), asthma, and other diseases (49–54). These conditions are typically associated with accumulations of LMW-HA [reviewed in Ref. (18)].

LMW-HA may also promote immune dysregulation at these sites. We have reported that LMW-HA inhibits the function of Foxp3+ regulatory T-cells (Treg) (38), a cell type that plays a major role in suppressing autoimmunity (55). Other TLR agonists are known to have similar effects on Treg (56).

Recently (57), we reported that autoimmune insulitis in autoimmune type 1 diabetes (T1D) was associated with islet-specific deposition of HA. Using human T1D tissue samples from cadaveric organ donors obtained through the Juvenile

Diabetes Research Foundation (JDRF) National Pancreatic Organ Donor (nPOD) program, we discovered that HA deposits were present in islets from recent-onset T1D donors but not in non-diabetic controls. These T1D-associated HA deposits were also associated with local alterations in hyaladherins, including reduced levels of intra-islet TSG-6 and I α I and increases in mRNA of versican, a pro-inflammatory hyaladherin (57). We have made similar observations in animal models of autoimmune diabetes, including non-obese diabetic (NOD) mice (58) and DORmO mice. Together with recently published histologic and biochemical analyses by our group and others, of islet ECM in non-diabetic human and murine islets (59–62), these data implicated HA and the islet ECM in the onset of T1D.

Along with insulitis, HA is highly abundant within demyelinated lesions in multiple sclerosis (MS) and in experimental autoimmune encephalomyelitis (EAE) (63). It is produced by local astrocytes (63, 64), and is known to contribute to EAE by promoting the extravasation of leukocytes (65) and inhibiting oligodendrocyte maturation (66, 67). Lymphocyte infiltration into the CNS is known to precede HA production by astrocytes in EAE, suggesting that astrocytes may produce HA in response to inflammatory factors produced by lymphocytes (63, 64, 68, 69).

HA has also been implicated in other autoimmune diseases, including rheumatoid arthritis (70, 71), lupus (72), Sjögren’s syndrome (73), and Hashimoto’s thyroiditis (74). There is further evidence that targeting HA receptors, including CD44, may be beneficial in several animal models of autoimmunity, including the NOD mouse model of autoimmune diabetes and the collagen-induced arthritis model of rheumatoid arthritis (75–77), though these effects may result from effects on lymphocyte trafficking or apoptosis rather than effects on the local ECM milieu.

HA IN CANCER

There is extensive communication between the tumor microenvironment and cancer cells (78, 79). This communication is thought to govern critical cellular processes in metastasis, including angiogenesis, proliferation, and stimulation of tissue-degrading proteases (80). Consistent with this, *in vivo* and *in vitro* data from different origins and various malignancy grades revealed a positive correlation between tumor aggressiveness and stromal HA expression (81–83).

Different expression patterns of HASes are seen during tumor progression. Aggressive ovarian and breast cancer cells express high levels of HA synthase 2 (HAS2) and lower levels of HA synthase 3 (HAS3) compared to non-aggressive cancer cells (84, 85). Indeed, HAS expression levels are inversely correlated with breast cancer staging grades and patient survival (86). HAS expression patterns may be somewhat cancer specific; for example, metastatic prostate and colon cancer express higher levels of HAS3 than HAS2. HA synthase 1 (HAS1) on the other hand was expressed only at very low levels in these tumors (87, 88).

HA forms inter- and intra-molecular organizations, creating a viscous milieu well suited for tumor growth and metastasis. This HA-rich tumor matrix provides structural integrity, maintenance of homeostasis, release of growth factors, cytokines, and nutrients

essential for proliferation (10). HA plays an important role in cancer in intracellular signaling cascades associated with tumor growth (89), tumor cell adhesion (90), neovascularization (91–93), and metastasis (90). Many of these pro-tumorigenic effects are attributable to HA fragments.

Conversely, HMW-HA was recently implicated in the inhibition of tumor progression (94). Tian et al. found that naked mole-rat fibroblasts secrete HMW-HA, which is over five times larger than human or mouse HA. This HMW-HA accumulates in naked mole-rat tissues. Interestingly, once HMW-HA is removed by either knocking down HAS2 or overexpressing hyaluronidase 2 (HYAL2), the naked mole-rat cells become susceptible to malignant transformation and form tumors.

HA is also known to influence the susceptibility of tumors to chemotherapeutic agents (95). HA-evoked anti-cancer drug resistance may be of a physico-mechanical nature as a dense ECM limits the delivery and distribution of therapeutic agents (96) and enzymatic depletion of HA is being explored as a means to improve drug delivery (97). Indeed, hyaluronidase, an enzyme that degrades HA, has been used in tumor therapy in combination with chemotherapeutic agents for over two decades (98).

Another reported approach to facilitate the delivery of chemotherapeutic agents through HA is to use the large volumetric domain of HA to entrain small chemotherapeutic drugs within the HA matrix. The resultant HA/drug formulation accumulates in the microvasculature of the tumor, forming a microembolism that increases drug retention at the tumor site and allows for active tumor uptake through HA receptors (99). As a result, a Phase II clinical trial of specific HA formulations of three anti-cancer drugs have been undertaken (100).

Taken together, these data suggest that HA may create a permissive environment for tumor growth and metastasis.

4-METHYLBELLIFERONE

In light of these contributions of HA to inflammation, autoimmunity, and to tumor growth and metastasis, there has been great interest in identifying pharmacologic tools to inhibit HA synthesis. One agent that has received much attention is 4-MU (**Figure 1**).

4-MU is a derivative of coumarin. Other coumarin derivatives, phenprocoumon (Marcumar®) and warfarin (Coumadin®), are used in preventive medicine to reduce cardiovascular events due to its anticoagulatory mechanism. Coumarin hydroxylated in position seven is known as umbelliferone and is a natural molecule in plants worldwide. Known representatives of *umbellifera* are lovage (*Levisticum officinale*) and chamomile (*Matricaria recutita*).

4-MU is umbelliferone methylated at position four. It has the IUPAC name 7-hydroxy-4-methylcoumarin and the international free name (INN) hymecromone. It has the molecular formula C₁₀H₈O₃, a molecular weight of 176.2 kDa, the CAS number is 90-33-5, and a pKa of 7.79. The melting point of 4-MU is 194–195°C. 4-MU is soluble in methanol with heating, DMSO, and in glacial acetic acid. It is slightly soluble in ether or chloroform and practically insoluble in water.

4-MU is known for its fluorescent properties and has an excitation wavelength of 380 nm and an emission wavelength of 454 nm in water. It is colorless at pH 7.0 and exhibits a blue fluorescence at pH 7.5. In light of these properties, it has been used extensively as a pH-sensitive fluorescent indicator in multiple experimental settings.

4-MU-MEDIATED INHIBITION OF HA PRODUCTION

The other major experimental use of 4-MU is for HA inhibition. 4-MU has been shown to inhibit HA production in multiple cell lines and tissue types both *in vitro* and *in vivo* (101–108).

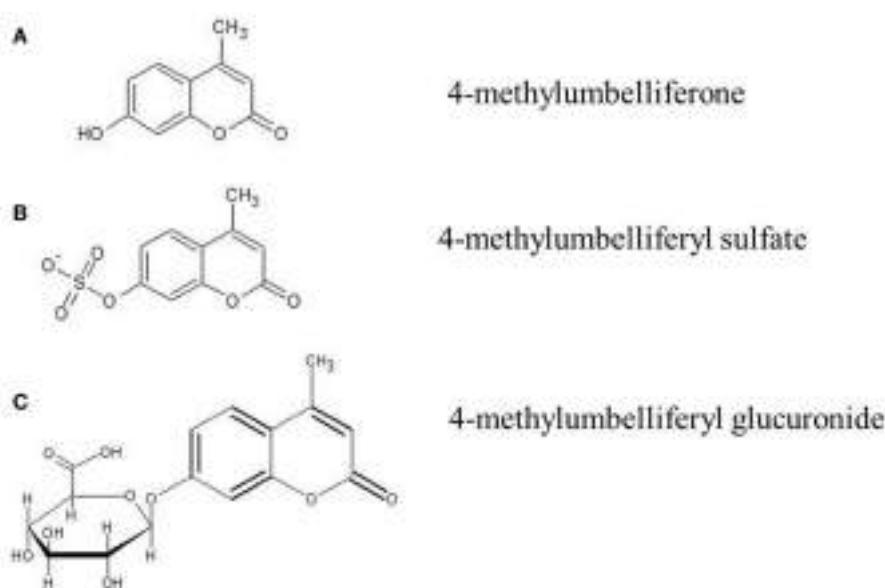


FIGURE 1 | Molecular structure of 4-MU and its metabolites. (A) 4-Methylumbelliferone (4-MU), **(B)** 4-methylumbelliferyl sulfate (4-MUS), **(C)** 4-methylumbelliferyl glucuronide (4-MUG).

4-MU is thought to inhibit HA production in at least two ways. First, 4-MU is thought to function as a competitive substrate for UDP-glucuronosyltransferase (UGT), an enzyme involved in HA synthesis (106). HA is produced by the HAS1, HAS2, and HAS3 from the precursors UDP-glucuronic acid (UDP-GlcUA) and UDP-N-acetyl-glucosamine (UDP-GlcNAc). These are generated by the transfer of an UDP-residue to *N*-acetylglucosamine and glucuronic acid via the UGT. The availability of UDP-GlcUA and UDP-GlcNAc thereby controls HA synthesis (109). However, when 4-MU is present, it covalently binds through its hydroxyl group at position four to glucuronic acid via the UGT. As a consequence, the concentration of UDP-GlcUA declines in the cytosol and HA synthesis is reduced (Figure 2). 4-MU thereby reduces the UDP-GlcUA content inside the cells and inhibits HA synthesis.

Second, 4-MU reduces expression of HAS mRNA expression (105) as well as mRNA for UDP-glucose pyrophosphorylase and dehydrogenase (110). It is unclear how this second mechanism works or how selective it is for these mRNAs.

4-MU EFFECTS ON OTHER GAGs

4-MU is commonly described as a specific inhibitor of HA synthesis. However, its impact on other GAGs has not been definitively established, to our knowledge.

It was recently reported that 4-MU exerts at least some of its actions via regulation of UDP-glucose dehydrogenase (UGDH), a key enzyme required for both HA and sulfated-glycosaminoglycan (sGAG) production (111). However, other GAGs, such as chondroitin and heparin sulfates, were less sensitive to UDP-GlcUA deficiency. This was suggested to be because they are synthesized in the Golgi apparatus, which has transporters with a very high affinity that pump in UDP sugars from the cytosol that might render inhibition by a competitive substrate such as 4-MU less efficient. In contrast, HA is synthesized at the cytoplasmic membrane.

4-MU EFFECTS ON TUMORS AND CANCER CELLS

The first described use of 4-MU in the context of HA was in 1995 when Nakamura et al. published their study about 4-MU in

human skin fibroblasts (112). The postulated 4-MU mechanism was described years later in 2004 by Kakizaki and his group (106).

By far, the greatest experience with 4-MU is in cancer cell lines and *in vivo* models. In 2006, the first *in vivo* study investigating the effect of 4-MU on pancreatic cancer was published (113). More *in vitro* and *in vivo* studies have followed on this subject (114–129). These are detailed in Table 1. The consensus of these studies is that 4-MU inhibits the proliferation, migration, and invasion of multiple cancer cell types, both *in vitro* and *in vivo*.

Most of these effects are consistent with what is known about the physiologic roles of HA in normal growth and differentiation and how many tumors establish HA-rich matrices to promote their own growth and metastasis. For example, consistent with HA's role in cell survival pathways, 4-MU treatment is associated with growth arrest and apoptosis of tumor cells (120). Indeed, the apoptotic effect of 4-MU on smooth muscle cells could be rescued with exogenous HA (139). Consistent with the established role of HA in angiogenesis, 4-MU treatment is reported to suppress the new blood vessel growth required for metastases (103, 110).

However, it is not obvious that all of the effects of 4-MU treatment are directly related to HA inhibition. For example, 4-MU was recently reported to inhibit growth of an ovarian tumor cell line via suppression of thymidine phosphorylase (TP) mRNA (127).

In summary, the use of 4-MU to inhibit cancer progression is an active frontier in oncology research, with extensive data in animal models and *in vitro* cell lines supporting further investigation. However, while one can be optimistic about the potential for adjunctive benefit of 4-MU in cancer therapy, much remains unknown and crucial human clinical studies have yet to be done.

4-MU EFFECTS ON INFLAMMATION AND AUTOIMMUNITY

There have been more limited investigations into the impact of 4-MU and HA synthesis inhibition in inflammation and autoimmunity. McKallip et al. reported that 4-MU treatment prevented lung injury and reduced inflammatory cytokine levels in mouse models of staphylococcal enterotoxin-mediated (135) and lipopolysaccharide-mediated acute lung injury (136). 4-MU has also been used to inhibit HA production by several

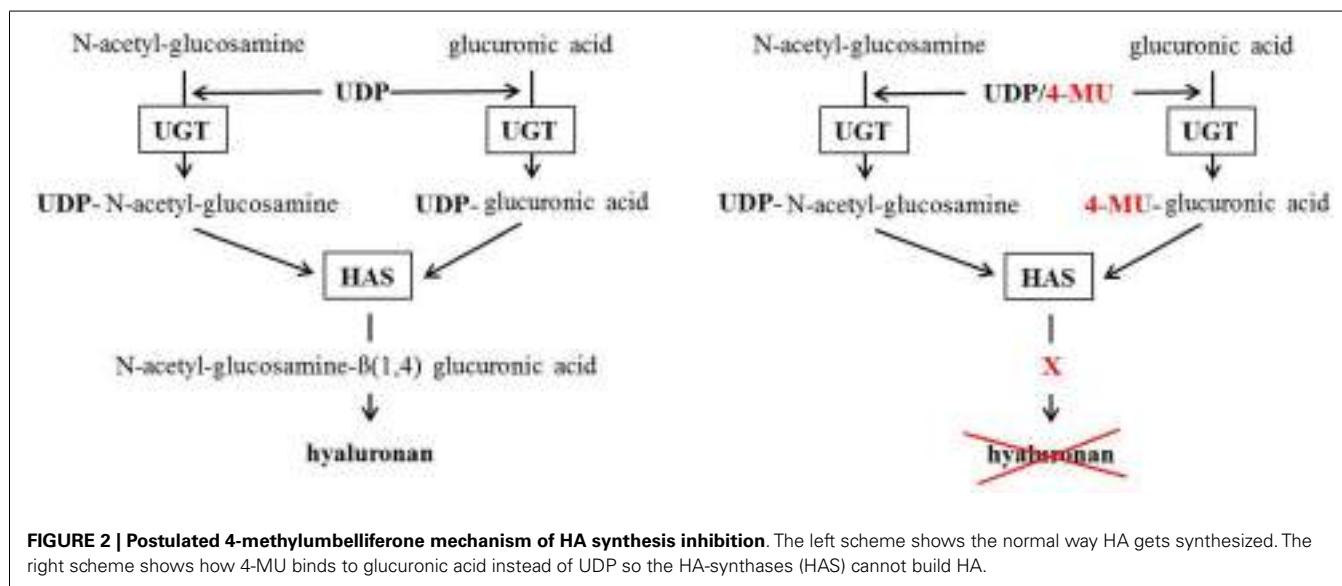


Table 1 | List of experimental studies using 4-MU, broken down by disease type.

Cancer	Inflammation	Autoimmunity
Pancreatic cancer (113–115)	Non-infectious inflammation (130–132)	CNS autoimmunity (133)
Prostate cancer (134)	Infectious inflammation (135, 136)	Autoimmune arthritis (70)
Skin cancer (107, 116–118)		
Esophageal cancer (129)		
Breast cancer (119–121)		
Liver cancer (122, 123)		
Bone cancer/metastases (126, 128, 137, 138)		
Leukemia (124, 125)		
Ovarian cancer (127)		

human pathogens and their interactions with human cells *in vitro* (140, 141).

4-MU has also been shown to have protective effects on non-infectious inflammation, including renal ischemia and reperfusion (130), and airway inflammation secondary to cigarette smoke (131). 4-MU was shown to restore normoglycemia and promote insulin sensitivity in obese, diabetic mice via increased production of adiponectin (132).

4-MU has also been reported to ameliorate disease in a limited number of mouse models of autoimmune disease. Specifically, 4-MU treatment was beneficial in the collagen-induced arthritis model where it improved disease scores and reduced expression of matrix metalloproteases (MMPs) (70). More recently, 4-MU treatment was demonstrated to prevent and treat disease in the EAE model where it increased populations of regulatory T-cells and polarized T-cell differentiation away from pathogenic, T-helper 1 T-cell subsets and toward non-pathogenic T-helper 2 subsets (133).

These effects point toward a potential role for 4-MU in immune modulation. We have reported that 4-MU treatment prevented cell–cell interactions required for antigen presentation (108) and others have described inhibitory effects on T-cell proliferation (102). These effects are consistent with established roles for HA and its receptors in T-cell proliferation, activation, and differentiation (3, 142, 143). These data also align well with the known effects of 4-MU in lymphoma studies (124, 125).

One pressing question is why 4-MU is anti-inflammatory in multiple systems whereas HA itself has both pro-and anti-inflammatory attributes. One hypothesis is that 4-MU may lead to a preponderance of HMW-HA polymers, with typically anti-inflammatory properties over HA fragments, with typically pro-inflammatory properties. This model assumes that loose HA fragments are more readily cleared than HMW-HA, which is more likely to be integrated into stable matrices and perhaps therefore less subject to rapid turnover. A related notion is that most of the increase in HA production that occurs at times of inflammation is pro-inflammatory in ways that HA produced at

times of homeostasis is not. These differences could be mediated either at the level of the different HASes or via cotemporaneous production of hyaladherins. These hypotheses remain to be tested.

There are also indications that 4-MU treatment may make some models of inflammation worse. For example, 4-MU treatment was also associated with worse atherosclerosis in ApoE-deficient mice fed a high-fat diet (104). It is tempting to speculate that HA plays roles in barrier function in some tissues such that its loss leads to enhanced exposure to bacteria or inflammatory mediators.

HYMECROMONE

4-MU is already an established therapeutic currently used in humans. Called “hymecromone,” it is used in multiple countries mainly for its choleric and biliary antispasmodic activity (144–147). Despite being a coumarin derivative, hymecromone does not possess anticoagulant properties. In Europe, hymecromone is an approved drug for use in humans for biliary dyskinesia (original European Union reference date 07/27/1960). For example, in Italy, hymecromone is marketed as a generic named Cantabilin® with a current marketing authorization via the Italian Medicines Agency (AIC no. 02130002) [“Cantabilin® (hymecromone Tablets) (Italian Package Insert)” 2013].

CLINICAL EXPERIENCE WITH HYMECROMONE (4-MU)

The typical approved dosing regimen for adults is 300–800 mg three times/day by mouth (900–2400 mg/day). It is generally available as a tablet with dose strengths of 300–400 mg. Hymecromone is currently not approved for any indication in the U.S., and therefore requires an Investigational New Drug (IND) application from the Food and Drug Administration (FDA) for clinical studies conducted in the U.S.

Several clinical trials in humans, including randomized placebo-controlled, have been published on hymecromone and all demonstrated excellent safety during short-term administration of approved doses (148–154) (Table 2). Taken together, at least 182 patients have been exposed in clinical trials and no serious adverse events from hymecromone were reported. The longest reported duration of administration of hymecromone was a multiple-dose study of oral administration of hymecromone at 1200 mg/day (400 mg three times/day) for 3 month in 20 participants with biliary dyskinesia (152). The tolerability and safety of longer durations of chronic administration is not known, yet this will be necessary to formally establish given the potential need for chronic long-term treatment duration as a therapy in inflammatory or autoimmune conditions.

Overall, the most common side effects during hymecromone treatment are diarrhea or other mild gastrointestinal symptoms [“Cantabilin® (hymecromone tablets) (Italian Package Insert)” 2013]. The diarrhea occurs in 1–10% of patients and appears to be dose-dependent. One study reported a dose of 2400 mg/day (800 mg three times/day) continued for more than 7 days would be expected to result in unacceptable diarrhea. However, this was a study in patients who had undergone bile duct surgery including insertion of a T-drain into the common bile duct. Therefore, whether patients with a normal biliary system would experience the same level of diarrhea at this dose is unknown.

Table 2 | Clinical trials using hymecromone (4-MU) in humans.

Reference	Patient type	Study type	n	Dose	Duration	Adverse events/notes
(153)	Patients requiring cholecystectomy, age >14	Double-blind, randomized, placebo-controlled	25	2400 mg/day × 7.5 days then 1200 mg × 7 days	2 weeks	Decreased drain output and need for post-op analgesics, two pts with mild headaches in treatment group, three with decreased appetite and diarrhea in placebo group
(149)	Post-cholecystectomy dyspepsia, age >60, mean 58.5 years	Double-blind, randomized, placebo-controlled	15	600 mg BID	3 weeks	N/A
(152)	Biliary dyskinesia	Randomized controlled trial vs. tiropamide 300 mg	20	1200 mg daily	3 months	N/A
(149)	Patients requiring cholecystectomy, age 29–84	Placebo-controlled, randomized	13	1600 mg/day	3 weeks	N/A
(155)	Healthy, age 21–35	Pharmacokinetics	8	400 mg IV, 800 mg IV, 600 mg PO solution, 1200 mg PO solution, 1200 mg tablets	Once	N/A
(150)	Healthy, age 22–30	Prospective, double-blind, randomized cross-over study	20	400 mg IV	Once, after meal	N/A
(148)	Healthy	Placebo-controlled, multicenter, randomized	61	600 mg with lunch, 600 mg with dinner	2 weeks	N/A
(154)	Healthy, age 20–37	4-methylumbelliferone PO and IV	20	800 mg × 1 (PO and IV)	Once, with meal	N/A

Also of value from a safety perspective is a single dose study in healthy volunteers given as an intravenous dose of 400 and 800 mg (145). Side effects other than those related to intravenous (IV) injection for the 400 mg IV dose included minor dizziness and nausea (four of eight subjects) and “cold sweat” for 5 min (two of eight subjects). After the 800 mg IV dose, side effects reported included “bad after taste” (one of six subjects), nausea and dizziness (three of six subjects), and emesis (two of six subjects). This safety data is of significance as the bioavailability after oral dosing is <3%. Therefore, the systemic exposures after these IV doses were substantially higher than the exposure after typical oral doses. While the safety data at these higher exposures after IV dosing are severely limited given only single dose exposure, it may hint at the tolerability of higher oral doses of hymecromone in humans, which may be necessary for new indications of the drug if higher systemic exposures are required. The overall safety of hymecromone is further supported by animal data noted in the Italian Medicines Agency “package insert” which notes, “acute toxicity has proved to be very low: the LD50, for oral administration is 7593 mg/kg in mice and 6220 mg/kg in rats. Protracted oral administration in the range of 800–2400 mg/kg/day for 3 months and in the rat 400–1000 mg/kg/day for 4 months, has shown excellent tolerability...” [“Cantabilin® (hymecromone tablets) (Italian Package Insert)” 2013]. Contraindications to taking hymecromone include pregnancy and lactation given the lack of safety data

in these groups [“Cantabilin® (hymecromone tablets) (Italian Package Insert)” 2013].

Taken together, the clinical experience to date suggests hymecromone is a safe and well-tolerated oral medication. The safety of oral hymecromone doses as high as 2400 mg/day and treatment durations as long as 3 months have been demonstrated in humans and can serve as a benchmark for early stage clinical trials exploring new indications.

CLINICAL PHARMACOLOGY OF HYMECROMONE

Hymecromone is extensively metabolized and <1% of a given dose is excreted unchanged in the urine (155, 156). Metabolism of the drug occurs via conjugation to either a glucuronic acid, 4-MUG, or a sulfate (4-MUS) (Figure 1). The glucuronide is the predominant pathway and accounts for over 90% of its metabolism (155, 156). Following conjugation of glucuronic acid to hymecromone, the resulting more hydrophilic metabolite, 4-MUG, is eliminated in the bile and urine (156). Biliary eliminated 4-MUG likely undergoes further enterohepatic recirculation with reabsorption of the metabolite from the intestine and ultimate elimination in the urine via the kidney. This is supported by a healthy volunteer pharmacokinetic study in which 93% of a single intravenous dose of hymecromone was eliminated as the 4-MUG metabolite in the urine (155). However, the precise contribution of enterohepatic recycling in the disposition of hymecromone and its metabolite is not well studied.

Glucuronidation of hymecromone is catalyzed by the UGTs which are a large superfamily of over 20 proteins involved in the Phase II biotransformation of lipophilic xenobiotics and endogenous compounds (157, 158). UGTs are expressed in a wide range of tissues, however, for the purposes of drug biotransformation, the most clinically relevant are located in the liver and intestine (159, 160). Interestingly, hymecromone is a promiscuous molecule in that it is a substrate of most of the major hepatic and intestinal UGTs involved in drug metabolism (158). Consequently, the intestine and liver are very efficient in the metabolism of hymecromone. Pharmacokinetic studies in animals have demonstrated the extraction of hymecromone by the gastrointestinal system (pre-hepatic) to be ~40% and extraction by the liver as high as 97% (156). As a result of this high extraction, the fraction of an administered oral dose of hymecromone that reaches the systemic circulation (post-hepatic) as unchanged drug (i.e., the bioavailability) is very low. In a pharmacokinetic study of hymecromone in healthy volunteers, the systemic bioavailability of hymecromone after oral dosing was <3% (155). As a treatment for biliary colic, the low bioavailability of hymecromone after an oral dose is less of a pharmacokinetic liability. Indeed, the high extraction by the liver may actually be beneficial as the drug is able to concentrate in the hepatic and biliary system.

If first-pass metabolism is bypassed by giving the dose IV, the systemic exposure achieved can be more than 10–30-fold higher than after the same dose given orally (155). However, due to the high clearance of hymecromone, systemic concentrations will decrease rapidly after an IV dose and peripheral exposures will likely be quite low by 4–6 h after a dose (apparent terminal half-life of ~1 h).

The pharmacokinetics of the hymecromone metabolite, 4-MUG, are not well studied. In healthy volunteers, the systemic exposure of 4-MUG after an IV dose was shown to be higher than that of hymecromone (155). Pharmacokinetic data in humans after oral dosing on systemic exposure of 4-MUG are lacking. However, animal data from our group has demonstrated that the median plasma concentration of 4-MUG compared to hymecromone was more than 3,000-fold higher in Balb/C mice on 5% oral hymecromone chow. This animal data highlights the potential importance of understanding 4-MUG pharmacokinetics during oral hymecromone therapy given the expected much higher exposures of the metabolite relative to the parent in peripheral tissues other than the intestine and liver. Future clinical studies of hymecromone in humans would benefit from a more thorough understanding of the pharmacokinetics of the 4-MUG metabolite including whether it is a potentially active moiety.

THE THERAPEUTIC OUTLOOK FOR REPURPOSING HYMECROMONE

The existing *in vitro* and *in vivo* data suggest that hymecromone may have utility as a component of therapeutic regimens directed against HA-producing cancers. There is less data at present to support this strategy in settings of chronic inflammation and autoimmunity but the potential is there as well. However, significant unresolved questions about safety, dosing, and mechanism remain.

While hymecromone has a long and relatively reassuring safety record, many questions remain about its potential repurposing for cancer treatment and other applications. These indications may require much higher dosages than those currently used to treat biliary spasm, introducing the potential for additional side effects. Certainly, the potent effects seen with 4-MU on tumor proliferation, angiogenesis, and migration could have detrimental effects on other tissues. There may also be unanticipated issues related to these novel applications. For example, in mouse models, 4-MU treatment has been linked to a reduced ability to renally excrete electrolytes and fluids (to diuresis) in response to rapid hydration (161). One could envision how this might be problematic if 4-MU were used in conjunction with chemotherapies that are renally cleared.

Long-term hymecromone treatment, rather than the more intermittent use associated with treatment of biliary spasm, might also be associated with unanticipated consequences. For example, we reported that 4-MU treatment was associated with worse atherosclerosis in ApoE-deficient mice fed a high-fat diet (104).

Several clinical pharmacology considerations must also be addressed. The large first-pass metabolism and rapid clearance of hymecromone are obstacles to achieving and maintaining high drug concentrations. This is particularly a concern in peripheral tissues (i.e., pancreas, skin, brain, etc.). Targeting conditions in the intestine and liver will pose less of a problem as these organs likely experience much higher exposures after oral dosing.

Understanding how experimental studies in animal models are likely to relate to human drug dose need and concentration is also likely to be important. Notably, the metabolism and drug disposition of 4-MU in mice may be very different than in humans.

Certainly the successful development of hymecromone will demand robust, pharmacokinetic studies of hymecromone and its metabolites in humans. Such detailed pharmacokinetic understanding will help develop dosing strategies including appropriate dose strength and frequency. These studies will set the stage for evaluation of this promising therapy in human clinical trials.

In summary, there is potential for hymecromone to be developed and repurposed as a safe, long-term adjunctive therapy for cancer treatment or other potential indication. Hymecromone's long and reassuring clinical track record, its oral route of delivery, and the exciting *in vitro* and *in vivo* data in mice all support further exploration of this therapeutic strategy. However, substantial pharmacologic and safety issues must be addressed in order to facilitate the translation of hymecromone into the clinic.

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Lipid raft-mediated regulation of hyaluronan–CD44 interactions in inflammation and cancer

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Hyaluronan is a major component of the extracellular matrix and plays pivotal roles in inflammation and cancer. Hyaluronan oligomers are frequently found in these pathological conditions, in which they exert their effects via association with the transmembrane receptor CD44. Lipid rafts are cholesterol- and glycosphingolipid-enriched membrane microdomains that may regulate membrane receptors while serving as platforms for transmembrane signaling at the cell surface. This article focuses on the recent discovery that lipid rafts regulate the interaction between CD44 and hyaluronan, which depends largely on hyaluronan's size. Lipid rafts regulate CD44's ability to bind hyaluronan in T cells, control the rolling adhesion of lymphocytes on vascular endothelial cells, and regulate hyaluronan- and CD44-mediated cancer cell migration. The implications of these findings for preventing inflammatory disorders and cancer are also discussed.

Keywords: extracellular matrix remodeling, oligosaccharides, cholesterol, membrane raft, membrane dynamics, transmembrane signaling, a disintegrin and metalloproteinase, ectodomain shedding

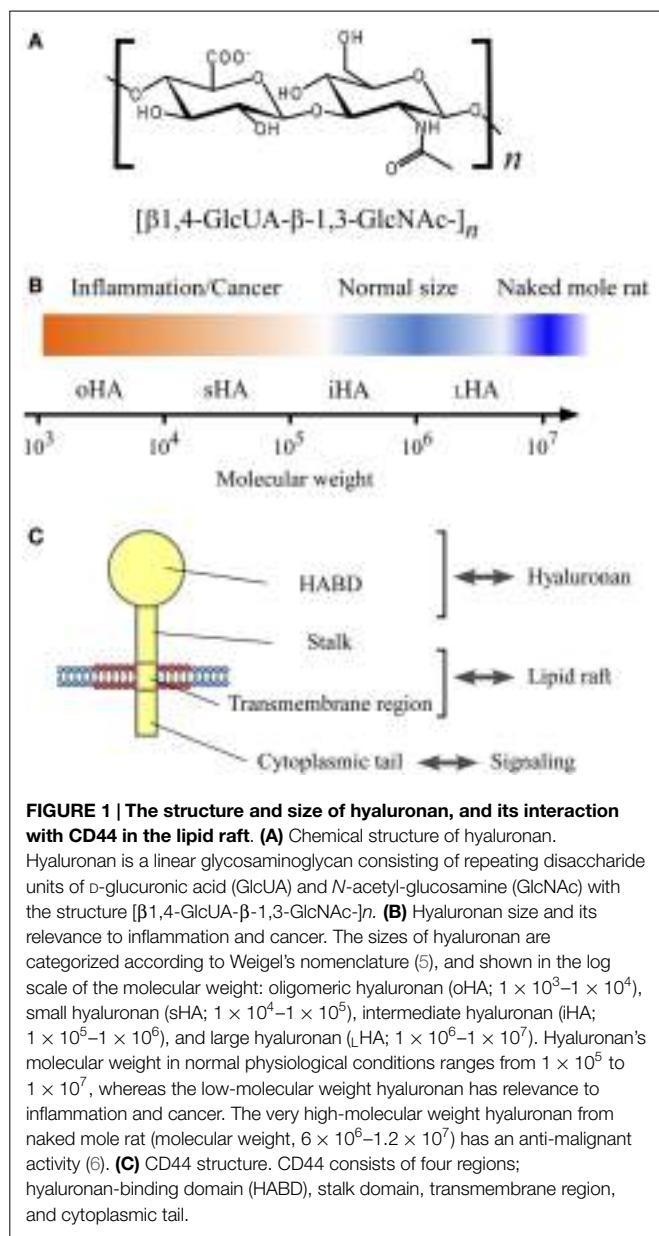
Introduction

Hyaluronan is a linear glycosaminoglycan consisting of repeating disaccharide units of D-glucuronic acid (GlcUA) and N-acetyl-D-glucosamine (GlcNAc) with the structure [β 1,4-GlcUA- β -1,3-GlcNAc-] n , and a physiological molecular weight (relative molecular mass) ranging from 1×10^5 to 1×10^7 with polydispersity (1) (Figure 1). Hyaluronan was first purified from bovine vitreous humor in 1934 (2). It is now known to be ubiquitous in vertebrate tissues, with particular abundance in connective tissues, such as synovial fluid, Wharton's jelly in the umbilical cord, and the vitreous humor of the eye, where it plays a mechanical role determined by its viscous features. While hyaluronan is traditionally regarded as a space filling, structural molecule involved in lubricating joints or holding connective tissues in place (3), it also functions as a microenvironmental cue in inflammation and cancer (4).

Hyaluronan: A Size-Dependent Bioactive Molecule

Structure and Physicochemical Properties

Hyaluronan has a simple structure that lacks a core protein linkage or sulfation. It is synthesized as a large, negatively charged linear polymer with multiple carboxyl groups on GlcUA residues. Both the GlcUA and GlcNAc residues are in the β configuration, which allows their bulky groups, including the hydroxyl and carboxyl groups, to reside in sterically unhindered equatorial positions, and thus hyaluronan forms particularly stable tertiary structures in aqueous solution that exhibit remarkable hydrodynamic properties, including non-Newtonian viscosity and water retention.



In dilute solution, hyaluronan forms an expanded random coil due to the mutual repulsion of its carboxyl groups, and at higher concentrations it forms an entangled meshwork, the size of which depends on its concentration and molecular weight, and on the ionic strength and pH of the solution (7). At physiological ionic strengths, hyaluronan's polyanionic structure causes the partition and diffusion of monovalent ions, such as Na^+ and Cl^- as well as the divalent cation Ca^{2+} at a nearly ideal Donnan equilibrium (8).

Biosynthesis

While most glycosaminoglycans are synthesized in the Golgi apparatus, hyaluronan is synthesized at the cell surface, from uridine 5'-diphosphate (UDP)-GlcUA and UDP-GlcNAc by hyaluronan synthases (HASs), a class of membrane-integrated glycosyltransferases (EC 2.4.1.212). There are three HAS isoforms in mammals, such as HAS1, HAS2, and HAS3 (9), which have

different tissue- and cell-specific expression patterns and K_m values for their substrates; they also synthesize hyaluronan of different sizes *in vitro* (10). Various growth factors, including epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and transforming growth factor- β (TGF- β), induce the transcription of HAS genes and enhance hyaluronan synthesis (11, 12). Dysregulated HAS expression or activity is sometimes associated with tissue injury and immune diseases (13).

Degradation

Hyaluronan is enzymatically degraded mainly by hyaluronidases. The mammalian hyaluronidases (EC 3.2.1.35) are endo- β -acetyl-hexosaminidases, which hydrolyze the hexosaminidic β 1,4-linkages between GlcNAc and GlcUA residues (14). Six hyaluronidase-like sequences are present in the human genome; the five genes, i.e., HYAL1, HYAL2, HYAL3, HYAL4, and SPAM1 genes, which encode Hyal-1, Hyal-2, Hyal-3, Hyal-4, and PH-20, respectively, and a pseudogene PHYAL1 that is transcribed but not translated (15). Among these isoforms, Hyal-1 and Hyal-2 are predominantly active in somatic tissues (16). Hyal-1 is an acid-active lysosomal enzyme, and catalyzes the hydrolytic degradation of hyaluronan with any molecular weight, generating predominantly tetrasaccharides (17). Hyal-2 is an acid-active glycosylphosphatidylinositol (GPI)-anchored enzyme, and digests hyaluronan polymers to products with a molecular weight of approximately 2×10^4 , i.e., 100 saccharides (18). In addition to enzymatic degradation, hyaluronan can be depolymerized by reactive oxygen species generated by oxidative stress (and/or reactive nitrogen species), which cause random cleavage of the endoglycosidic linkages (19).

The degradation of large hyaluronan to low-molecular weight hyaluronan occurs at sites of inflammation including atherosclerosis, rheumatoid arthritis, and tumorigenesis, and low-molecular weight hyaluronan promotes inflammation (20). Low-molecular weight hyaluronan arises by the action of hyaluronidases, and the upregulation of expression and activity of hyaluronidases have been noticed in such inflammation conditions (13). Reactive oxygen species accumulate at sites of inflammation, where low-molecular weight hyaluronan can arise also by oxidative degradation.

Size

Hyaluronan is a major component of extracellular matrix and plays important roles in development and tissue remodeling. Under normal physiological conditions, hyaluronan has a high average molecular weight ($>1 \times 10^6$) and exhibits immunosuppressive effects: high-molecular weight hyaluronan suppresses septic responses to lipopolysaccharides (21), inhibits lymphocyte-mediated cytosis (22), and has anti-angiogenic effects (23). Under pathological conditions, such as inflammation and cancer, extracellular matrix remodeling is upregulated. In this situation, the hyaluronan is more polydispersed, showing a preponderance of low-molecular weight forms (13). In general, low-molecular weight hyaluronan is highly immunostimulatory, inflammatory, and angiogenic.

Table 1 summarizes the biological activities associated with different sizes of hyaluronan. The terms used in the literature

TABLE 1 | Size-dependent biological activities of hyaluronan.

Cell type	Hyaluronan size ^a	Receptor	Activity	Reference
oHA (1×10^3–1×10^4)				
Human glioblastoma	$6, 8, 10, 12\text{-mer}, 6.9 \times 10^3$	CD44	Enhance CD44 shedding and cell migration	(24)
Human ovarian carcinoma	2.5×10^3 (4 ~ 20-mer)	CD44	Inhibit the RTK–CD44 association	(25)
Human peripheral nerve sheath tumor	2.5×10^3 (4 ~ 20-mer)	CD44	Inhibit the BCRP–CD44 association	(26)
Human breast carcinoma	2.5×10^3 (6 ~ 20-mer)	CD44	Inhibit lactate influx	(27)
Rat glioma	2.5×10^3 (6 ~ 20-mer)	CD44	Suppress growth	(28)
Human prostate, colon, and breast carcinoma	2.5×10^3 (6 ~ 20-mer)	CD44	Inhibit the activation of RTKs	(29)
Human colon carcinoma	2.5×10^3 (6 ~ 20-mer)	CD44	Inhibit ErbB2 phosphorylation	(30)
Human colon, mouse mammary carcinoma	2.5×10^3 (6 ~ 20-mer) ($8 \times 10^4, 2 \times 10^6$)	CD44 –	Suppress PI3K/Akt cell survival pathway No effect	(31)
Human breast cancer	2.5×10^3 (6 ~ 20-mer)	CD44	Abrogate CD44 clustering and stimulate ERK	(32)
Rat fibroblast	6-mer, 10-mer	CD44	Inactivate ERM	(33)
Mouse and human glioma	10-mer	CD44	Enhance hyaluronan synthesis	(34)
Mouse brain capillary EC	12-mer	CD44	Induce differentiation	(35)
Rat dermal fibroblast	6-mer, 8-mer (4×10^4)	CD44, RHAMM CD44, RHAMM	Stimulate wound repair Inhibit wound closure	(36)
Bovine aortic EC	1.4×10^3 – 4.5×10^3	CD44, RHAMM	Activate PLC γ 1, Src, and ERK	(37)
Human dermal microvascular EC	4–6-mer	TLR4	Increase chemokine production	(38)
Mouse Lewis lung carcinoma	4–6-mer (4×10^6)	Unknown ^b –	Induce MMP expression No effect	(39)
Human dendritic cells	4–14-mer (8×10^4 – 2×10^5 , 2×10^5 – 1×10^6)	Unknown ^c –	Induce production of TNF- α , IL-1 β , and IL-12 No effect	(40)
sHA (1×10^4–1×10^5)				
Human vascular SMC	2×10^4 – 5×10^5 (4×10^6)	CD44 CD44	Stimulate cell-cycle progression Inhibit cell-cycle progression	(41)
Human cervical cancer	2.3×10^4 (9.2×10^5)	CD44 –	Enhance chemokinesis No effect	(42)
Mouse macrophage cell line	2.5×10^4 – 7.5×10^4 (8×10^5 – 1.2×10^6)	CD44 CD44	Facilitate GAS phagocytosis Limit GAS phagocytosis	(43)
Human colon carcinoma	3.5×10^4 ($4.7 \times 10^3, 2 \times 10^6$)	TLR4 –	Induce H β D2 expression No effect	(44, 45)
HEK293 transfected	8×10^4 – 1.8×10^5 ($<6.6 \times 10^3, >4.4 \times 10^5$)	HARE –	Activate NF- κ B-mediated gene expression No effect	(5)
iHA (1×10^5–1×10^6)				
Mouse macrophage cell line	4.7×10^5 (6×10^6)	CD44 –	Induce chemokine production No effect	(46)
Human primary monocyte	5×10^4 – 6×10^5 , 2×10^5 ($4 \times 10^3, 2.5 \times 10^6$)	TLR4 –	Stimulate arachidonic acid release No effect	(47)
LHA (1×10^6–1×10^7)				
Naked mole rat fibroblast	6×10^6 – 1.2×10^7 (3×10^3)	CD44 CD44	Transformation resistant Transformation susceptible	(6)

BCRP, breast cancer resistance protein/ABCG2; EC, endothelial cells; ERK, extracellular signal-regulated kinase; ERM, ezrin/radixin/moesin; GAS, group A Streptococcus; HARE, hyaluronic acid receptor for endocytosis; H β D2, human β -defensin 2; IL-12, interleukin-12; IL-1 β , interleukin-1 β ; MMP, matrix metalloproteinase; NF- κ B, nuclear factor- κ B; PI3K, phosphoinositide 3-kinase; PLC γ 1, phospholipase C γ 1; RHAMM, receptor for hyaluronan-mediated motility; RTK, receptor tyrosine kinase; SMC, smooth muscle cell; TLR4, Toll-like receptor 4; TNF- α , tumor necrosis factor- α .

^aMolecular weight or number of saccharides (mer).

^bThe effect of hyaluronan was independent of CD44, RHAMM, and TLR4 (39).

^cThe effect of hyaluronan was independent of CD44 and RHAMM (40).

to describe hyaluronan's sizes are confusing and inconsistent. Therefore, this article uses the system proposed by Weigel (5), in which hyaluronan's sizes are categorized according to the log of the molecular weight: oligomeric hyaluronan (oHA; 1×10^3 – 1×10^4), small hyaluronan (sHA; 1×10^4 – 1×10^5), intermediate hyaluronan (iHA; 1×10^5 – 1×10^6), and large hyaluronan (LHA; 1×10^6 – 1×10^7) (Figure 1B). As shown in Table 1, low-molecular weight hyaluronans (oHA, sHA, and iHA) generally exhibit inflammation- and cancer-promoting activities (5, 6, 24–47). The other effects of low-molecular weight hyaluronan on gene expression are well summarized elsewhere (13). Notably, studies in the naked mole rat (*Heterocephalus glaber*), an

extraordinarily long-lived rodent with low cancer incidence, show that while low-molecular weight hyaluronan has pro-malignant or pro-inflammatory effects, very high-molecular weight hyaluronan (6×10^6 – 1.2×10^7) has an anti-malignant activity (6) (Figure 1B). Another study shows that oligomeric hyaluronan of 6–40 saccharides, which is frequently found in tumor-bearing patients, enhances cleavage of the hyaluronan receptor CD44 in malignant tumor cells, and concomitantly upregulates CD44-dependent tumor cell migration, whereas larger polymers of hyaluronan fail to enhance CD44 cleavage and migration (24). Collectively, low-molecular weight hyaluronan tends to function as a "danger signal" (48).

Receptors for Hyaluronan

The major cell-surface receptor for hyaluronan is CD44, a widely distributed type-I transmembrane glycoprotein that is implicated in a wide variety of biological processes, including cell adhesion and migration, as well as in inflammation and cancer (49). CD44 mediates the adhesion and dissemination of immune and cancer cells through its association with hyaluronan (50, 51) (**Figure 1C**). In addition to hyaluronan, CD44's interaction with certain growth factors also plays important roles in cancer progression (52). Receptor for hyaluronan-mediated motility (RHAMM)/CD168 is another major hyaluronan receptor expressed in a variety of cell types, and it plays important roles in tissue injury and repair and in tumor cell motility (53). Other hyaluronan receptors include lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1) (54) and hyaluronan receptor for endocytosis (HARE)/stabilin-2 (55). LYVE-1 is mainly restricted to the endothelium of lymph nodes and lymphatic vessels, while HARE is expressed in sinusoidal endothelial cells of the liver, spleen, and lymph nodes, and it mediates the systemic clearance of hyaluronan from the vascular and lymphatic circulatory systems.

Lipid Rafts

Lipid Raft Structure

The plasma membrane is a dynamic mixture of proteins and lipids that forms the boundary and interface between the intracellular space and the cellular environment. The traditional model of cellular membrane structure was the fluid mosaic model proposed by Singer and Nicolson, in which globular proteins float in a lipid bilayer with an amphipathic structure (56). Later, non-homogeneously distributed assemblies of lipids were found in the plasma membrane of many cell types, and the model was improved by Simons and van Meer, who suggested the existence of small domains called lipid rafts (57). In the understanding of the lipid raft model, cholesterol- and sphingolipid-enriched microdomains of the plasma membrane assumed a biophysical state resembling a liquid-ordered (L_o) phase floating within a liquid-disordered (L_d) membrane phase (58). In that model, the representative proteins with raft affinity were GPI-anchored proteins. The finding that GPI-anchored proteins were isolated in a low-density detergent resistant membrane (DRM) fractions contributed to the expectation of their residence in lipid rafts (59).

Since then, accumulating evidence has improved the understanding of lipid rafts, also called membrane rafts, and rafts are currently viewed as fluctuating nanoscale assemblies enriched in sphingolipid, cholesterol, and proteins that can be stabilized to coalesce, forming platforms that function in membrane signaling and trafficking (60).

Lipid Raft Function

The most important properties of lipid rafts are that they are small, dynamic, and heterogeneous, and can selectively recruit certain classes of proteins (61, 62). However, the underlying mechanism for the formation and functionality of lipid rafts has been largely unclear. Using single-molecule fluorescence tracking, Kusumi and colleagues recently found that GPI-anchored proteins formed dynamic, transient homodimer rafts in the plasma membrane, in a

manner dependent on the interactions between their ectodomain protein portions (63). The homodimer formation seems to be the basic units for the organization and functions of membrane raft domains containing GPI-anchored proteins. Schütz and colleagues observed the relation between the physical state of the membrane domains and the partition of GPI-anchored proteins, and showed that GPI-anchored proteins do not reside in ordered domains (64). This report suggests that the phase partitioning is not a fundamental element of GPI-anchored protein organization in the plasma membrane, and also suggests the heterogeneity in the structure and function of membrane rafts.

Proteins with raft affinity include doubly acylated proteins such as Src family kinases, palmitoylated type-I transmembrane proteins, such as CD44 (65, 66), and receptor tyrosine kinases with two transmembrane subunits, such as insulin receptor (67) and EGF receptor (68). Lipid rafts are implicated in many physiological cellular processes, such as protein membrane trafficking and signal transduction (62, 69). Cholesterol depletion is often used as a method for investigating the role of lipid rafts *in vitro*, although these studies are limited by non-specific effects. Nevertheless, these studies indicate that cholesterol is a crucial component of cell membranes that contributes to the organization of lipid rafts, and particularly to lipid rafts that contain large numbers of cancer-related signaling and adhesion molecules.

Hyaluronan–CD44 Interaction and Lipid Rafts in Cancer

The dynamics of extracellular matrix production, degradation, and remodeling are carefully regulated during organ development; the dysregulation of extracellular matrix turnover and maintenance leads to abnormal cell behaviors and to failure of organ homeostasis and function, one of the most severe clinical outcomes in cancer (70). Altered cell adhesion and enhanced cell migration are the most prominent features of malignant tumor cells (71). The migratory properties of invasive tumor cells are affected by the interaction of their adhesion molecules with the surrounding extracellular matrix, and by growth factor signaling (72). The proteolytic cleavage and release (shedding) of membrane proteins' ectodomain is a critical regulatory step in both physiological and pathological processes (73, 74). It was recently reported that oligomeric hyaluronan induce CD44 shedding from tumor cells (24).

Ectopic hyaluronan production is a frequent feature of colorectal, gastric, and breast cancers (75–77). Under normal physiological conditions, hyaluronan exists as a long polymer with a molecular weight of around 1×10^5 – 1×10^7 (1), whereas low-molecular weight hyaluronan is frequently detected in certain pathological conditions, such as inflammation (78) and cancer (79, 80), possibly due to the dysregulated expression of HASs and hyaluronidases. Hyaluronidase expression in prostate cancer tissues increased with tumor grade and metastasis, suggesting that prostate tumor cell-derived hyaluronidase might help the accumulation of low-molecular weight hyaluronan (80).

A prominent abnormality of certain malignant tumor cells, e.g., gliomas, is overexpression of the EGF receptor, and EGF induces CD44 shedding, that concomitantly enhance

hyaluronan-mediated cell migration (81). PDGF and bradykinin also induce CD44 shedding, indicating that the Rho family of small GTPases plays crucial roles in the regulation of CD44 cleavage (81). TGF- β induces CD44 shedding in breast cancer cells (82), and this cleavage is MT1-MMP-dependent as previously described (83, 84). Granulocyte-colony stimulating factor (G-CSF) stimulates the MT1-MMP-mediated CD44 proteolysis in hematopoietic progenitor cells (85). Although the molecular mechanisms of the intracellular signaling in the tumor microenvironment that lead to CD44 shedding have been partially clarified (81, 86), the mechanism that triggers CD44's shedding at the membrane is not understood.

There is growing interest in targeting lipid rafts for cancer prevention and treatment, because of their role in regulating various steps of cancer progression, including cancer cell migration and invasion (87), and because cancer-related proteins were listed in an unbiased proteomics analysis of these structures (88). Cell adhesion is a key factor in the metastatic spread of cancer cells, and regulating this process holds promise as an important therapeutic intervention for cancer. CD44 is the principal cell adhesion receptor expressed in cancer cells and implicated in cancer cell migration, invasion, and metastasis (89). Several reports recently demonstrated that CD44 is present in lipid rafts (90–100) (Table 2), and the role of lipid rafts in cancer cell adhesion and migration is being elucidated.

Lipid rafts play a pivotal role in CD44's localization and function (97). Cholesterol depletion from human glioma cells using methyl- β -cyclodextrin (M β CD), an agent frequently used to disrupt lipid rafts, results in increased CD44 shedding, which was mediated by a transmembrane protease a disintegrin and metalloproteinase 10 (ADAM10). The CD44 shedding induced by cholesterol depletion is also seen in other tumor cells, such as pancreatic cancer cells. CD44 shedding can also be induced by a polyene macrolide antibiotic filipin that binds cholesterol and disperses it in the membrane, thereby disrupting lipid rafts by a different mechanism from M β CD. The cholesterol-lowering medication simvastatin also enhances CD44 shedding; it also blocks the stimulation of glioma cell migration by oligomeric hyaluronan or EGF. Taken together, these results suggest that

cholesterol-lowering causes disordered CD44 localization, raft-dependent CD44 shedding, and the suppression of tumor cell migration that is dependent on hyaluronan's size. CD44's affiliation with lipid rafts is likely to occur through its palmitoylation, which may play a role in breast cancer malignancy (95).

In addition to CD44, several hyaluronan-related proteins, Hyal-2, HAS3, and toll-like receptor 4 (TLR4), have been reported to be associated with lipid rafts in cell membranes (93, 101–103) (Table 2). These membrane proteins are also likely to be involved in the regulation of lipid raft-associated interactions between hyaluronan and CD44. In addition, CD147 was found to regulate the lipid raft-associated CD44 function in cancer cell invasion (94, 104).

Potential Roles of Hyaluronan–CD44 Interactions in Inflammation

The recruitment of lymphocytes from circulating blood to inflammatory sites or secondary lymphoid organs involves complementary receptor-ligand interactions between the lymphocytes and vascular endothelial cells. A multistep series of sequential receptor engagements enables the lymphocytes' recognition of the endothelial surface and their subsequent extravasation (105). This process begins with the establishment of transient adhesive interactions that result in the rolling of lymphocytes along the endothelium under blood flow, and rolling is mediated by interactions between CD44 and hyaluronan (106, 107). The CD44–hyaluronan interaction is required for the extravasation of activated T cells from circulating blood to inflammatory sites (108). There is also evidence that the hyaluronan-binding ability of CD44 is correlated with the suppressor activity of CD4 $^{+}$ CD25 $^{+}$ regulatory T cells (109).

CD44 does not bind hyaluronan constitutively in most immune cells, although CD44 is the principal receptor for hyaluronan in immune cells (89). Considering the ubiquitous distribution of CD44 and hyaluronan, tight regulation of the hyaluronan-binding ability of CD44 is likely to play a critical role in immunological responses: CD44 on resting T cells does not bind hyaluronan, but can be induced to bind it when the T cell is activated by antigen via the T-cell receptor (108–112). Various post-translational modifications on CD44, including glycosylation (113–115), chondroitin sulfate addition (116, 117), and sulfation (118, 119), are reported to affect its hyaluronan-binding ability. However, the membrane-based regulation of CD44's hyaluronan-binding ability has not been clarified. A recent study demonstrated that the hyaluronan-binding ability of CD44 in T cells is upregulated by membrane cholesterol depletion, which causes CD44 to be dispersed from lipid rafts, although the effect is small (120). Cholesterol depletion also enhances the frequency of rolling adhesion under physiological flow conditions, suggesting that the CD44's ligand-binding ability is governed by its cholesterol-dependent localization to lipid rafts.

Perspectives

Epidermal growth factor receptor is one of the first reported growth factor receptors that exhibit raft affinity, and EGF induced

TABLE 2 | Hyaluronan-related proteins associated with lipid rafts.

Protein	Cell type	Function in lipid rafts	Reference
CD44	Mammary adenocarcinoma	NHE1 activation	(93)
	Mammary adenocarcinoma	EGFR signaling	(94)
	Mammary adenocarcinoma	Cell migration	(95)
	Colon adenocarcinoma	Src-integrin signaling	(96)
	Glioblastoma	Cell migration	(97)
	Lung adenocarcinoma	Lamellipodia formation	(98)
	Lymphoma	Cell adhesion	(99)
	Myofibroblast	EGFR signaling	(100)
Hyal-2	Mammary adenocarcinoma	ECM degradation	(93)
	Mammary adenocarcinoma	N/A	(101)
HAS3	Mammary adenocarcinoma	Cell-surface protrusion	(102)
TLR4	Monocytic cell line	Cellular activation	(103)

ECM, extracellular matrix; EGFR, epidermal growth factor receptor; HAS3, hyaluronan synthase 3; Hyal-2, hyaluronidase-2; NHE-1, Na $^{+}$ -H $^{+}$ exchanger 1; TLR4, toll-like receptor 4.

the coalescence of EGF receptor-containing rafts with different type of lipid rafts that contain GPI-anchored proteins (68). This coalescence of different types of nanoscale assemblies possibly leads to the formation of functional platforms for transmembrane signaling and the initiation of the internalization of EGF receptors. In the case of hyaluronan receptor CD44, the function of hyaluronan in the regulation of lipid rafts may be in a similar way as proposed for EGF. As oligomeric hyaluronan can displace large hyaluronan from cells (4), it may modulate the raft coalescence that leads to form signaling platforms toward inflammation and cancer progression. Competitive binding assay showed that the minimum length of hyaluronan that can compete large hyaluronan binding to CD44 is 6-mer, and the nuclear magnetic resonance spectroscopy confirmed that 6-mer is the shortest oligomeric hyaluronan to give essentially full perturbation of CD44's spectra (121). The structure of CD44's hyaluronan-binding domain (HABD) solved by X-ray crystallography revealed that CD44 forms two different conformations upon binding to hyaluronan (122). To understand the molecular mechanism and associated energetics underlying the hyaluronan–CD44 binding interaction, Guvench group performed extensive all-atom explicit-solvent molecular dynamics (MD) simulations employing the adaptive biasing force free-energy methodology (123). They determined a clear description for the conformation-dependent affinity switching of the hyaluronan–CD44 interactions by MD simulation. These results should help the development of novel

small compounds to therapeutics in inflammation and cancer by modulating hyaluronan–CD44 interactions, which may regulate the functionality of lipid rafts.

There has been growing interest in lipid rafts, and the lipid raft is a potential novel target in inflammation and cancer therapy (66, 124). Targeting hyaluronan–CD44 axis is one of the principal ways, and the lipid raft-targeted delivery of hyaluronan-grafted liposomes could have important applications in cancer therapy (125, 126). The modulation of CD44's partition to lipid rafts may also offer potential avenues in inflammation and cancer therapy. Thus, the regulation and manipulation of hyaluronan–CD44 interactions through lipid rafts have potential applications for the prevention of inflammatory disorders and cancer.

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Cancer microenvironment and inflammation: role of hyaluronan

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The role of inflammation in the development of cancer was described as early as the nineteenth century. Abundant evidence supports the preposition that various cancers are triggered by infection and chronic inflammatory disease whereas, evading immune destruction has been proposed as one of the new “hallmarks of cancer.” Changes of the tumor microenvironment have been closely correlated to cancer-mediated inflammation. Hyaluronan (HA), an important extracellular matrices component, has become recognized as an active participant in inflammatory, angiogenic, fibrotic, and cancer promoting processes. This review discusses how HA and specific HA-binding proteins participate in and regulate cancer-related inflammatory processes.

Keywords: cancer microenvironment, inflammation, hyaluronan, RHAMM, CD44

CANCER MICROENVIRONMENT

The role of inflammation in the development of cancer was described as early as 1863, by Rudolf Virchow, who hypothesized that cancer arises from inflammatory sites, “lymphoreticular infiltration” (1). In the last decades, Virchow’s postulation has been supported by abundant evidence that various cancers are triggered by infection and chronic inflammatory disease (2). On the other hand, an inflammatory response is also detectable in tumors that are not causally related to inflammation (3). Following cell transformation to a malignant state, the inflammatory mediators are involved in tumor growth, by stimulating the proliferation of tumor cells and by evading immunosurveillance. Notably, the inflammation orchestrated by the tumor is aberrant and promotes the recruitment and/or the induction of cells that, besides having a role in the direct promotion of the tumor progression, are also endowed with immunosuppressive properties. Indeed, evading immune destruction has been proposed as one of the new “hallmarks of cancer” (4).

The consecutive steps of tumor growth, local invasion, intravasation, extravasation, and invasion of anatomically distant sites as well as immunosuppression are obligatorily perpetrated through specific interactions of the tumor cells with their microenvironment (3, 5). Extracellular matrices (ECMs) represent a complex network of proteins and glycosaminoglycans (GAGs), which define the structure of tissues *in vivo* and are critically important for cell growth, survival as well as differentiation, and key

to various disease processes including inflammation and cancer (6–10). During cancer progression, significant changes can be observed in the properties of ECM components, which deregulate the behavior of stromal cells, promote tumor-associated angiogenesis and inflammation, and lead to generation of a tumorigenic microenvironment (11–14).

Hyaluronan (HA), an important ECM component, has become recognized as an active participant in inflammatory, angiogenic, fibrotic, and cancer promoting processes. HA and its binding proteins regulate the expression of inflammatory genes, the recruitment of inflammatory cells, the release of inflammatory cytokines and thus, ultimately can attenuate the course of inflammation (15). Surprisingly, HA is a relatively simple molecule being an anionic, non-sulfated GAG in the 5000–20,000,000 Da molecular weight range. It is a polymer of disaccharides composed of alternating *N*-acetylglucosamine (GlcNAc) and glucuronic acid (GlcA) units (16). HA is unique among GAGs because it neither contains sulfate groups nor is it covalently linked with a core protein (17). This GAG is synthesized by three types of integral membrane proteins denominated HA synthases: HAS1, HAS2, and HAS3. The HAS enzymes synthesize different HA sizes by repeatedly adding glucuronic acid and *N*-acetylglucosamine to the nascent polysaccharide while it is extruded through the cell membrane into the extracellular space (18). Specifically, HAS1 and HAS2 produce very high molecular weight HA (HMWHA) up to 2000 kDa (19). The degradation of HA within tissues, on the other hand, is performed by enzymes known as hyaluronidases (HYAL). In humans, there are at least seven types of hyaluronidase-like enzymes with HYAL1 and 2 being the most important. HYAL hydrolyzes the $\beta(1-4)$ glycoside bond between *N*-acetyl-D-glucosamine and D-glucuronic acid, which results in the production of fragments of different sizes (20). It is noteworthy that the size of HA chains affects its biological functions. Indeed, oligosaccharides that result from HA degradation and low-molecular-weight HA

Abbreviations: DAMP, damage-associated molecular-pattern; ECMs, extracellular matrices; GAGs, glycosaminoglycans; HA, hyaluronan; HAS, hyaluronan synthases; HMWHA, high molecular weight HA; LMWHA, low molecular weight hyaluronan; HYAL, hyaluronidases; RHAMM, receptor for hyaluronan-mediated motility; ICAM-1, intercellular adhesion molecule 1; MAPKs, mitogen-activated protein kinases; MMP, matrix metalloprotease; TNF, tumor necrosis factor; TNF-alpha, tumor necrosis factor-alpha.

(LMWHA), defined as fragments in the 5–500 kDa range (20) are able to induce the processes of inflammation and angiogenesis. HMWHA (1000–2000 kDa), on the other hand, is present in intact tissues and is antiangiogenic as well as immunosuppressive (21–23).

HYALURONAN ACCUMULATION AND TURNOVER IN CANCER TISSUE

The alteration of HA deposition in various malignancies has been well established (13, 24). Thus, a significant number of studies show that HA deposition is elevated in various types of cancer tissues including colon, breast, lung, and prostate cancer (25–27). The magnitude of the HA accumulation both around the tumor cells and in the surrounding stroma strongly correlates with the aggressiveness of cancers by enhancing processes involved in malignant growth, like cell proliferation, invasion, metastasis, and tumor–stroma interactions (13, 24, 28). It is widely accepted that HAS mRNA levels determine the synthesis of HA (29, 30). The mechanisms, however, of HA accumulation vary. Thus, it has been suggested that fibroblast growth factor receptor (FGFR) activation induces accumulation of HA within the ECM, through HAS upregulation (31, 32). Furthermore, abnormal pre-mRNA splicing, leading to intracellular or extracellular HA synthesis by HASs, is suggested to contribute to the initiation and progression of various types of cancer (33). Importantly, an increased HYAL expression has been associated with tumor progression in a number of cancer types (34, 35). It is noteworthy that, tumor tissues are characterized by increased production of reactive oxygen species (ROS) resulting from increased metabolic activity, enhanced activity of NADPH oxidase (NOXs), or mitochondrial dysfunction of tumor tissues (12, 36). GAGs are very susceptible to ROS-induced degradation either via $\bullet\text{-OH}$ radical action, which is a product of ONOO^- decomposition (37) or through radical $\bullet\text{NO}$ action. Importantly, the balance between radical $\bullet\text{NO}$ and $\bullet\text{O}_2$ radical determines which GAG component of the ECM is destroyed and this selective degradation may be important in regulating specific aspects of the disease processes (38, 39). Therefore, on one hand, there is an established upregulation of HA deposition in tumor tissues whereas simultaneous overexpression of HYALs and overproduction of ROS induces HA degradation. Indeed, taking into account HA-size-dependent biological effects, this complex turnover pattern is in fact suggested to confer tumorigenic potential (40). The generation of various HA fragments sizes and their highly specific action on tumor cell functions has been widely established (24, 41–43). The majority of reports up to date indicate that LMWHA fragments support tumor growth and dissemination whereas, HMWHA is suggested to have anti-tumor effects (24, 44, 45). Indeed, excess deposition of HA was found to suppress tumor growth in the absence of HYAL. Thus, the overexpression of HAS in prostate carcinoma cells that are characterized by very low endogenous HA deposition and HAS expression significantly reduces tumor growth kinetics in both the subcutaneous (46, 47) and the orthotopic primary injection site (48). In contrast, results obtained in the fibrosarcoma cell model suggest that HMWHA may be pro-proliferative and enhance motility (49). This may be an unusual property of fibrosarcoma tumors that is opposite to effects observed in tumors that originate within

the epithelial compartment. In line with the established pattern of HA effects, the majority of reports suggest that HMWHA protects the integrity of the endothelial barrier. HMWHA was shown to decrease permeability in cancer lymphatic endothelial cell monolayers (45) and actually promote enhancement of vascular integrity, indicative of anti-metastatic effects (44). Opposite effects of LMWHA have been documented (44, 45). A recent report, however, indicates that the augmentation of CXCR4 signaling by HMWHA resulted in increased vessel sprouting and angiogenesis in a variety of assays (50). When interpreting data relevant to HA action, it is important to note that in general HA signaling is cancer type/cell line-specific as HAS3-dependent HA synthesis has been found to suppress cell proliferation by elevating cell cycle inhibitor expression and suppressing G1- to S-phase transition (51) whereas, LMWHA inhibits colorectal carcinoma growth by decreasing tumor cell proliferation and stimulating immune response (42).

IMMUNOLOGICAL ASPECTS OF HA IN CANCER PROGRESSION

Intriguingly, a recently proposed driver model for the initiation and early development of solid cancers associated with inflammation-induced chronic hypoxia and ROS accumulation focuses on HA action. Namely, inflammation-induced chronic hypoxia can ultimately result in the production and export of HA, which will be degraded into fragments of various sizes, serving as tissue-repair signals, which lead to the initial proliferation of the underlying cells (52). In addition, HA degradation products have the ability to induce specific gene expression programs for proteases and cytokines that are necessary for inflammation and matrix remodeling. Several studies have shown that HA fragments activate innate immune responses by interacting with TLR2 and TLR4 and inducing inflammatory gene expression in a variety of immune cells (53–55). There appear to be a feedback regulation here as, proinflammatory cytokines induce HA synthesis and monocyte adhesion in human endothelial cells through HAS2 and the nuclear factor $\kappa\text{-B}$ (NF- κB) pathway (56). As regarding tumor cells, exposure of human melanoma cells to HA fragments leads, via TLR4, to NF- κB activation followed by enhanced expression of matrix metalloprotease (MMP) 2 and interleukin (IL)-8, factors that can contribute to melanoma progression (57). In a recent study, LMWHA (but not HMWHA) was found to preferentially stimulate a physical association between CD44 and TLRs followed by a concomitant recruitment of AFAP-110 and MyD88 into receptor-containing complexes in breast tumor cells. This results in MyD88/NF- κB nuclear translocation, NF- κB -specific transcription, and target gene IL-1 β and IL-8 expression. Therefore, LMWHA signaling events lead to proinflammatory cytokine/chemokine production in the breast tumor cells (58). Another example of contrasting LMWHA and HMWHA effects is illustrated by a study performed on human SW-1353 chondrosarcoma cells. HMWHA antagonized the effects of IL-1 β by increasing PPAR γ and decreasing cyclooxygenase (COX)-2, MMP-1, and MMP-13 levels. Furthermore, in this model, HMWHA promoted Akt, but suppressed mitogen-activated protein kinases (MAPKs) and NF- κB signaling, indicating anti-inflammatory

effects. In contrast, chondrosarcoma cells had overall stimulatory responses to oligo-HA as regarding inflammatory genes (59). Inflammation establishes a tissue microenvironment, which tolerates tumor growth and metastasis by setting immunosuppressive mechanisms (60). Therefore, inflammation not only induces carcinogenesis but also makes immune cells incapable of destroying tumor cells (61). It is indicative that LMWHA fragments are able, in a TLR4/IFN- β -dependent pathway, to accelerate the elimination of inflammatory neutrophils by promoting their apoptosis (62). Moreover, in the tumor microenvironment, HA fragments can reprogram neutrophil action. Thus, tumor cell-derived HA fragments through TLR4/PI3K signaling induce early activation and longevity of tumor neutrophils, which in turn stimulate the motility of malignant cells. This skewed inflammatory mechanism represents an example of the positive regulatory loop between tumors and their stroma during neoplastic progression (63). On the other hand, LMWHA treated dendritic cells increased IFN- γ production, and secreted lower levels of the immunosuppressive IL-10 coupled with higher proliferation rates and increased motility. Moreover, these preconditioned dendritic cells elicited induced immunity in a murine colorectal cancer model (42).

Recently, the importance of HA-coated extracellular vesicles in carcinogenesis has been suggested. HA is suggested to be carried on the surface of these vesicles in tissues and body fluids, creating beneficial environments by itself, or by associated molecules, for the invasion and metastasis of cancer cells (52, 64). HA transferred by these vesicles could putatively contribute to cancer-related inflammatory processes.

HA RECEPTORS IN INFLAMMATION

Biological functions of HA are mediated by its molecular interaction with HA-binding proteins, called hyaladherins, and as a result, gain new biological identities (65, 66). In more detail, HA binds to its specific cell-surface receptors, including CD44, receptor for hyaluronan-mediated motility (RHAMM), and intercellular adhesion molecule 1 (ICAM-1), activating the transduction of a wide range of intracellular signals (67, 68). These HA receptor interactions are implicated in both physiological and pathological conditions, as they regulate cellular processes such as morphogenesis, wound healing, and inflammation (68–70). CD44 is a cell-surface glycoprotein encoded by a single gene, although there are a number of isoforms expressed in a number of cells and tissues as a result of alternative splicing (71). Importantly, it has been shown that some splice variants such as CD44v-9 and CD44v-6 are involved in tumor metastasis (72, 73) even though CD44 expression pattern cannot always be correlated with malignant progression (74). Moreover, inflammation and malignancy are often associated with sequential proteolytic cleavage of CD44 resulting in a soluble extracellular part of CD44 that most likely regulates cell migration, and to a CD44 intracellular domain that translocates to the nucleus and promotes transcription of different genes including the CD44 gene itself (75, 76). Noteworthy, at sites of inflammation, a concomitant increase in HA synthesis and release of inflammatory mediators can increase the binding avidity of CD44 for HA. Post-translational modifications of CD44 have also been implicated in the transition of an “inactive” low affinity state to an “active” high affinity state of the

CD44–HA binding capacity. Other molecules, also produced in the inflammatory milieu, including IL-2, tumor necrosis factor (TNF), and chemokines including MIP-1 β , IL-8, and RANTES, can stabilize and increase HA–CD44 interactions (77, 78). Another inflammatory marker, SHAP protein that corresponds to the heavy chains of inter-alpha-trypsin inhibitor family molecules circulating in blood, also stabilizes HA–CD44 interactions (79). Moreover, recently it was suggested that HAS1-dependent HA coat is induced by inflammatory agents and glycemic stress, mediated by altered presentation of either CD44 or HA and can offer a rapid cellular response to injury and inflammation (80). Such interactions are important for the regulation of CD44-mediated leukocyte migration to sites of inflammation (78, 81) as well as monocyte/macrophage retention and activation in inflammatory sites (82). Moreover, an alternate immune evasion mechanism, based on the interaction between CD44 on lung cancer cells and extracellular HA has been proposed. In this study, CD44/HA interactions, which reduce both Fas expression and Fas-mediated apoptosis of the cells, result in decreased susceptibility of the cells to T lymphocytes-mediated cytotoxicity through Fas–FasL pathway (83). On the other hand, engagement of CD44 was found to upregulate Fas ligand expression on T cells leading to their activation-induced cell death (84). The versatility of HA/CD44 interactions are illustrated by HA-mediated CD44 interaction with RhoGEF and Rho kinase, which promotes Grb2-associated binder-1 phosphorylation and phosphatidylinositol 3-kinase signaling leading to cytokine (macrophage-colony stimulating factor) production and breast tumor progression (85). There appears to be a backfeed interaction between inflammatory mediators and CD44 expression as, tumor necrosis factor-alpha (TNF-alpha), a major inflammatory cytokine, abundant in the ovarian cancer microenvironment was found to differentially modulate CD44 expression in ovarian cancer cells (86). Some reports, however, propose that CD44 negatively regulates *in vivo* inflammation mediated by TLRs via NF- κ B activation, which ultimately leads to proinflammatory cytokine production (87).

Receptor for hyaluronan-mediated motility has been suggested to contribute to “cancerization” of the tumor microenvironment through its wound repair functions including inflammatory cues (69, 88). It is hypothesized that RHAMM could be a member of the damage-associated molecular-pattern (DAMP) molecules, which function as proinflammatory signals (89). This is corroborated by data showing that RHAMM expression was strongly positively correlated to severe infection in immune atopic diseases (90). Moreover, central to the inflammation process, macrophage chemotaxis was found to be upregulated in a RHAMM- and HA-dependent manner (91). Indeed, upon utilization of a RHAMM mimetic peptide, which specifically blocks HA signaling a strong reduction of inflammation and fibrogenesis in excisional skin wounds was determined (69). Furthermore, RHAMM has been identified as an immunologically relevant antigen, strongly expressed in several hematologic malignancies, and associated with both cellular and humoral immunity (92, 93). Indeed, persistent RHAMM expression and decreasing CD8+ T-cell responses to RHAMM in the framework of allogeneic stem cell transplantation or chemotherapy alone might indicate the immune escape of leukemia cells (94).

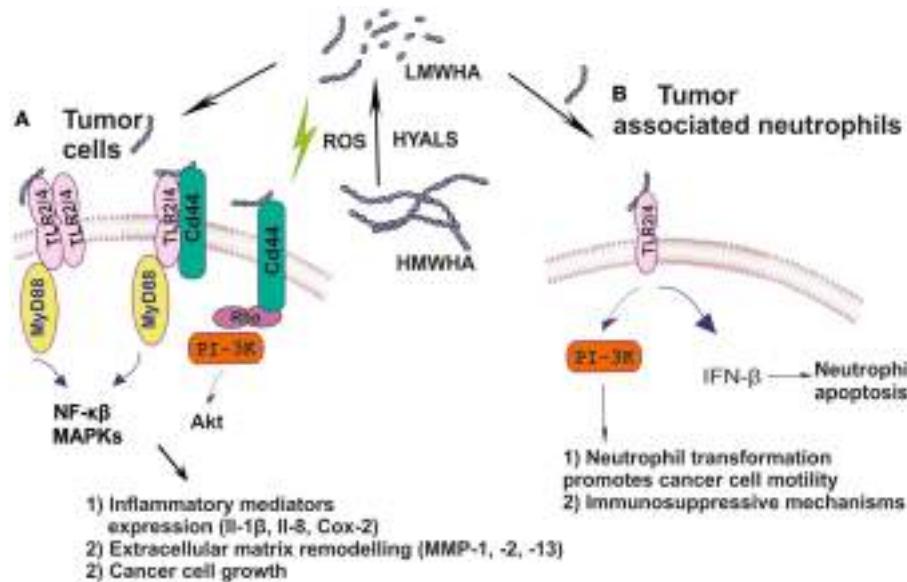


FIGURE 1 | (A) HA fragments through specific interactions with hyaladherins activate intracellular pathways including MAPKs, NF- κ B, and PI3K/Akt, which support tumor cell growth; **(B)** HA by modulating TLR2/4 downstream signaling reprograms inflammatory cells to create a tumor-permissive environment.

These aspects of RHAMM/HA signaling can be utilized as targets of novel cell-based strategies in cancer. Thus, vaccination with a highly immunogenic peptide, which was derived from RHAMM and, respectively, denominated R3, has demonstrated positive and safe effects in generating CD8+ cytotoxic cellular responses and anti-tumor traits in patients with myelodysplastic syndrome, acute myeloid leukemia, multiple myeloma, as well as chronic lymphocytic leukemia (95, 96).

ICAM-1 is of great importance for immune response, inflammation, and wound healing. Indeed, it is a key molecule for leukocyte adherence and transendothelial migration with significant HA participation in this regulation (68, 97). ICAM-1/HA interactions have been implicated in various inflammatory processes. Thus, a reduction of ICAM-1 expression, mediated by HA may have an anti-inflammatory role in a rat model of severe non-bacterial cystitis (98). Indeed, the anti-inflammatory effect of HMWHA is suggested to be perpetrated through interactions with more than one hyaladherin, including ICAM-1 (99). These authors show that the inhibition of inflammation promoting cathepsin K and MMP-1 activities is accomplished through joint TLR4, CD44, and ICAM-1 actions. Up to date, however, ICAM-1/HA interactions have not been examined in cancer-induced inflammation. The cancer inflammation-related effects of HA and its respective receptors are schematically depicted in Figure 1.

CONCLUSION AND PERSPECTIVES

The tumor microenvironment plays a key role in cancer progression. Specifically, HA-rich tumor microenvironments regulate important host–tumor interactions and have significant impact on cancer-related inflammatory processes. One area, which holds promise for cancer immunotherapy, is the manipulation of immune responses, ultimately providing a therapy that

might “switch” back on the immune system to target the tumor cell. Therefore, by determining the mechanisms through which inflammatory HA fragments are generated in cancer and the respective role of HA receptors will enable us to understand better the contribution of inflammation in malignant disease and perhaps reveal new therapeutic strategies.

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Hyaluronan, inflammation, and breast cancer progression

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Breast cancer-induced inflammation in the tumor reactive stroma supports invasion and malignant progression and is contributed to by a variety of host cells including macrophages and fibroblasts. Inflammation appears to be initiated by tumor cells and surrounding host fibroblasts that secrete pro-inflammatory cytokines and chemokines and remodel the extracellular matrix (ECM) to create a pro-inflammatory “cancerized” or tumor reactive microenvironment that supports tumor expansion and invasion. The tissue polysaccharide hyaluronan (HA) is an example of an ECM component within the cancerized microenvironment that promotes breast cancer progression. Like many ECM molecules, the function of native high-molecular weight HA is altered by fragmentation, which is promoted by oxygen/nitrogen free radicals and release of hyaluronidases within the tumor microenvironment. HA fragments are pro-inflammatory and activate signaling pathways that promote survival, migration, and invasion within both tumor and host cells through binding to HA receptors such as CD44 and RHAMM/HMMR. In breast cancer, elevated HA in the peri-tumor stroma and increased HA receptor expression are prognostic for poor outcome and are associated with disease recurrence. This review addresses the critical issues regarding tumor-induced inflammation and its role in breast cancer progression focusing specifically on the changes in HA metabolism within tumor reactive stroma as a key factor in malignant progression.

Keywords: hyaluronan, breast cancer, inflammation, tumor microenvironment, RHAMM/HMMR, CD44, macrophage

Hyaluronan as a Component of Cancerized Stroma in Human Breast Cancer

During breast cancer growth, tumor cells interact with their surrounding stroma to create an environment resembling that found during wound healing with increased inflammation, angiogenesis, and stromal remodeling. Both tumor cells and fibroblasts produce pro-inflammatory chemokines and cytokines, which recruit and activate innate immune cells including neutrophils and macrophages (1). Macrophages are recruited by tumor cells as a key component of the inflammatory microenvironment and have been strongly implicated in breast cancer growth and progression in patients (1–4). Together, the tumor cells, fibroblasts, and inflammatory cells produce factors that remodel the extracellular matrix (ECM), leading to the formation of a “cancerized”

microenvironment that sustains tumor growth and promotes malignant progression. The ECM is composed of proteins and proteoglycans/glycosaminoglycans that provide structural support and facilitate tissue organization. In addition, specific components of the ECM contribute to cell survival, proliferation, migration, angiogenesis, and immune cell infiltration. A major ECM component of the stroma is hyaluronan (HA), a member of the glycosaminoglycan family of polysaccharides. HA is synthesized at the cell surface as a large linear anionic polymer (up to 10^7 Da) by multiple cell types in healing wounds and in tumors. There are three distinct isoenzymes (HA synthases, HAS 1–3) that synthesize HA (see below). Understanding the role that HA plays in contributing to breast carcinoma-induced inflammation has important implications for the design of therapeutic approaches targeting both the tumor cells and the pro-tumorigenic functions of the cancerized stroma (5).

A number of studies have demonstrated that HA regulates tumor cell migration and invasion *in vitro*, and tumor growth and progression *in vivo* (5–7). Cell culture studies show that invasive breast cancer cells synthesize and accumulate larger amounts of HA than normal tissue and preferentially express more HAS2 mRNA than less aggressive tumor cells (8). Furthermore, HAS2 promotes breast cancer cell invasion *in vitro* (9). Overexpression of HAS2 in mammary epithelial cells of MMTV-Neu transgenic mice increases tumor HA production and enhances growth of mammary tumors (10). HAS2-overexpressing tumors exhibit enhanced angiogenesis and stromal cell recruitment. These results demonstrate that increased HA in the tumor microenvironment supports mechanisms of neoplastic progression.

Although carcinoma cells synthesize HA, stromal HA levels are increased in breast cancers predicting that stromal cells are also a rich source of this biopolymer. Similar to the contributions of HA fragments in wound healing, HA fragmentation leads to the generation of angiogenic fragments that act on endothelial cells to promote blood vessel formation (11). As described below, HA regulates inflammatory cell functions located within the tumor microenvironment. The combined effects of HA on both tumor and host cells as well as evidence that elevated accumulation of peri-tumor stromal HA is linked to reduced 5-year survival (12), provide strong evidence that HA participates in the generation of a pro-tumorigenic “cancerized” stroma (5).

In-depth analysis of the HA staining patterns within tumors shows an enrichment of HA in the stroma at the leading edge of the tumor, and detailed clinical study of these HA levels and localization in patient samples support a relationship between high-stromal HA accumulation and poor patient survival (12). While most HA is likely synthesized by stromal cells, a subset of breast cancers also stain for HA in the tumor parenchyma and this is correlated with lymph node positivity and poor differentiation (12). Furthermore, these tumors tend to be negative for the hormone receptors estrogen receptor (ER) and progesterone receptor (PR) (12).

Hyaluronan accumulation has additionally been compared in early and later stage breast tumors, specifically in ductal carcinoma *in situ* (DCIS), DCIS with microinvasion and invasive carcinoma, to determine if altered HA production is linked to early as well as later stage invasion events in breast cancer. HA levels of DCIS

associated with microinvasion and later stage invasive carcinoma are significantly increased when compared to pure DCIS (13). RHAMM/HMMR, which promotes migration and invasion of breast cancer lines, is also elevated in breast cancer, particularly at the invasive front of tumors and in tumor cell subsets (14, 15). Collectively, these results suggest that HA performs a number of functions in progressing tumors and in particular contributes to invasion in early and later stage breast cancer.

More recently, HA staining and CD44 expression have been examined in HER2-positive breast tumors. High levels of stromal HA staining in this breast cancer subtype have been linked to specific clinical correlates, including lymph node-positive breast cancer and reduced overall survival (16). Elevated CD44 expression, which occurs in tumor parenchyma and to a lesser extent in stromal cells, is associated with HER2-positive breast cancers and linked to reduced overall survival in this breast cancer subtype. A number of studies have also examined expression levels of HA synthases in breast cancer tissues. Expression of all of the HAS isoenzymes (HAS 1–3) have been detected in the tumor parenchyma and stroma of breast tumors (17). Expression of tumor cell HAS1, but not HAS2 or HAS3, was found to correlate with reduced overall survival when breast cancer patients were not sorted into subtypes. In this study, expression of all three HAS proteins in the stroma corresponds with reduced overall survival (17). However, HAS2 expression is particularly linked to triple negative and basal-like breast cancer subtypes and its elevated expression is associated with reduced overall survival of these cancer patients (18).

Together, these studies suggest pro-tumorigenic roles for increased levels of HA in breast cancer (19, 20) and predict possible mechanisms through which HA might facilitate tumor initiation and progression. For example, the increase in tumor cell HA may provide a self-protective coat, minimizing recognition by immune cells and helping to reduce damage by reactive oxygen and nitrogen species. Increased levels of HA may also facilitate mitosis and invasion of surrounding tissue. However, recent studies demonstrating that fragmentation of HA within damaged tissues alters biological properties of the intact biopolymer and that HA receptors differ in their recognition of HA polymer sizes suggest a much more complex mode of regulation (see below). These more recent studies emphasize the importance of defining both changes in HA levels and the extent of HA fragmentation for understanding mechanisms by which the peri-tumor stroma, in particular the inflammatory status of the tumor-associated stroma, influences breast cancer initiation and progression.

Regulation of HA Synthesis and Fragmentation

Elevated HA synthesis in adults is most often associated with a response to tissue damage or disease. These increases result from both transcriptional and post-transcriptional control of HA synthesis. HA synthesis is catalyzed by one or more of three HA synthase isoenzymes (HAS 1–3) (21, 22), which are unique among other glycosyltransferases since they are localized at the plasma membrane rather than in the Golgi (23). Primary structures of all three enzymes predict that they span the plasma

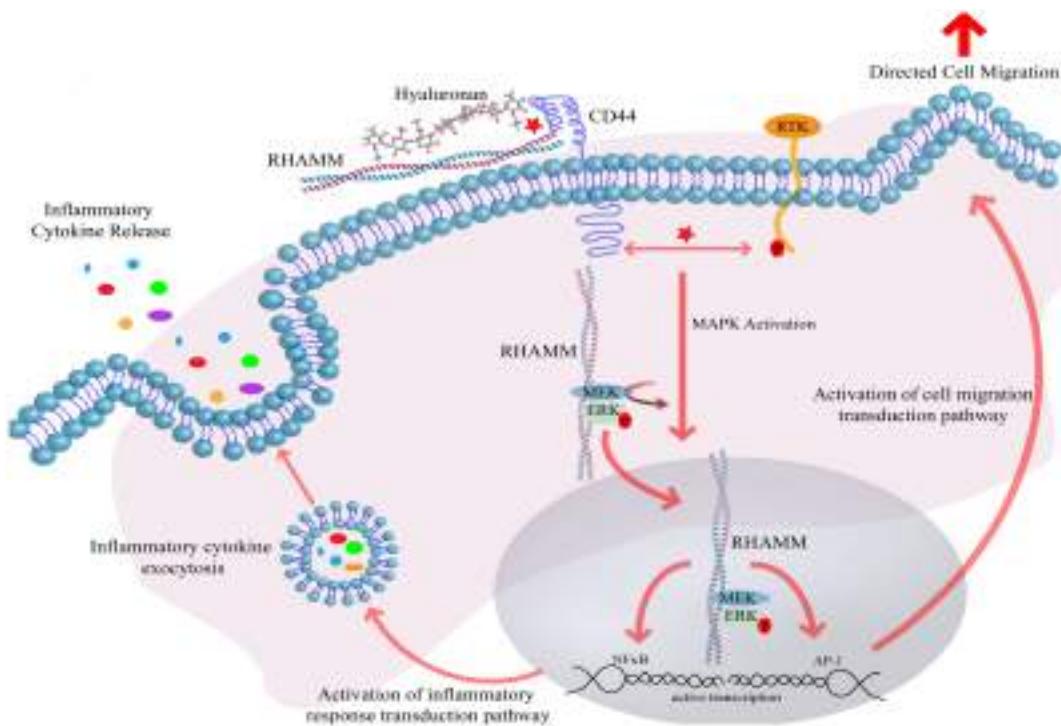


FIGURE 1 | Hyaluronan induces receptor-mediated signaling through interaction with cell-surface HA binding proteins.

Interaction of HA with CD44 and RHAMM induces CD44 receptor clustering and intracellular RHAMM-regulated MAPK activation, resulting in ERK phosphorylation and downstream activation of the transcription effectors AP-1 and NF κ B. Active transcription of AP-1 and NF κ B target

genes ultimately result in the induction of directed cell migration and release of inflammatory cytokines. Abbreviations: MEK, MAPK ERK kinase; ERK, extracellular regulated kinase; RHAMM, receptor for hyaluronan-mediated motility; CD44, cluster of differentiation 44; AP-1, activator protein 1; RTK, receptor tyrosine kinase; NF κ B, nuclear factor kappa B.

membrane several times (21). The three isoforms contain cytoplasmic catalytic sites that sequentially add the activated UDP-D-glucuronic acid and UDP-N-acetyl-D-glucosamine to the growing HA polymer, which is then extruded through pores in the plasma membrane, likely created by formation of HAS oligomers (21, 22). Released HA polymers are captured by extracellular HA binding proteoglycans such as versican, along with other ECM protein components and cell-surface receptors (5, 6, 24). High-molecular weight polymers of HA are thought to function like other ECM components, in part, by providing a multivalent template to organize ECM proteoglycans and to cluster HA receptors thus “organizing” plasma membrane components. Clustering leads to subsequent cytoskeletal re-organization, the efficient assembly and activation of signaling pathways and ultimately changes in the cellular transcriptome (Figure 1).

Hyaluronan synthesis is controlled by multiple distinct but overlapping mechanisms. HA synthesis is partially regulated by intracellular levels of the HAS substrates UDP-GlcA and UDP-GlcNAc. These are produced by complex pathways that control their levels and/or availability within cells. For example, the compound 4-methyl-umbelliflorone (4-MU) inhibits the synthesis of HA by depleting cytoplasmic UDP-GlcA, (25). Although 4-MU theoretically could limit substrate availability to multiple glycosyltransferases, its inhibitory effect appears to be localized to limiting substrate availability for HAS isoforms associated

with the inner plasma membrane. By contrast, the majority of glycosyltransferases are resistant to the inhibitory effects of 4-MU since they are located in the Golgi, which is not permeable to 4-MU (23). The genes encoding HAS isoforms are located on distinct chromosomes and their expression is regulated by distinct transcriptional and post-transcriptional mechanisms (23, 26, 27). Numerous wound and tumor-associated cytokines and growth factors promote HA synthesis, including TGF β , PDGF, FGF2, EGF, and TNF α [Ref. (23) and references therein]. It is important to emphasize that regulation of HAS isozyme expression and activity can be cell and tissue specific. Thus, careful analysis is needed when considering HA-related mechanisms in different pathologies. For example, HAS2 transcription can be regulated via EGFR/STAT3 pathways, PKA/CREB pathways (23), or TNF α or IL-1 β -induced activation of NF- κ B. The latter pathway is particularly relevant to the impact of up-regulated HA synthesis in the context of inflammation. While transcriptional control is a major mechanism for regulating HA synthesis, post-translational modification of HAS isoforms also affects their activity and/or cell-surface localization. For example, ErbB2/ERK1, 2 signaling activates and phosphorylates the three HAS isoforms implicating this as a mechanism for up-regulated HA synthesis in HER2/neu-positive tumor subtypes (28). HAS proteins can also be covalently modified by O-GlcNAcylation, which modifies trafficking and/or subcellular localization of the enzymes to the

plasma membrane (23). Finally, early evidence from analyzing the naked mole rat genome shows that activating mutations of HAS2 can be selected for, that increase not only the production but also the predominant size of HA synthesized by this HAS isoform (29).

Large, native HA polymers clearly participate in the architectural maintenance and hydration of homeostatic adult tissues. However, recent evidence demonstrates that HA fragmentation is a critical contributing factor in the physiology of wounds and cancerized stroma (27, 30, 31). While larger HA polymers appear to be anti-inflammatory and anti-tumorigenic, HA fragments and oligomers are pro-inflammatory and pro-tumorigenic. This has led to the concept that HA fragmentation is one of the initial “danger signals” sensed by cells to initiate efforts that limit tissue damage through promoting tissue inflammation and repair (5). This cycle of increased synthesis and fragmentation appears to be hijacked by tumor cells and their stromal partners to sustain inflammation, which contributes to malignant progression. The mechanisms by which HA fragmentation contributes to such tissue pathology are not well understood. One proposed function is that LMW fragments alter or disrupt the cellular “organizing” properties of HMW HA by inhibiting the HA-induced clustering of cell-surface receptors such as CD44 and affecting signaling (6, 24, 32). Direct pull down assays of cellular extracts using beads coupled to HA oligomers have demonstrated that tumor cell and wound RHAMM can bind LMW HA fragments (33). This scenario predicts that cell-surface RHAMM, displayed in response to cellular stress, is one HA receptor that “senses” HA fragmentation and thus serves to initiate cellular responses to tissue damage possibly by affecting CD44 clustering (5). These previous studies point to the importance in determining both the level of HA and the ratio of HMW HA to LMW fragments noted previously (34, 35) and in an accompanying manuscript in this issue (36).

Hyaluronan fragmentation within tissues results from the increased expression of one or more hyaluronidases (Hyals) and from oxidative/nitrosative damage. Hyals function as endo- or exoglycosidases to cleave HA polymers (27, 30). Hyal1 and Hyal2 are most often associated with damaged or tumor-associated stroma undergoing remodeling (27). *In vitro* analysis of hyaluronidases indicates that their activity results in unique fragmentation patterns. For example, although both Hyal1 and Hyal2 can catalyze degradation by cleaving β -(1,4) linkages, they differ in that Hyal1 degrades HA into small fragments (hexasaccharides and tetrasaccharides) whereas Hyal2 appears to produce predominantly larger (i.e., 20 kDa) fragments (37). Both Hyal1 and Hyal2 have pH optima in the acidic range and are associated with processing HA that has been internalized into endocytic vesicles. However, low pH within localized stromal microenvironments facilitates extracellular Hyal-mediated HA degradation (27).

Hyaluronan is also fragmented by reactive oxygen and nitrogen species (ROS/RNS) such as hydroxyl radicals ($\bullet\text{OH}$), peroxynitrite/peroxynitrous acid ($\text{ONOO}^-/\text{ONOONH}_2$), and hypochlorite anion (OCl^-). Iron, derived from tissue associated heme or ferritin, is one important contributor in catalyzing the formation of both hydroxyl radicals and superoxide anions (O_2^{\bullet}). This mechanism is contributed to by infiltrating polymorphonuclear leukocytes, monocytes, and activated macrophages (38). HA is

extensively cleaved by any of these reactive species, and they are therefore important mechanisms for HA fragmentation within inflamed tissues. Although assessment of HA fragmentation by these mechanisms have largely been defined using *in vitro* analyses (39), it is clear that this degree of HA fragmentation occurs in skin wound tissue and in human milk (34, 35).

Cellular Receptors for Hyaluronan

Although a number of HA receptors have been identified, the two that have been best characterized and are to date most relevant to inflammation and breast cancer are CD44 and RHAMM (5, 27). Other receptors implicated in cellular responses to HA, TLR2, and TLR4, are discussed in more detail below. Interactions between HA and CD44 lead to ligand-induced clustering, and activation of intracellular signaling pathways such as ERK1, 2, Akt, and FAK. The binding of HA by CD44 occurs through interactions with an amino terminal “link” domain, similar to those found in several other types of HA binding proteins, in particular, extracellular HA binding proteoglycans such as versican, aggrecan, and link protein. RHAMM binds HA through structurally distinct domains (BX₇B motifs where B is a basic amino acid residue and X are non-acidic residues) that differ from link domains (5, 27). While CD44 expression is ubiquitous, RHAMM is normally not detected in most homeostatic tissues, but expression increases in response to injury and thus seems to be primarily important for restoring homeostasis following injury (5). Null RHAMM mice are viable but exhibit defects in tissue response to injury including vascular damage and excisional wound healing (40). RHAMM may also be required for robust female fertility in mice (41). Interaction of HA with CD44 is often associated with increased cell motility and invasion, although numerous reports have demonstrated that CD44 can also modify growth and therapeutic resistance of tumor cells (6, 24). As with CD44, RHAMM is displayed on cell surfaces. However, unlike CD44, RHAMM surface expression is tightly regulated, occurring under conditions of cellular stress. Thus, RHAMM is largely a cytoplasmic protein whose surface localization is regulated by mechanisms similar to other non-conventionally exported cytoplasmic and nuclear proteins and that regulates signaling cascade activation through co-receptor functions with integral receptors such as CD44 (5). Cell surface and intracellular RHAMM are also involved in stimulating cell motility and invasion. Intracellular RHAMM co-distributes with interphase microtubules and a splice variant of human RHAMM has been detected in nuclei (14, 42). RHAMM expression increases in G2/M of the cell cycle, associating it with mitosis and modifying cell-surface RHAMM blocks cells in G2M (43). This is consistent with more recent reports indicating that RHAMM is a critical contributor to mitotic spindle formation and regulation of proper chromosomal segregation and genomic stability (44). Both CD44 and cell-surface RHAMM also function as co-receptors for activating transmembrane tyrosine kinases (including EGFR, c-MET, and PDGFR) and ERK1,2 (Figure 1).

Both CD44 and RHAMM regulate the intensity and/or duration of such signal transduction pathways as ERK1, 2, which are initiated by growth factors (40, 45). Intracellular RHAMM functions as a scaffold protein that directly binds to ERK1 and

forms complexes with ERK1, 2, and MEK1. This has been proposed to be one mechanism by which RHAMM helps to increase the intensity and/or duration of oncogenic ERK1, 2 signaling pathways (46, 47). One consequence of HA, CD44, RHAMM-mediated increases in the duration of ERK 1, 2 activation is the alteration of the transcriptome of cells within the cancerized stroma (Figure 1). These changes in gene expression have an impact on the activation of transduction pathways related to cell migration and the expression and export of inflammatory mediators. In turn, the persistent activation of these pathways in cancerized stroma enhances pro-tumorigenic inflammation and breast tumor progression. Thus, this represents one major mechanism by which biological “information” encoded within HA can lead to pro-tumorigenic or “cancerized” alterations in stroma.

Positive paracrine and autocrine feedback loops between tumor and stromal cells can be initiated by inflammatory mediators such as IL-1 α and TGF β that increase HA synthesis and expression of both RHAMM and CD44, which collectively sustain cell migration and invasion within cancerized stroma. Thus, the aberrant upregulation of CD44 or RHAMM in cancerized stroma is a nefarious consequence of sustained ERK 1, 2 activation, further aggravating persistent oncogenic signaling (46, 47). Since CD44 and RHAMM functionally cooperate under certain conditions (40), targeting RHAMM may be an effective way to specifically limit the function of CD44 in breast tumors.

LYVE-1, another cell-surface HA receptor associated with cancerized stroma (48–50), was first identified as a surface marker expressed by lymphatic endothelium and has been proposed to serve in HA transport from interstitial tissue to lymph (51). However, studies addressing the obligatory importance of LYVE-1 in promoting normal lymphangiogenesis have yielded conflicting results (52, 53). In tumors, the density of stromal LYVE-1 positive lymphatic vessels is a negative prognostic indicator in breast cancer patients with invasive ductal carcinomas (54). Furthermore, *in vitro* studies suggest that HA and LYVE-1 promote adhesion of breast cancer cells to fibroblasts, predicting these interactions contribute to adhesion or dissemination of tumor cells (55). Nevertheless, a mechanistic role for LYVE-1 in poor prognosis of breast cancer has yet to be demonstrated. One possible mechanism is suggested by the expression of LYVE-1 in cancer-associated macrophages (56) but a causative role for this HA receptor in inflammation has yet to be established.

Effects of Hyaluronan on Innate Immune Cells in Cancerized Stroma

The generation of a pro-tumorigenic inflammatory environment during breast cancer initiation and progression requires recruitment of inflammatory cells, including neutrophils and macrophages. Once recruited to the tumor site, these cells become activated and secrete factors that are normally involved in proliferation, angiogenesis, and stromal remodeling during tissue repair (1). Macrophages residing within the tumor parenchyma and the tumor reactive stroma are prognostic of poor outcome in breast cancer patients (57). Macrophages in a wound-healing context are characterized as pro-inflammatory (M1) or anti-inflammatory (M2) (58). Pro-inflammatory macrophages are involved in the

initial stages of wound healing and are characterized by the expression of NF- κ B-regulated pro-inflammatory cytokines, including IL-1 β and IL-12 as well as mediators contributing to pathogen destruction, including reactive oxygen species. Anti-inflammatory macrophages are important for the resolution phase of the wound-healing process and they are characterized by expression of anti-inflammatory cytokines, including TGF β and IL-10 as well as factors that promote tissue remodeling including the MMPs. Profiling and functional studies demonstrate that macrophages within the tumor microenvironment express a range of both pro- and anti-inflammatory factors depending upon tumor type and stage. For example, macrophages associated with early stages of tumorigenesis have high levels of NF- κ B activation and subsequently express pro-inflammatory factors, such as IL-1 β and IL-6 (59). As tumors become increasingly aggressive, tumor-associated macrophages express high levels of immunosuppressive cytokines, such as IL-10 and TGF β (58). Tumor-associated macrophages also produce factors that are established promoters of breast cancer growth and progression including EGF, VEGF, and MMP-9 (60). Thus, it is clear that tumor-associated macrophages reside in a functional continuum that is regulated by specific factors within the tumor microenvironment. However, the specific factors within the microenvironment that macrophages are responding to and driving these responses are not well understood.

A primary function of monocytes and macrophages in wound-healing environments is to produce reactive oxygen intermediates, which contribute to pathogen killing during wound healing (58). High levels of reactive oxygen species, found in both wound healing and tumor environments, are known to fragment HA, which then induce expression of pro-inflammatory genes (38, 61, 62). Recent studies of human breast cancer samples demonstrate that high numbers of CD163 positive macrophages correlate with increased levels of HA synthases and HA accumulation within tumors (63). Based on the links between HA and macrophages during wound healing, it is likely that HA in the tumor microenvironment may regulate macrophage function.

Indeed, HA modulates expression levels of pro-tumorigenic cytokines and chemokines in macrophages. Specifically, HA induces expression of the pro-inflammatory cytokine IL-1 β in macrophages (64). Numerous studies have implicated IL-1 β in breast cancer initiation and progression. Expression of IL-1 β is increased in tumor and stromal cells in 90% of ER negative invasive breast carcinomas (65, 66). In addition, high levels of serum IL-1 β correlate with recurrence in breast cancer patients (67). Finally, IL-1 β may also be involved in premalignant breast cancer based on studies that show increased IL-1 β expression in pre-invasive DCIS (65, 68). Mechanistically, increased IL-1 β within the tumor microenvironment leads to enhanced expression of cyclooxygenase-2 (COX-2), which contributes to the formation of early stage lesions and is a well-established tumor promoter (69). Increased IL-1 β also leads to mammary tumor growth and metastasis in part through inducing regulation of myeloid derived suppressor cells (MDSCs), which promote an immunosuppressive environment (70). Taken together, these studies suggest that modulation of pro-inflammatory cytokines by HA in the tumor microenvironment represents a potential

mechanism through which HA might contribute to tumor growth and progression.

The precise mechanisms by which elevated levels of stromal HA modulate pro-inflammatory responses are not well understood. Similar to the wound-healing environment, both increased levels of hyaluronidases (71, 72) and reactive oxygen or nitrogen species, including nitric oxide are present in breast tumors (39, 73), predicting elevated HA fragmentation in the tumor microenvironment. *In vitro* studies demonstrate that increased HA fragmentation is correlated with elevated hyaluronidase expression by breast cancer cells (74). Studies focusing specifically on hyaluronidase 1 (Hyal1) demonstrate that enhanced expression of Hyal1 in breast cancer cells induces tumor cell proliferation, migration, invasion, and angiogenesis (75). Furthermore, knock-down of Hyal1 in breast cancer cells reduces cell growth, adhesion, and invasion in culture as well as decreased tumor growth *in vivo* (72). Breast cancer cells lacking ER expression typically produce more hyaluronidases than estrogen positive cells and this correlates with invasion *in vitro* (15). LMW HA fragments, but not total HA levels, detected in the serum of breast cancer patients also correlates with the presence of lymph node metastasis (74). In addition, Hyal1 expression in non-invasive ductal hyperplasias correlates with subsequent development of invasive breast carcinoma (76). These studies indirectly establish a link between breast cancer and HA fragmentation (**Figure 2**), although studies analyzing the accumulation of HA fragments in experimental or clinical breast cancer tissues are still lacking. Because HA fragments are pro-inflammatory, it is reasonable to assume that they contribute to production of inflammatory cytokines, chemokines, and proteases by tumor-associated macrophages (27). In contrast to LMW fragments, HMW HA suppresses expression of many of the above pro-inflammatory cytokines in macrophages (77). This opposing function of native HA suggests that both the level and the distribution ratio of different size HA fragments may dictate inflammatory cell phenotypes within cancerized stroma. Development of new technologies to isolate and characterize HA polymers and fragments from tissues will be key for developing a mechanistic understanding of the biological complexities associated with HA metabolism (35).

In addition to regulating pro-inflammatory cytokine production, HA can modulate the expression of anti-inflammatory cytokines. Analysis of macrophage responses to tumor cell conditioned media demonstrates that tumor cell-derived HA stimulates production of IL-10 by macrophages (78). IL-10, an anti-inflammatory cytokine, is a potent mediator of immunosuppression in the tumor microenvironment through inhibition of T cell activation (79). Recent studies have demonstrated that increased IL-10 in the breast cancer microenvironment leads to therapeutic resistance through multiple potential mechanisms. For example, increased levels of IL-10 lead to the suppression of CD8⁺ T cell responses in response to chemotherapy (**Figure 2**) (4). Furthermore, IL-10 has been found to act directly on breast cancer cells to promote survival in response to chemotherapy involving a STAT3/bcl-2 mechanism (80). Thus, it is possible that HA contributes to immunosuppression and therapeutic resistance through modulation of IL-10 in the tumor microenvironment.

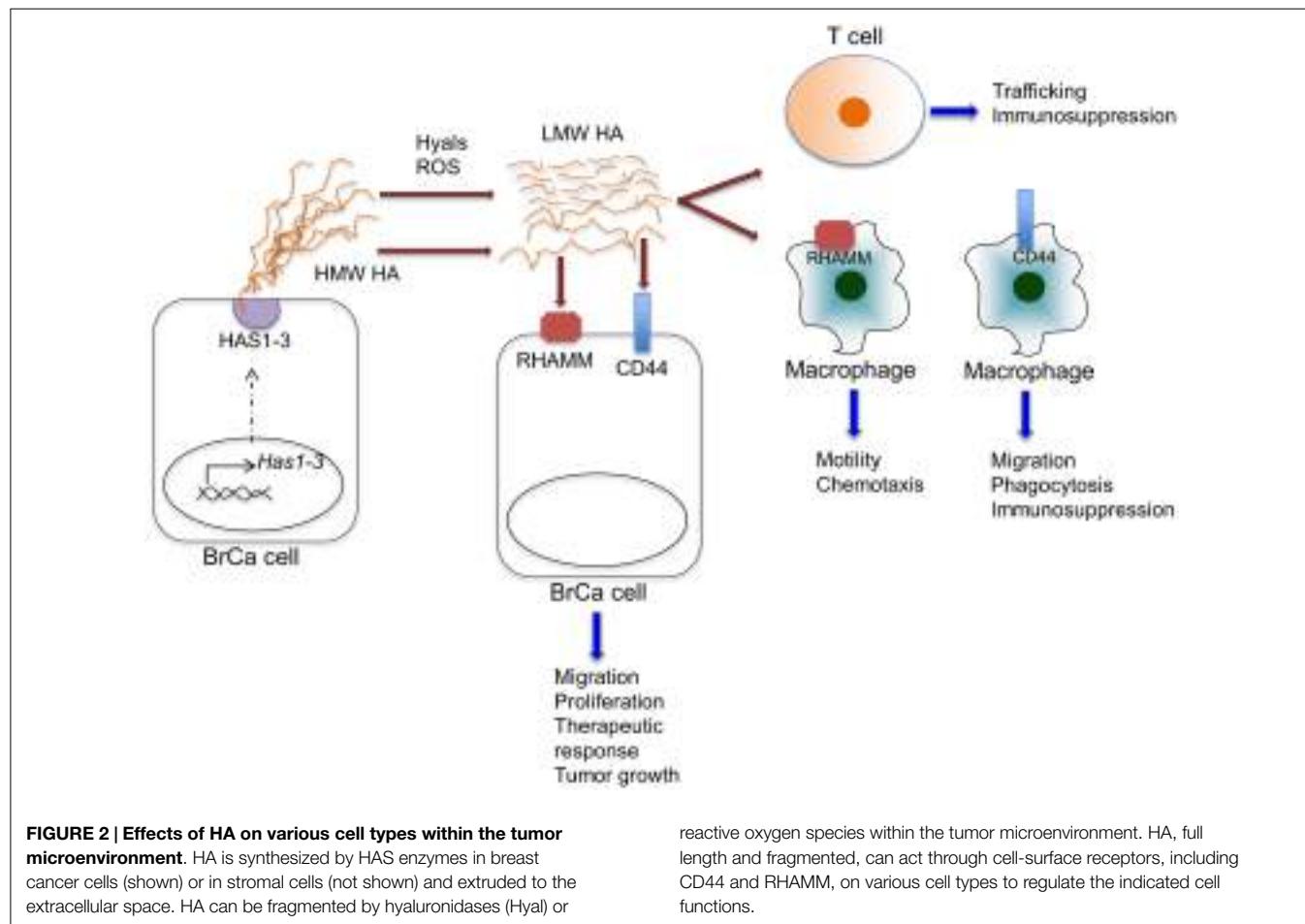
Hyaluronan also controls expression of chemokines, including IL-8/CXCL8 (81). Chemokines are pro-inflammatory cytokines that play an essential role in leukocyte recruitment and cell trafficking. These secreted proteins interact with cell-surface G-protein-coupled receptors to induce cytoskeletal rearrangement, adhesion to endothelial cells, and directional migration of cells to specific tissue sites (82). For example, IL-8 binds its receptors, CXCR1 and CXCR2, to stimulate neutrophil chemotaxis (67). IL-8 is overexpressed in breast cancers and contributes to tumor initiation and growth through promoting migration and invasion of breast cancer cells. More recently, studies have implicated IL-8 in the regulation of breast cancer stem cell invasion (83). Macrophage chemokines that are regulated by HA, including CXCL2 and CXCL12, have similarly been implicated in breast cancer progression (27) and have been shown to promote migration and invasion of these cancer cells (84, 85). The CXCL12/CXCR4 axis is particularly important for homing of breast cancer cells to metastatic sites, including bone and lung (86).

In another positive feedback loop, HA production is also modulated by pro-inflammatory signaling pathways. For example, both IL-1 β and TNF α induce HA production in endothelial cells in an NF- κ B-dependent manner (87). We have also demonstrated that HA synthesis is enhanced in tumor cells through an IL-6/STAT3-dependent mechanism (88). Furthermore, inflammatory macrophages express hyaluronidases (89) and ROS (58), which potentially fragment HA into pro-inflammatory polymers. These results predict that HA and pro-inflammatory cytokines act reciprocally to sustain inflammation.

Contributions of Hyaluronan Receptors and Binding Proteins to Inflammation

A major challenge in the mechanistic understanding of HA in breast cancer-associated inflammation is to link HA metabolism with specific contributions of HA receptors CD44, RHAMM, and LYVE-1, which are all expressed by macrophages (78, 90–92). CD44 has been examined for its ability to regulate macrophage migration and phagocytosis (93). In the context of modulating macrophage responses to tumor cells, functional studies demonstrate a link between CD44:HA binding and generation of immunosuppressive macrophages. Specifically, blocking the ability of HA to bind to monocytes either through blocking HA:CD44 binding or using an HA-specific blocking peptide inhibits tumor cell conditioned media promoted formation of immunosuppressive macrophages (78).

While RHAMM has not been examined specifically in the context of tumor-associated macrophages, recent studies have started to elucidate its potential functions during response to injury. RHAMM expression is induced in macrophages following chemically induced lung injury (94) and in excisional skin wounds (34) and blocking RHAMM function in these injuries reduces the level of tissue macrophages (31, 34). Additional studies have demonstrated that RHAMM regulates macrophage chemotaxis in response to TGF β in the context of surfactant protein A-mediated inflammation in the lung (92). While not specifically addressed, these studies predict the potential of RHAMM for



promoting HA-mediated macrophage motility and chemotaxis in tumor-associated inflammation.

While the contributions of HA interactions with LYVE-1 to macrophage functions are even less well understood, recent interest in LYVE-1 as a marker of tumor-associated macrophages suggests that further studies of these interactions are warranted (90). Given the numerous effects of HA on macrophage recruitment and function, a focus on the roles of HA receptors in mediating tumor-associated macrophage functions will likely dramatically increase understanding of the mechanisms driving macrophages to promote breast tumor progression.

Studies have also suggested a link between HA and toll-like receptor (TLR) signaling in macrophages (27, 95). Specifically, LMW HA induces expression of pro-inflammatory cytokines and chemokines, mediated in part by TLR2 and/or TLR4 (27, 95). Additional published studies using blocking antibodies have suggested that the TLR-mediated effects may require interactions with CD44 (96). TLR signaling has been implicated in breast cancer progression, as TLR4 is expressed at high levels on invasive breast cancer cells and knock-down of TLR4 leads to reduced cell proliferation and survival (97). *In vivo* studies have suggested that TLR4 agonists can inhibit mammary tumor metastasis (98, 99). By contrast, recent studies using a potential TLR4 agonist demonstrated enhanced survival of mice in a model of tumor resection, suggesting that the contributions of

TLR4 to breast cancer progression are complex (100). Recent studies have suggested that breast cancer cell-derived exosomes modulate inflammatory cytokines in macrophages potentially involving both TLRs and CD44 (101). While direct interactions between HA and TLRs in breast cancer cells have not been established, additional studies examining HA and TLR signaling in both tumor cells and the microenvironment are warranted.

Tumor necrosis factor-stimulated gene-6 (TSG-6), which is an extracellular HA binding protein, is synthesized and secreted at sites of inflammation (102). TSG-6 binds HA with high affinity via a link module and enhances binding of HA to CD44 (103). TSG-6 also contributes to HA cross-linking, which has been implicated in adhesion and rolling of leukocytes (104). In the context of breast cancer, TSG-6 is up-regulated in breast cancer cells following ionizing radiation, suggesting a potential role for TSG-6 when tissue is damaged (105). It will be interesting to determine the contributions of TSG-6 to HA remodeling and function within the breast cancer microenvironment.

Effects of Hyaluronan on Adaptive Immune Cells in Cancerized Stroma

In addition to innate immune cells, adaptive immune cells are also prevalent within the breast cancer microenvironment. Immune

cell profiling studies have demonstrated that breast cancer with high levels of macrophages and Th2 T cells are associated with worse outcome than those with high levels of Th1 cells (106). More recently, studies have demonstrated that the presence of infiltrating T cells and B cells predict better response to neoadjuvant chemotherapy in breast cancer patients (107). Understanding the regulation and function of adaptive immune cells during both tumor progression and therapy is a rapidly growing focus of research in the breast cancer field.

While the potential role of HA on tumor infiltrating lymphocytes has not to our knowledge been reported, HA is known to contribute to the regulation of T cell trafficking (Figure 2). Studies have demonstrated that upon activation, T cells adhere to and migrate on native HA (108). Other studies show that HA:CD44 interactions on T cells can contribute to activation-induced T cell death (109). This response occurs following exposure to HMW, rather than LMW HA, suggesting an additional anti-inflammatory role for HMW HA. Finally, HMW HA has also been found to promote the immunosuppressive functions of regulatory T cells (Tregs) (110). Exposure of Tregs to HMW HA leads to prolonged expression of Foxp3, a transcription factor that is required for Treg function. Collectively, these studies predict an important role for HA in the regulation of T cell recruitment and/or function.

Targeting HA Metabolism as a Potential Therapeutic Strategy in Breast Cancer

Given these links of HA and its receptors with breast cancer progression, targeting HA metabolism represents a potential therapeutic approach for treatment of breast and other cancers. There are multiple potential points in the HA metabolic pathway that could potentially be targeted including HA synthesis, accumulation, degradation, and/or HA:receptor interaction. Use of 4-MU, an inhibitor of HA synthesis, is a common approach for blocking HA synthesis in experimental models of breast cancer and is described in detail in another article in this Research Topic (111). Numerous studies have demonstrated that inhibition of HA synthesis using 4-MU reduces breast cancer tumor cell proliferation and migration (88, 112, 113). Furthermore, treatment of tumor bearing mice with 4-MU reduces tumor growth (114, 115). Treatment of mice bearing bone metastatic lesions with 4-MU reduces HA accumulation and growth of osteolytic lesions (116, 117). 4-MU is well-tolerated in both animal models suggesting that blocking HAS catalytic function represents a viable therapeutic strategy. While the efficacy of targeting HA synthesis alone remains to be determined in human cancers, we have recently demonstrated that reducing HA synthesis combined with targeted therapy enhances therapeutic response (88). These studies highlight the importance of combinatorial targeting of both tumor cell specific oncogenic signaling pathways and pro-tumorigenic alterations in the tumor microenvironment in new therapeutic approaches.

Elimination of HA in the tumor microenvironment using hyaluronidases has also been explored as a potential therapeutic strategy for some cancers, including pancreatic cancer, and is currently being tested in clinical trials (118–120). Treatment

of breast cancer cells with bacteriophage hyaluronidase inhibits growth, migration, and invasion in culture (121). Recombinant hyaluronidase, which eliminates stromal HA, allows increased drug access to tumor cells (118–120). Studies suggest that recombinant human hyaluronidase (rHuPH20) improves subcutaneous delivery of antibody-based targeted therapies such as trastuzumab, currently used for treatment of HER2-positive breast cancer (122). HA is a normal component of the breast stroma that provides structural support and contributes to epithelial morphogenesis (123). Whether eradication of HA and/or the generation of fragments due to the hyaluronidase activity negatively affects breast tissue architecture remains to be determined.

Additional approaches to inhibiting HA function in tumors include interfering with HA:receptor interactions. CD44 expression correlates with specific subtypes of breast cancer, including triple negative and endocrine resistant breast cancers (124, 125). Furthermore, HA-CD44 interactions promote invasion and therapeutic resistance (7, 124, 125). Thus, developing targeted therapies that specifically inhibit this interaction could lead to viable therapies for treating breast cancer subtypes that currently have limited therapeutic options. Nevertheless, the use of a humanized monoclonal antibody (Bivatuzumab) in clinical trials of patients with squamous cell carcinomas showed early promise. However, it had a dose related toxicity in some patients and caused the death of one patient causing the trial to be terminated prematurely (126) raising concerns about this therapeutic approach. Furthermore, since there are multiple structural variants of CD44, it may be difficult to develop a complete array of humanized antibodies that can target this structurally complex group of proteins.

An alternative approach, which may be less toxic than Bivatuzumab will be to develop and utilize HA binding peptides that can specifically block HA-stimulated signaling and inflammation. Early efforts along this line using a 12mer phage display resulted in a peptide termed PEP-1, which was identified by sequential binding of 12mer-displaying phage to immobilized HA (127). PEP-1 has been shown to reduce gastric stem cell proliferation (128) and reduce *H. pylori*-induced gastric epithelial proliferation *in vivo* (128). Finally, PEP-1, in combination with the selective activation of the adenosine A2 receptor, inhibits arthritis-associated inflammation (129, 130). While the PEP-1 was effective in these studies, it was not demonstrated to inhibit interactions with a specific HA receptor. More recently, we have developed a unique HA binding “RHAMM mimetic” peptide using a 15mer (P-15) based phage display approach (34). This 15mer approach is unique from PEP-1 in several respects. Unlike PEP-1, P-15 contains a BX₇B HA binding motif found in RHAMM, it binds HA, in particular HA fragments with high affinity, can inhibit HA binding to RHAMM but does not block HA binding to CD44. It inhibits HA-stimulated migration of RHAMM^{+/+} fibroblasts but has no effect on the migration of RHAMM null fibroblasts. P-15 reduces inflammation, angiogenesis, and fibroplasia of RHAMM^{+/+} but not RHAMM^{-/-} excisional wounds. Peptides or mimetics similar to P-15 may offer an effective alternative therapy since specific blockade of RHAMM can also limit CD44 signaling.

Summary

In summary, there is clear evidence that alterations in HA are associated with malignant progression of breast cancer. Based on the known pro-inflammatory properties of HA fragments during wound healing and the increased levels of HA associated with the peri-tumor stroma in breast cancers, it is likely that HA contributes to the generation of a pro-tumorigenic inflammatory environment. This is supported by the recently identified links between HA levels in the tumor stroma and infiltration of macrophages. Analyzing the presence and function of HA fragments within the tumor microenvironment will provide insights into changes in HA metabolism during tumor growth and progression. As described in an accompanying article in this issue (36), advances have been made in the isolation of HA from tissues and analysis of HA fragmentation and addressing these questions is now feasible. Identifying the specific HA receptors involved in mediating recruitment and activation of inflammatory cells, such as macrophages, into the tumor environment and

determining how HA regulates adaptive immune cells will lead to a better understanding of how alterations in HA contribute to host immune responses to breast cancer. Agents that limit aberrant HA synthesis, fragmentation, or block specific HA:receptor interactions is very likely to yield advances in the development of new therapies to limit relapse and recurrence in patients receiving tumor cell targeted therapies.

Author Contributions

KS, MC, PT, ET, and JM contributed to the drafting and revising of this manuscript. All authors approved this manuscript.

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The role of CD44 in the pathophysiology of chronic lymphocytic leukemia

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CD44 interactions with hyaluronan (HA) play a key role in various malignancies, supporting tumor cell migration, adhesion, and survival. In contrast to solid tumors, the expression of CD44 standard and variant forms and their functional interplay with HA is less understood in hematological malignancies. Chronic lymphocytic leukemia (CLL) is a highly abundant B-cell malignancy with a well coordinated balance between cell cycle-arrest and proliferation of tumor subpopulations. The long-term survival and proliferation of CLL cells requires their dynamic interactions with stromal and immune cells in lymphoid organs. Interactions of HA with CD44 and HA-mediated motility receptor (RHAMM) contribute to CLL cell localization, and hence CLL pathophysiology, by shaping homing, interstitial migration, and adhesion of the tumor cells. CD44 can complex with key prognostic factors of CLL, particularly CD38 and CD49d, bridging the gap between prognosis and cellular function. Here, we review the current evidence for the individual and associated contributions of CD44 to CLL pathophysiology, the dynamic functional regulation of CD44 upon CLL cell activation, and possible therapeutic strategies targeting CD44 in CLL.

Keywords: CD44, chronic lymphocytic leukemia, microenvironment, homing, hyaluronan

INTRODUCTION

The tumor microenvironment, shaped by interactions between malignant and non-malignant cells, is influential for tumor formation and progression of various cancers. Chronic lymphocytic leukemia (CLL) is a disease of mature B lymphocytes and is manifested by progressive accumulation of these malignant cells in blood, bone marrow (BM), and lymphoid tissues (1). Characteristically, CLL follows extremely variable clinical courses with survival times ranging from months to decades, making it necessary to classify the patients according to prognostic risk (2). Besides genomic aberrations such as 17p deletion, 13q deletion, trisomy 12, and 11q deletion, a most important and long-established prognostic marker is the mutational status of the B-cell receptor (BCR) immunoglobulin variable heavy chain (IgVH) genes (2–4). Patients with CLL cells that express IgVH genes without significant levels of mutation (<2% difference from germline gene counterpart, “unmutated”) follow a more aggressive clinical course with shorter times to first treatment and overall survival than patients harboring IgVH gene mutations ($\geq 2\%$ difference from germline, “mutated”) (5). Other common prognostic parameters are the extent of expression of CD38 and zeta-chain-associated protein kinase 70 (ZAP-70), earlier suggested as surrogate markers for the IgVH mutation status (6). However, both have independent prognostic power, too.

CD49d, despite being the newest among the prognostic markers, is the strongest flow cytometry-based predictor of overall survival and treatment-free survival in CLL (7). Following the first reports on the poor outcome of patients with an expression

of CD49d on $\geq 30\%$ of the tumor cells (8, 9), its high prognostic relevance has been unequivocally confirmed by several groups (10–15). Expression of CD38 and CD49d is associated in about 80% of samples (12, 16) and the molecules can form macromolecular complexes with CD44 (17, 18).

It is well established that the CLL pathophysiology relies on the lymphoid tumor microenvironment. Unusual for tumor cells, CLL cells circulating in the peripheral blood are cell cycle arrested. *Ex vivo*, CLL cells rapidly die from apoptosis if not co-cultured with immune or stromal cells, suggesting that the malignant cells are in constant need of supportive signals from the lymphoid microenvironment (19). It is therefore believed that at least a subpopulation of the peripheral blood CLL pool is able to recirculate into lymphoid organs in order to receive signals for proliferation and survival. Moreover, retention in these organs appears to favor onset and progression of CLL. Consequently, therapeutic targeting the microenvironmental interactions and lymphoid localization of the malignant cells emerges as a most successful strategy to permanently control CLL. This is impressively reflected by the clinical success of novel drugs such as ibrutinib and idelalisib that inhibit downstream signals of the BCR and retention molecules (20–23). Notably, the mode of action of ibrutinib and idelalisib is likely dual, they antagonize tumor cell proliferation in a NF- κ B dependent manner (24, 25) and disrupt CLL cell retention in lymphoid organs. Particularly, during the first period of treatment with these drugs, a redistribution of CLL cells from the lymphoid organs into the peripheral blood of patients can be observed (21, 26, 27), obviously depriving the tumor cells of supportive signals.

Despite this recent therapeutic progress, the detailed mechanisms that underlie the communication of CLL cells and accessory cells in the lymphoid microenvironment are still far from understood. Adhesion molecules and homing receptors orchestrate the localization and retention of CLL cells in lymphoid proliferation areas where CLL cells receive activation and protection signals. The glycoprotein CD44 can direct microenvironmental communication and intracellular signaling for growth and motility in many types of cancers (28). On hematopoietic cells, CD44 is universally expressed (28). The CD44 gene encodes different CD44 variant (CD44v) isoforms, which are generated by alternative splicing. The standard isoform of CD44 (CD44s) lacks the entire variable region. Hyaluronan (HA), the main ligand of CD44, is bound via a conserved BX₇B binding motif (in which B represents Arg or Lys and X7 represents any seven non-acidic amino acids, but includes an additional Arg or Lys) present in the extracellular part of CD44 (28). The binding ability of the ubiquitously expressed molecules CD44 and HA needs to be strictly controlled. This can be achieved by posttranslational modifications such as glycosylations, CD44v expression, or CD44 clustering (28). In CLL, an external activation stimulus leads to increased CD44v expression and N-linked glycosylation, which induces CD44–HA binding (29). Concordantly, many studies have implicated CD44v rather than CD44s in tumor progression, dependent on the stage of progression and type of tumor (28).

In CLL, elevated CD44s and CD44v serum levels have been suggested as markers for disease progression and potential functional contributions to the pathophysiology have been discussed; however, the underlying biological mechanisms remain elusive. With some aspects controversially described, it has become necessary to further examine and more deeply understand the role of CD44 in this disease. Here, we discuss the prognostic role of CD44 and CD44v, its involvement in localization of CLL cells in lymphoid organs and tumor cell survival, and its suitability for therapeutic exploitation.

HA RECEPTORS AND CLL PROGNOSIS

CD44 is described to form a complex with the prognostic markers CD49d and CD38, outlined in the introduction (18, 30). However, first reports on an individual prognostic role of CD44 in CLL were already published in the early 1990s (31), long before this complex was found. Despite this early discovery, the existing data are not completely consistent. In 1993, de Rossi and colleagues distinguished three groups of CLL patients, depending on either high, intermediate or low CD44 surface expression, defined in relation to the CD44 expression on T-cells. In this study, patients of the CD44-high group presented with an increased incidence of diffuse BM infiltration, which is a negative prognostic marker itself (31–33). Illogically, the follow up study of the same group identified these CD44-intermediate/high classified patients as good clinical outcomes (34). Subsequently and more consistent to the early findings, Eisterer and colleagues confirmed the prognostic value of CD44 by immunohistochemistry of BM specimen. CD44-high patients presented with advanced disease, a diffuse pattern of BM infiltration, and reduced survival within the observation period (35). Much later, Herishanu et al. (36) suggested that IgVH unmuted CLL cases express higher CD44 expression (36). We did not

find any differences in the intensity of CD44s expression in low and high risk patients, stratified according to IgVH mutation status, CD38, ZAP-70, or CD49d expression (29). This was confirmed by Fedorchenko et al. (37) when grouping patients according to IgVH mutational status or ZAP-70 expression.

The reason of these diverging observations remains unclear but one could hypothesize a differential activation status of the samples. We found that CD44 surface expression of CLL cells is induced upon their stimulation with activated T-cells or CD40 Ligand (CD40L) (29). In addition, several variant isoforms of CD44, known as markers for tumor progression in various malignancies (28), are transcribed and expressed at the surface upon activation (29).

In resting CLL cells, however, surface expression of CD44v is only detectable in the minority of CLL cases (38). These cases differ from the CD44v low expressing cases in regard to disease progression, lymphocyte doubling time, and therapy requirement (39). We found transcripts of CD44v3, v5, v6, v7, v8, v9, and v10 in unstimulated CLL cells, and a robust upregulation of CD44v3 and v6 upon CLL cell activation (29).

Soluble CD44, lacking the transmembrane region (40, 41) is found in serum due to shedding events (40, 42). High serum levels of CD44s, elevated in approximately half of CLL samples, are significantly associated with high tumor burden and the presence of other unfavorable prognostic markers such as high beta2-microglobulin levels (38, 43). The correlation is stable in time, treatment independent, and allows separation of two distinct patient groups with differential survival times (38). While de Rossi and colleagues did not observe any differences in CD44v in serum of CLL patients compared to healthy donors (38), a later study by Eisterer and colleagues identified elevated serum CD44v6 levels being associated with advanced disease defined by lymph node involvement and splenomegaly, and therapy requirement (44). This divergence was attributed to differential sensitivities of the statistical tests used. Nevertheless, independent analyses are required to solve these issues, particularly in case of CD44v6.

In CLL, little is known on the role of the second major HA-binding molecule RHAMM. One report describes a prominent expression of RHAMM and its splice variant RHAMM^{-exon 4} in advanced CLL (45). As RHAMM expression was missing in peripheral blood mononuclear cells (PBMCs) from healthy individuals, it was suggested as a tumor-associated antigen (TAA) in CLL (45). A follow up study provided evidence of an additional prognostic role of RHAMM expression among CLL patients with mutated IgVH genes (46).

MIGRATION AND LOCALIZATION

The CD44 molecule was originally defined as a lymphocyte homing receptor that can be bound by the Hermes class of antibodies (47–49). Homing hereby means the rapid process, in which circulating hematopoietic cells actively cross the blood/endothelium barrier to enter the tissue (50). BM homing of normal progenitor cells is dependent on CD44 expressed on these cells and HA displayed on the BM endothelium (51–53). Moreover, CD44 participates in homing and engraftment of various tumor cells (54–57).

The contribution of CD44 to homing of CLL cells to BM and secondary lymphoid organs has not been dissected yet. However, we have previously established the integrin VLA-4, a heterodimer of the negative prognostic marker CD49d and the beta1 integrin subunit CD29, as the chief orchestrator of CLL BM homing (12, 58). Moreover, it was also shown that interaction of E-selectin with a specific glycoform of CD44 (HCELL) induces VCAM-1 binding of VLA-4. Thereby, HCELL ligation triggers inside-out upregulation of VLA-4 adhesiveness via G-protein dependent signal transduction leading to firm adhesion and subsequent transendothelial migration of human mesenchymal stem cells (59). Notably, in CLL, CD44v and VLA-4 constitute a cell surface docking complex for matrix metalloproteinase 9 (MMP-9) (in the pro and active form) (30). Here, proMMP-9 does not act as a protease upon docking to this surface receptor complex but fulfills functions in promoting CLL cell survival (60). MMP-9 lacks a transmembrane domain and is therefore dependent on cellular binding sites for all directed functions (61). Most recently, it was observed that high proMMP-9 expression and binding to these sites inhibits migration and reduces the homing capacity of CLL cells, suggesting a cooperation of VLA-4 and CD44(v) with MMP-9 (in the pro and active form) leading to CLL cell retention in lymphoid organs (62).

Consistent with this idea of CD44-mediated stop signals, we discovered that upon CLL cell activation by T-cells in lymphoid organs, high avidity CD44-HA interactions are formed due to induction of CD44v, most prominently CD44v6, harboring N-linked glycosylations. These interactions result in reduced cellular motility and lock CLL cells to immobilized HA. Thus, activation results in stop signals to migrating CLL cells by inducing strong cellular adhesion to the substrate, which may subsequently allow proliferation (29) (Figure 1). Since MMP-9 is particularly bound to CD44v rather than CD44 (30), it will be interesting how the suggested functions of MMP-9 in CLL are modulated by the activation-induced CD44v expression (29) and contribute to proliferation.

The suggestion of CD44(v) as a retention signal of interstitial motility – a process completely different from homing – is in line with early reports on the involvement of RHAMM rather than CD44 in IL-8-triggered motility of CLL cells on HA (63). This is consistent to our findings that blocking CD44 does not interfere with motility of resting CLL cells under shear free conditions but antagonizes their HA binding and adhesion, once they are activated (29).

CD44 AND CLL CELL SURVIVAL

Human CD44 expression is increased by microenvironmental stimulation of CLL cells not only by CD40L-induced activation (29, 37) but also by the presence of feeder cells, known to provide prosurvival signals and early activation (64, 65). Activated CLL cells are protected against spontaneous and drug-induced apoptosis (66–68). Several previous studies suggested that CD44 is part of the survival signaling in CLL (37, 64, 69, 70). The addition of blocking anti-CD44 antibodies to CLL co-cultures with follicular dendritic cells reduced the survival of CLL cells, paralleled by decreased levels of the anti-apoptotic protein myeloid cell leukemia sequence 1 (Mcl-1) (64). Recently, Federochenko and

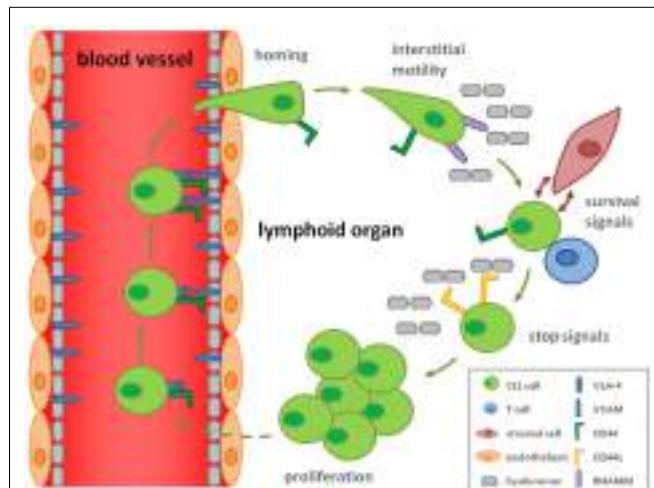


FIGURE 1 | Hypothetical model how CD44 and CD44v contribute to the CLL cell life cycle. By the ability of CD44 to complex with VLA-4 (CD49d/CD29), a key molecule for homing of CLL cells, CD44 may influence the homing process. Interstitial migration in the lymphoid organs is CD44 independent but mediated by RHAMM binding to hyaluronan. Interactions with T-cells and hyaluronan-displaying stromal cells secure CLL cell survival and activate the malignant cells. Activation is responsible for a rearrangement from CD44s to CD44v expression enhancing the affinity for hyaluronan, which induces a stop signal for the CLL cell. This retention allows CLL cell proliferation.

colleagues recapitulated the inhibitory effect of CD44 blockage or downregulation on Mcl1 protein levels *in vitro* and *in vivo*. To study the impact of CD44 on murine leukemogenesis, the authors used CD44 gene deletion, crossing CD44^{-/-} animals with Eμ-TCL1 transgenic (tg) mice (37), which represent a well established murine model for CLL (71). In these mice, a CD5/CD19-double positive clonal B-cell hyperplasia arises in the peritoneal cavity and the disease subsequently spreads into other organs (spleen, BM, LNs, blood), with an overt leukemic phase starting from 8 to 10 months (71, 72). Eμ-TCL1 tg CD44^{-/-} mice displayed a reduced peripheral blood tumor load at 12 months and significantly reduced spleen weights (37) compared to Eμ-TCL1 tg CD44^{+/+} mice. The CD44 deficient murine CLL cells had marked signs of apoptosis, e.g., increased expression of cleaved caspase-3, suggesting a role of CD44 in tumor cell survival in the spleen microenvironment.

Notably, a novel humanized anti-CD44 mAb, RG7356, was recently found to induce apoptosis particularly in ZAP-70 positive CLL cells, in a caspase-dependent manner (70). The effects of this antibody occurred independent of complement and immune-effector cells and were attributed to ligation of CD44, altering its potential complexing with ZAP-70. This involvement of ZAP-70 in CD44-mediated CLL survival signaling and its physical complexing with CD44 clearly impacts on BCR signaling. ZAP-70 is known as an enhancer of BCR signaling upstream of survival and proliferation signals such as protein kinase B (Akt) and extracellular-signal-regulated kinases (ERKs) (73), which in turn induce anti-apoptotic proteins such as Mcl-1 and Bcl-xL (74). In consequence, the data may also suggest alterations of

the known complex of MMP-9 with CD44 and CD49d (VLA-4) (60) dependent on the BCR reactivity, which is shaped by ZAP-70.

THERAPY

Therapeutically, CD44 is difficult to exploit due to its high variability and ability to complex with different partners in which CD44 function is apparently influenced. It is therefore not surprising that CD44 is not easily druggable, with some cases of previous failures (clinical trial identifier: NCT02254031; NCT02254044). The anti-CD44v6 antibody bivatuzumab (previously BIWA 4) coupled with a non-radioactive cytotoxic drug mertansine, for example, was used in studies against breast neoplasms (NCT02254005) and squamous cell carcinomas of the head and neck (HNSCC) (NCT02254018) (75–77). The death of one patient terminated the HNSCC trial (NCT02254044) whereas in the breast cancer study the antibody was found in non-tumor tissue as well and was therefore stopped (NCT02254031).

Nevertheless, several recent approaches could be advanced from preclinical status to testing in clinical trials. One promising candidate is the Å6 peptide (Ac-KPSSPSEE-amide), which is derived from the non-receptor binding domain of urokinase plasminogen activator and known to share a homologous sequence with CD44 (78, 79) (see also article by Finlayson in this volume). This homologous sequence (120-NASAPPEE-127) is found in the HA-binding site and is therefore present in all CD44 isoforms independent of alternative splicing events (80, 81). In preclinical studies, treatment with the Å6 peptide significantly decreased tumor growth and metastasis in a breast cancer mouse model without direct evidence of cytotoxicity or anti-proliferative activities toward the tumor (78). Instead, tumor and endothelial cell migration was clearly impaired by the peptide suggesting its impact on tumor invasion, metastasis, and angiogenesis. Similarly, Å6 reduced lymph node metastasis in a prostate cancer model (82). Notably, Å6 inhibited the migration of a subset of ovarian and breast cancer cell lines *in vitro* by inducing high adhesion of the CD44-expressing cells to an HA substrate and altering CD44 conformation (79), obviously locking the cells to substrates of HA, abundantly found, e.g., in LNs. First clinical trials demonstrated that Å6 was well tolerated (83, 84), resulting at least in an increased time to clinical disease progression of women with epithelial ovarian, fallopian tube, or primary peritoneal cancer in clinical remission (85) (NCT00083928). Currently a phase 2 trial is under way to determine the safety, tolerability, and efficacy of Å6 in CLL patients (NCT02046928).

A second promising candidate in CLL is the anti-CD44 antibody RG7356 (also known as RO5429083 or ARH460-16-2), a humanized antibody targeting a glycosylated, extracellular constant region of CD44 (86). As outlined above, this apoptosis-inducing antibody exerts a particular influence on BCR signaling in CLL and may be promising in light of the current success of all BCR-downstream-signal-targeting drugs.

Two clinical trials are underway to examine the pharmacokinetics, pharmacodynamics, safety, and efficacy of RG7356 in acute myelogenous leukemia (AML) patients and patients with metastatic and/or locally advanced CD44-expressing solid tumors.

SUMMARY AND OPEN QUESTIONS

In summary, CD44 emerges as a key molecule of CLL cell interactions with the lymphoid microenvironment, shaping malignant cell positioning, and in consequence survival and proliferation in a fine-tuned manner. Nevertheless, some open questions remain on the mode of CD44 regulation in dependence of the activation status of the cells and the respective complex partner, such as CD49d/VLA-4. In addition, little is known on the second chief receptor interacting with HA, RHAMM. It is conceivable that RHAMM and CD44 fulfill distinct functions of cell migration and retention in CLL, which should be addressed in more detail in future. A deeper understanding of the functional regulation of CD44-HA interactions by splicing events and posttranslational modifications might help solving the existing controversies of its role in prognosis and survival. More functional studies as well as comprehensive patient cohorts and a clear clinical stratification of the patient groups would allow addressing these issues with sufficient statistical power and also assisting in the choice of the appropriate type of CD44 antagonizing therapy in CLL.

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The where, when, how, and why of hyaluronan binding by immune cells

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Hyaluronan is made and extruded from cells to form a pericellular or extracellular matrix (ECM) and is present in virtually all tissues in the body. The size and form of hyaluronan present in tissues are indicative of a healthy or inflamed tissue, and the interactions of hyaluronan with immune cells can influence their response. Thus, in order to understand how inflammation is regulated, it is necessary to understand these interactions and their consequences. Although there is a large turnover of hyaluronan in our bodies, the large molecular mass form of hyaluronan predominates in healthy tissues. Upon tissue damage and/or infection, the ECM and hyaluronan are broken down and an inflammatory response ensues. As inflammation is resolved, the ECM is restored, and high molecular mass hyaluronan predominates again. Immune cells encounter hyaluronan in the tissues and lymphoid organs and respond differently to high and low molecular mass forms. Immune cells differ in their ability to bind hyaluronan and this can vary with the cell type and their activation state. For example, peritoneal macrophages do not bind soluble hyaluronan but can be induced to bind after exposure to inflammatory stimuli. Likewise, naïve T cells, which typically express low levels of the hyaluronan receptor, CD44, do not bind hyaluronan until they undergo antigen-stimulated T cell proliferation and upregulate CD44. Despite substantial knowledge of where and when immune cells bind hyaluronan, why immune cells bind hyaluronan remains a major outstanding question. Here, we review what is currently known about the interactions of hyaluronan with immune cells in both healthy and inflamed tissues and discuss how hyaluronan binding by immune cells influences the inflammatory response.

Keywords: hyaluronan, CD44, inflammation, immune cells, leukocytes

INTRODUCTION

The function of our immune cells is to maintain homeostasis. When immune cells detect damage or infection, they respond by making an inflammatory response that is aimed at removing the threat. The ultimate goal is to repair the damage and return the tissue to its original state. The inflammatory process is a potent, fundamental, and normally protective immune mechanism. However, if it is not properly regulated, it can result in serious damage to the host and lead to a pathological state. In fact, inflammation is thought to be at the root of many chronic conditions, from heart attacks and strokes to arthritis and type-2 diabetes. Thus, it is important to understand the factors that drive and resolve inflammation in order to better treat inflammatory diseases and identify novel therapeutic targets and new predictors of treatment efficacy.

Our skin and mucosal surfaces provide the first line of defense, and any pathogen that breaches these barriers activates innate immune cells, which trigger an inflammatory response. Macrophages are innate immune cells that reside in our tissues and play a key role in maintaining tissue homeostasis. Their primary role is to remove dead and damaged cells, and to detect and destroy invading pathogens. Dendritic cells are also innate immune cells that become activated in response to pathogens

and migrate to the lymph node to activate antigen-specific adaptive immune cells (T and B lymphocytes). Once the pathogen and cell debris are removed, damaged cells and extracellular matrix (ECM) components are replaced, and tissue homeostasis is restored. One major constituent of the ECM is hyaluronan (HA), a large glycosaminoglycan under physiological conditions that becomes fragmented during infection and tissue damage, and is restored upon the resolution of inflammation. HA turnover is perturbed during inflammation and HA fragments accumulate extracellularly. These fragments are associated with propagating the inflammatory response, whereas full-length high molecular mass HA is associated with the resolution of inflammation. While all immune cells express the HA receptor, CD44, not many bind HA under homeostatic conditions. However, this changes when immune cells become activated. In this review, we discuss what is known about the interactions between immune cells and HA during homeostasis and inflammation.

HA TURNOVER DURING HOMEOSTASIS AND INFLAMMATION

HA is widely distributed throughout all the tissues in the body with up to 50% being present in the skin. HA is found at high levels in the umbilical cord (~4 mg/ml) and synovial fluid (~2 mg/ml); it is

prevalent in the vitreous of the eye (~100–400 µg/g of wet tissues) and the dermis of the skin (~500 µg/g wet tissue); and present at 10–100 ng/ml in the blood (1, 2). It is hygroscopic in nature and has viscoelastic properties making it a useful lubricant in joints. HA comprises repeating units of D-N-acetyl glucosamine and D-glucuronic acid. HA is often confined to specific areas within tissues, for example, HA is present around blood vessels and bronchioles in the lung (3). At homeostasis, HA production is balanced by its cellular uptake and degradation (4). Cellular HA synthases (HAS 1–3) and hyaluronidases (Hyal 1–3) mediate the turnover of HA [reviewed in more detail elsewhere (5–11)]. HA catabolism can occur locally by nearby cells involving CD44-mediated uptake, partial degradation by Hyal 2, and further degradation in the lysosome by Hyal 1 [(12, 13); see Figure 1]. Alternatively, HA can drain into the lymphatics and be degraded at a distant site such as the liver (2).

During inflammation, an increase in HA is accompanied by a decrease in chain length, possibly due to altered HAS and Hyal activities (3, 18) or to cleavage by reactive oxygen and nitrogen species produced by activated immune cells (23, 24). Macrophages are thought to be involved in HA uptake and the removal of HA fragments (19), and stromal cells are a major source of newly synthesized HA, see Figure 1. Upon the resolution of inflammation, HA production and cellular turnover return to normal and the high molecular mass form predominates again.

THE DIFFERENT FORMS OF HA

AT HOMEOSTASIS

HA as part of the extracellular matrix

Hyaluronan is secreted from the cell and forms pericellular or extracellular matrices presumably after cleavage and release from the cell surface. HA is a major component of the ECM and at homeostasis, extracellular HA is found predominantly in its high molecular mass form of over 1000 kDa (10, 25, 26). HA chains occupy a large hydrodynamic volume in solution (27) and can associate with collagen in extracellular matrices. Proteoglycans such as versican and aggrecan bind to HA and this could create a stable network under homeostatic conditions (14–16).

DURING INFLAMMATION

HA fragments

Both damage and inflammatory conditions can cause the fragmentation of HA, which is considered fragmented when its molecular mass falls below 500 kDa. Studies in the lung tissue have reported the detection of 500 kDa HA fragments after bleomycin-induced inflammation (19), 70 kDa fragments after cigarette smoke-induced chronic obstructive pulmonary disease (COPD) (18), and 100–200 kDa fragments in the bronchial alveolar lavage fluid (BALF) after ozone-induced airway hyper-responsiveness (20). There is evidence that HA fragmentation can result from degradation by reactive oxygen species (ROS) that are produced by neutrophils (23, 24), or by enzymatic cleavage by Hyal 1 or 2 that perhaps have escaped from dying cells. Hyal 2 and Hyal 1 both work optimally at acidic pH and break down HA to 20 kDa and small oligomers, respectively (28). Alternatively, an increase in HA fragments may be the result of HA synthases making smaller chains of HA (3, 18).

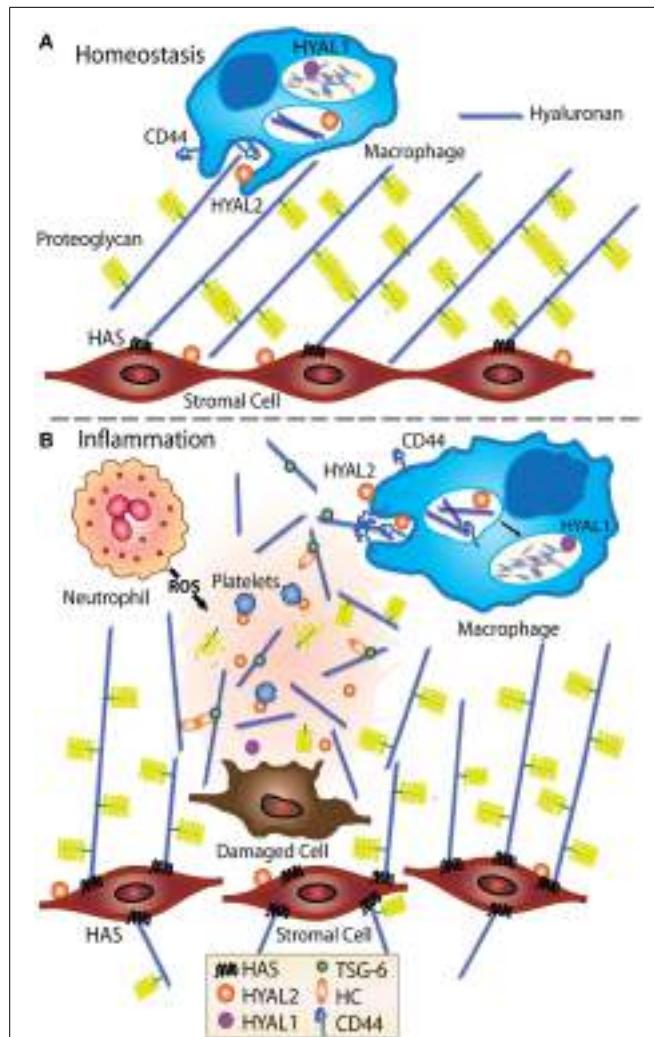


FIGURE 1 | Model showing HA turnover at homeostasis and during inflammation. At homeostasis (A), stromal cells produce ECM, including HA, which becomes decorated with proteoglycans such as versican (14–16), indicated by the yellow brush structures. HA is turned over in tissues, likely by CD44-mediated cellular uptake by fibroblasts and macrophages (17), then degraded by Hyal 1 and 2 (12, 13). (B) During inflammation, the levels of HA increase and the ECM becomes susceptible to damage and fragmentation (18–20). Inflammation induced HA binding proteins such as TSG-6 bind and crosslink itself or the heavy chain of IgL (HC) to HA (21, 22) and in some situations such as chronic inflammation, HA deposits may develop (3, 18). CD44-mediated uptake of HA fragments by macrophages is thought to play an important role in resolving inflammation (19).

HA oligomers

Under normal conditions, small oligomers of HA should only be present in the lysosome as HA is degraded into tetrasaccharides by Hyal 1. Hyal 1 has been reported in the serum, but neither the source nor its activity is known (28). Thus, one can predict that the smallest oligomers of HA would only be present under severe inflammatory conditions when there is significant cell death and release of the lysosomal contents. It is these smaller oligomers that are thought to activate dendritic cells (29, 30). As CD44 binds

to these oligomers with very low avidity, they may activate dendritic cells by interacting with other receptors such as TLR2 or 4, although this remains to be shown directly.

HA complexes

In vitro, pericellular cable-like structures of HA can be induced in cells in response to inflammatory stimuli such as polyI:C, a viral RNA mimic (31, 32), or to tunicamycin, an ER stress and N-glycosylation inhibitor (33). These HA cables are also positive for the inter-alpha-trypsin inhibitor (I α I) (32). The heavy chains (HC or serum HA-associated protein, SHAP) of I α I can become covalently bound to HA and this is thought to crosslink and stabilize HA (34). The human monocytic cell line U937 and human peripheral blood mononuclear leukocytes, which do not bind soluble HA, can bind to these cables (31, 32, 35), although only at 4°C as at 37°C the HA is taken up by the U937 cells. (36). HA cables have been induced in a variety of cell types *in vitro*, including smooth muscle cells from the lung (37) and colon (32), in airway (38) and renal epithelial cells (39), as well as epidermal keratinocytes (40). However, HA cables have yet to be described *in vivo*, although HA and I α I co-staining has been reported in a human case of inflammatory bowel disease (32).

TNF α -stimulated gene-6 protein (TSG-6) is a multifunctional protein that has a HA binding module and binds HA under acidic conditions (41). TSG-6 production is greatly increased with inflammation and is associated with tissue remodeling (41). TSG-6 is the enzyme that catalyzes the covalent addition of the HC from I α I to HA (21, 22, 42), which can lead to the crosslinking and aggregation of HA (34). Interestingly, TSG-6 and the attachment of the HC from I α I enhance the binding of HA to CD44 (43, 44). Furthermore, these HA–HC complexes are found as HA deposits *in vivo* during persistent inflammation in the lung and TSG-6 has been shown to promote these deposits (3, 45). However, the function of these HA–HC complexes in inflammation and tissue remodeling is still being explored.

HA BINDING BY IMMUNE CELLS AT HOMEOSTASIS

HA BINDING BY ALVEOLAR MACROPHAGES

Under homeostatic conditions, without infection or inflammation, the majority of developing and mature immune cells do not bind HA, as assessed by flow cytometry using fluoresceinated HA (Fl-HA, see **Box 1**). In fact, alveolar macrophages are the only immune cells that have been shown to bind high levels of HA under homeostatic, non-inflammatory conditions, in both rodents and humans [(46–48); see **Table 1**]. Alveolar macrophages reside in

Box 1 | Evaluation of HA binding by flow cytometry.

Hyaluronan from rooster comb (1000–1500 kDa) or commercially available HA of specific molecular mass is conjugated to fluorescent dyes, using the method of de Belder (52), or indirectly using a coupling reagent. Fluoresceinated HA (Fl-HA) used in flow cytometry provides a useful means to evaluate surface HA binding, HA uptake, and CD44-specific HA binding using HA-blocking CD44 mAbs such as KM81 or KM201 (53). To date, all experiments indicate that the HA binding on immune cells is mediated by CD44 [(54, 55), and reviewed in Ref. (56, 57)].

High molecular mass HA (>1000 kDa) binds to CD44 with a higher avidity than medium (~200 kDa) or low (<20 kDa) molecular mass HA fragments, and thus high molecular mass Fl-HA is routinely used to evaluate HA binding by immune cells. CD44 can bind monovalently to 6–18 sugars of HA, with a noticeable increase in avidity when the HA reaches 20–38 sugars in length, suggesting that divalent binding is occurring (58). The avidity will increase with increasing length as more CD44 molecules are engaged. Ultimately, the strength of Fl-HA binding depends on the size of HA as well as the amount, density, and type of CD44 at the cell surface. Flow cytometry allows us to determine relative HA binding abilities as it can distinguish cells that bind different amounts of Fl-HA. The pretreatment of cells with hyaluronidase (which is then washed away) can determine if CD44 is binding to endogenous HA and thus blocking the binding of Fl-HA. It is thus a useful technique to gain insights into the HA binding abilities of CD44. Commercial sources now provide specific molecular sizes of purified soluble HA that are low in contaminants and endotoxin. However, it is important to keep in mind that purified soluble chains of HA may not always be the form encountered *in vivo*.

Table 1 | HA binding ability of immune cells.

Cell type	Stimulation	Type of HA binding	HA receptor	Reference
Monocyte (human)	TNF α , LPS, IL-1, IFN- γ	Inducible	CD44	(59–61)
Alveolar macrophages (human, rodents)	None	Constitutive	CD44	(17, 46–48, 50)
Peritoneal macrophage (mouse)	LPS with IFN γ , or IL-4	Inducible	CD44	(62)
Bone marrow-derived macrophages (mouse)	LPS with IFN γ , TNF α , or IL-4	Inducible	CD44	(62)
Monocyte-derived DC (human)	CD40L expressing fibroblasts	Inducible	CD44	(63)
B cells (human, mouse)	PMA, IL-5, LPS	Inducible, a subset binds	CD44	(64–67)
T cells (mouse)	PMA/ionomycin, CD3 antibodies, specific antigen, or superantigen	Inducible, often a subset binds	CD44	(54, 55, 68)
CD4+ CD25+ T regulatory cells (human and mouse)	CD3 +/- CD28 activation	Inducible, a subset binds	CD44	(69, 70)
Neutrophil (mouse)	LPS induced liver inflammation <i>in vivo</i>	Binding to SHAP modified HA	CD44, not RHAMM	(71)
NK cells (mouse)	IL-2, IL-15	Inducible, a subset binds	CD44	(72)
Platelets (mouse)	None	Constitutive	CD44	(73)

the respiratory tract and alveolar space, between the epithelial layer and surfactant, where they are responsible for the uptake and clearance of pathogens and debris. In the absence of these macrophages, the immune response is exacerbated (49), indicating that these scavenger cells also have a role in limiting inflammation, perhaps by clearing debris and removing inflammatory stimuli. Alveolar macrophages take up HA in a CD44-dependent manner, which is then delivered to the lysosomes and subsequently degraded (17). HA is present in the connective tissue space during lung development, but is reduced as the number of CD44-positive macrophages increases (50). Fetal alveolar type II pneumocytes produce HA (51), which is thought to associate with the pulmonary surfactant. However, in adults, it is less clear if mature pneumocytes make HA and most of the HA in the lung tissue is found lining blood vessels and bronchioles (3, 50). There seems to be two possible explanations why alveolar macrophages constitutively bind HA: (1) to bind to the HA producing pneumocytes to help anchor themselves in the alveolar space or (2) to internalize HA or HA fragments and help keep the alveolar space free of debris.

THE HA BINDING STATUS OF DENDRITIC CELLS

There is very little direct evidence that immature or mature dendritic cells bind HA, HA fragments, or oligomers of HA, despite reports that HA fragments and small oligomers of HA stimulate dendritic cells to produce proinflammatory cytokines (29, 30, 74). Dendritic cells have been reported to express HA and HA-synthesizing and degrading enzymes (75), with human monocyte-derived dendritic cells expressing primarily HAS 3 (76). However, it is not clear whether CD44 on either immature or activated dendritic cells is capable of interacting with HA. Unlike macrophages, which stay in the tissues to fight infection and maintain homeostasis, activated dendritic cells migrate to the draining lymph node, where they present antigen to naïve T cells. A HA binding peptide, Pep-1, reduced dendritic cell clusters and antigen-induced T cell proliferation, however, Pep-1 acted on the T cells, suggesting that T cells make HA (75). Non-endotoxin tested HA upregulated costimulatory molecule expression on bone marrow-derived dendritic cells, which facilitated T cell proliferation, while CD44 on the T cells promoted clustering with the dendritic cells (74). However, since endotoxin is a common contaminant in some HA preparations and can have similar effects on dendritic cells, additional steps are needed to exclude an endotoxin effect. Supernatants from Th1 clones also promoted HA-dependent adhesion between human monocyte-derived dendritic cells and activated T cells or Th1 or Th2 clones, providing evidence for a dendritic cell-HA:CD44-T cell interaction (76). However, as we discuss below, naïve T cells express low levels of CD44 and have a low avidity for HA, making this mechanism unlikely to play a key role in the initial contact and activation of naïve T cells, unless dendritic cells produce a form of HA that enables naïve T cells to bind. It could, however, play a role in activating memory T cells, which express higher levels of CD44 and have a greater propensity to bind HA (54).

THE LACK OF HA BINDING BY NAÏVE T CELLS

Activated dendritic cells present antigen to naïve T cells, which stimulates their activation, proliferation, and differentiation into

effector T cells. Early studies showed that CD44 monoclonal antibodies (mAbs) could provide a costimulatory signal that together with a signal from the T cell receptor (TCR) activates T cells (77–79). As CD44 is present in lipid rafts along with the tyrosine kinase Lck (80), its crosslinking was thought to help Lck activation and bring it into contact with the TCR signaling complex (81). Although these antibodies crosslink CD44 and enhance TCR signaling, there is limited evidence that HA can do this, perhaps because naïve T cells have a very low avidity for HA. Data from naïve T cells isolated from C57Bl/6 mice show that naïve T cells express low levels of CD44 and do not bind Fl-HA (54, 55, 68). However, one study showed that immobilized HA could augment PMA or CD3-induced proliferation of human peripheral blood T lymphocytes and could augment IL-2 production from CD3-stimulated CD4 T cell clones (82). In this study, HA binding did not become apparent until 1–2 days after stimulation.

HA BINDING BY IMMUNE CELLS DURING INFLAMMATION

Activation of immune cells by proinflammatory cytokines, inflammatory stimuli, and by antigen recognition can all induce HA binding by CD44 [reviewed in Ref. (56); see Table 1]. HA binding in response to these stimuli is generally accompanied by an increase in CD44 expression and typically takes 2–3 days to reach maximal HA binding levels. Flow cytometry can indicate a shift in HA binding or identify a specific subset of binding cells (see Box 1 for more details). Table 1 shows the stimuli that induce HA binding in the various immune cells. Although neutrophils are major inflammatory phagocytic cells, these cells do not bind Fl-HA and are normally recruited to inflammatory sites independently of CD44 (83). However, as we discuss later, HC-modified HA provides a means of neutrophil recruitment to inflamed liver (71). Below, we will focus on the HA binding abilities of monocytes, macrophages, dendritic cells, and T cells during inflammation.

THE INDUCTION OF HA BINDING BY MONOCYTES AND MACROPHAGES

Human monocytes in the blood bind negligible amounts of soluble Fl-HA (59–61), but are induced to bind over a period of 2–3 days when activated *in vitro* by inflammatory cytokines such as TNF α and IL-1 β , and the inflammatory agent, LPS (see Table 1). Similarly, M-CSF-induced mouse bone marrow-derived macrophages and *ex vivo* peritoneal macrophages do not bind Fl-HA until induced by proinflammatory agents (62). Treatment of TNF α -induced human monocytes with IL-4 prevents the induction of HA binding (60), whereas IL-4 treatment alone induced HA binding on mouse bone marrow-derived macrophages (62). Both the transcription and post-translational modifications of CD44, such as glycosaminoglycan or carbohydrate addition, can influence HA binding. Decreases in sialylation and changes in the chondroitin sulfate modification to CD44 can modulate HA binding by human monocytes (84) and mouse macrophages, respectively (62). Macrophages are found in many locations in the body, with resident populations in the spleen, liver, skin, gut, lung and the alveolar space, brain, and the peritoneum, but only the alveolar macrophages bind substantial amounts of HA under homeostatic conditions. This suggests that the environment of the macrophage influences its ability to interact with HA, and raises the possibility that these macrophages may be induced to bind HA when

the environment or cytokine milieu changes upon infection or inflammation.

Why inflammation induces HA binding on monocytes and macrophages is not well understood. Possible explanations include: (1) activated monocytes and macrophages use HA as a substrate to aid in migration toward the site of infection; (2) an enhanced ability to bind HA or HA-HC complexes to retain activated immune cells in the tissue at sites of inflammation; (3) an enhanced ability to bind, take up, and degrade HA, HA fragments, or HA-HC complexes via CD44, thereby helping reduce inflammation and promote tissue repair; (4) HA binding provides a supportive environment for these cells, either by providing a direct survival signal to the cell or by creating a cytokine-rich environment that aids in their survival, proliferation, or function.

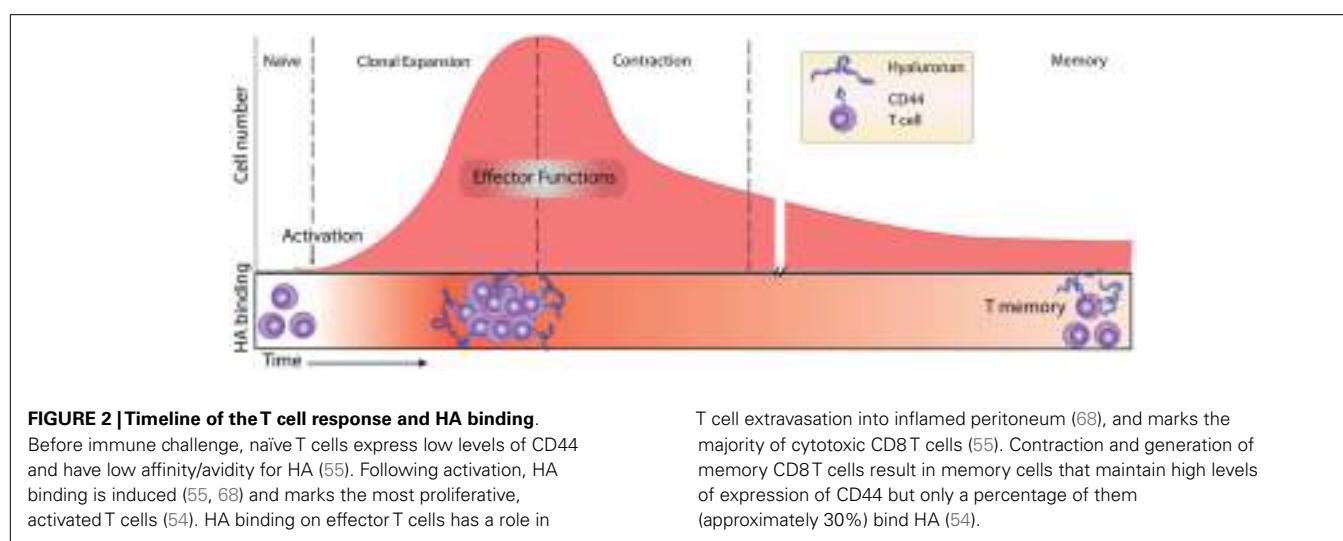
HA BINDING BY ACTIVATED T CELLS

In contrast to unstimulated naïve T cells, antigen-induced activation of T cells induces HA binding, coincident with an increase in CD44 expression, and this is now well-established both *in vitro* and *in vivo* [(54, 55, 68); see Figure 2]. *In vitro*, the induction of HA binding is transient, peaking at 2–3 days, whereas it is more sustained *in vivo*, reaching its maximum around 5–8 days (54, 55). The absence of any significant Fl-HA binding on naïve T cells until 2–3 days, after at least the first division, also argues against a model where HA on dendritic cells facilitates CD44-mediated naïve T cell adhesion and the initiation of T cell activation. As T cell activation and proliferation proceeds, HA binding increases, as does the ability of T cells to roll under flow on a HA substrate (85) and the ability of superantigen-activated T cells to extravasate to the inflamed peritoneum in an HA-dependent manner (86). Thus, one function of the upregulation of HA binding by activated T cells may be to guide the effector T cell to the site of infection. This later induction of HA binding after activation and some proliferation suggests a role beyond the initial contact and activation step. Notably, the strength of the signal received by the T cell dictates the level and percentage of T cells that are induced to bind HA, with cells binding the most HA being the most proliferative (54). Despite these correlations, no evidence was found to support HA-stimulated proliferation. However, in another study focusing on

CD4⁺ CD25⁺ T cells, HA was found to stimulate IL-2 production and sustain FoxP3 expression (87). 4-methylumbellifera, a compound that can inhibit HA synthesis (88, and also see article in this research topic), prevented T cell proliferation and IL-2 production, suggesting that HA synthesis by the T cell itself is important for this effect (89). Thus, there is some evidence that HA production and/or the binding of high molecular mass HA on activated T cells may enhance IL-2 production, which may either prolong T cell proliferation or, if acting on regulatory T cells (Tregs), may limit T cell activation. The outcome of an interaction of activated T cells with high molecular mass HA may also depend on the differentiation state of the T cell, as re-activated T cells exposed to high molecular mass HA undergo a rapid form of activation-induced cell death, as observed in human Jurkat T cells and a subpopulation of splenic T cells from mice (90).

HA BINDING BY EFFECTOR CD4 AND CD8 T CELLS

After naïve T cell activation and proliferation, CD4 T cells differentiate into various effector T cells (Th1, Th2, Th17, Tregs), and CD8 T cells become cytotoxic effector cells. Both CD4 and CD8 effector cells leave the lymph node and migrate to the infected tissue to fight the infection. After the initial induction of HA binding during the proliferative phase, it is less clear how long these cells retain their HA binding abilities. *In vitro* evidence indicates that after antigen-induced HA binding in 50% of ovalbumin- (Ova-) specific OT-I CD8 T cells 2 days post activation, only 6% retain HA binding at day 6 (54). *In vivo*, antigen-induced HA binding peaks 5 days post infection, marking approximately 50% of the OT-I CD8 T cells, and this drops to 14% by day 10, indicating that HA binding is more sustained *in vivo* although it also declines after the peak of the proliferative phase (54). In other mouse studies, the percent of HA binding cells peaked at day 7–8 and the HA-positive population contained the majority of the cytotoxic CD8 T effector cells (55), consistent with the HA-negative cells being naïve cells. However, since both soluble HA and HA-blocking CD44 mAbs had no effect on allogeneic killing, HA binding was not implicated in the killing function of these cells (55). After the peak of the response, many effector T cells undergo apoptosis during the contraction phase and only long-lived memory cells remain.



Following *Listeria*-Ova infection and the development of T memory cells *in vivo*, about 30% of the Ova-specific OT-I CD8 memory T cells in the bone marrow and spleen bind Fl-HA 30 days after infection [(54); see Figure 2].

CD4 T cells differentiated *in vitro* to become either Th1 or Th2 bind slightly higher levels of Fl-HA than naïve T cells, with Th2 cells binding slightly more than Th1, but the binding is still at a very low level (91, 92). Nevertheless, this low-level binding is sufficient to allow them to roll and adhere on TNF α -inflamed endothelium *in vivo* (91). In another study, CD44 promoted T effector cell survival from Fas-mediated contraction, and was required for the formation of Th1 memory cells, but the effect of HA was not examined (93). Thus HA binding by effector T cells may assist in their recruitment to inflammatory sites and the interaction of HA with CD44 in the tissues may provide a survival signal for the effector T cell.

HA BINDING BY ACTIVATED CD4 T REGULATORY CELLS

Tregs express CD4 and CD25, as do activated CD4 T cells, and so are further characterized by expression of the transcription factor, FoxP3 (94). Firman and colleagues activated CD4 $^{+}$ CD25 $^{+}$ T cells isolated from BalbC mice, separated them into HA binding and non-binding populations, and found that the HA binding fraction was functionally more suppressive (69). The activation of human peripheral blood CD4 $^{+}$ CD25 $^{+}$ T cells induced a subpopulation to bind HA and this correlated with the highest expression of CD44 and FoxP3 (70). The addition of 20 μ g/ml of high molecular mass HA (1.5×10^6 Da) maintained FoxP3 expression under limiting levels of IL-2, and enhanced their suppressive ability *in vitro*. At a higher concentration (100 μ g/ml), HA directly reduced CD4 T cell proliferation (70). Both high molecular mass HA and the crosslinking of CD44 provided a costimulatory signal that augmented IL-2, FoxP3 expression, and IL-10 production in the human CD4 $^{+}$ CD25 $^{+}$ T cell population (87). Furthermore, HA or CD44 crosslinking activated p38 and ERK1/2-dependent pathways that induced IL-10 producing regulatory T cells (TR1) from FoxP3-negative cells (95). Together, this suggests a role for high molecular mass HA in limiting T cell proliferation either indirectly via supporting Tregs or directly when given in higher amounts to CD4 T cells.

It is clear from the above sections that HA binding by T cells is not restricted to activated Tregs, suggesting a more general function for HA binding in activated CD4 and CD8 T cells. Since HA binding labels the most proliferative, functionally active T cells, HA may exert its effect by aiding in the production of IL-2 under limiting conditions. Alternatively, perhaps HA localizes activated T cells to a specific area in the lymph node, where they have optimal access to cytokines and growth factors, or maybe HA binding itself provides a survival signal for activated T cells.

THE FUNCTION OF HA BINDING BY IMMUNE CELLS

Previous reviews have discussed HA binding by immune cells (56, 85), and have detailed what is known regarding the role of HA (96) and CD44 in inflammation and inflammatory diseases (57, 97, 98). Many diseases involve an inflammatory component and it has become apparent that HA levels are increased in many tissues upon inflammation. Here, we describe the effects of HA interactions

with immune cells during inflammation with a particular focus on lung inflammation.

THE ROLE OF CD44 AND HA IN LEUKOCYTE RECRUITMENT TO INFLAMMATORY SITES

Upregulation of HA on microvascular endothelium facilitates T cell recruitment

At the first signs of damage or infection in a tissue, ensuing danger signals are received by macrophages that induce the secretion of inflammatory stimuli such as TNF α and IL-1 β , which act on the endothelium in the microvasculature. This leads to the upregulation of adhesion molecules that facilitate leukocyte recruitment to the inflamed tissue. HA is one adhesion molecule that is upregulated on microvascular endothelial cell lines (99). Under flow conditions, T cells can roll on HA via CD44 both *in vitro* (100, 101) and *in vivo* (91), implying that the upregulation of HA on activated endothelial cells will facilitate T cell recruitment to inflamed tissues. Indeed, a CD44-mediated interaction with HA is important for T cell extravasation into the peritoneum in a model of superantigen-driven T cell activation and inflammation (86). However, given the well-established roles of the selectin molecules in leukocyte recruitment (102), the CD44–HA interaction may provide an additional and possibly redundant mechanism to facilitate T cell extravasation. The fact that the majority of CD44-deficient leukocytes still reach inflammatory sites (57) supports this idea.

HC-modified HA facilitates CD44-mediated neutrophil recruitment to liver sinusoids

CD44- and HA-dependent rolling on endothelium is not a factor for neutrophil recruitment to inflammatory sites (83). However, CD44-mediated adhesion to HA is a key factor in neutrophil adhesion to inflamed liver sinusoids in endotoxemic mice (71). Leukocyte recruitment to liver sinusoids proceeds in the absence of rolling and involves both integrin- and CD44-dependent mechanisms (103). LPS recognition by liver endothelial cells induces the deposition of HC (SHAP) on HA that is constitutively expressed by the liver sinusoids, and this leads to CD44-dependent adhesion of neutrophils to HA (71, 104, and also see article in this research topic).

CD44-mediated HA binding facilitates eosinophil and Th2 cell recruitment to the allergic lung

In experimental pulmonary eosinophilia that is induced by administration of *Ascaris suum* extract, HA-blocking CD44 antibodies prevented lymphocyte and eosinophil recruitment into the BALF (105). A follow-up study using CD44-deficient mice and the house dust mite allergen concluded that the loss of CD44 affected Th2, but not Th1 recruitment to the BALF (92). This suggests that HA and CD44 play a role in the recruitment of eosinophils and Th2 cells in an allergic response in the lung.

A ROLE FOR HA FRAGMENTS AND DEPOSITS IN PROMOTING INFLAMMATION

The proinflammatory effects of HA fragments on immune cells

In vitro. Hyaluronan fragments were first reported to stimulate chemokine and proinflammatory cytokine expression and NF κ B-mediated iNOS expression in macrophage cell lines in the

1990s (106–109). At first, it was thought that CD44 mediated the effect of these HA fragments, which were quite large (470, 200–280 kDa), but later studies began to implicate the toll-like receptors, TLR2 and/or TLR4 (**Table 2**; **Figure 3**). While CD44 can bind HA fragments on activated cells, it is not clear if this alone leads to proinflammatory cytokine secretion. Instead, TLR4 (110), TLR2 (111), TLR2 and TLR4 (112), a complex of CD44 and TLR4 (113), or a combination of TLR4, CD44 and activation of the NLRP3-mediated inflammasome (114) have all been implicated in mediating HA fragment-induced proinflammatory signals (see **Figure 3**). TLR4 was required for HA oligomers to initiate dendritic cell maturation and proinflammatory cytokine production from both human and mouse *in vitro*-derived dendritic cells (29, 30) and 200 kDa HA fragments stimulated dendritic cell maturation in a CD44-independent manner (74). However, whether HA fragments can directly activate TLRs remains uncertain, as contaminants such as LPS can produce similar results and the direct binding of HA to TLRs has not yet been demonstrated. This needs to be determined to establish HA as a *bona fide* TLR ligand.

In **Table 2**, we summarize the reported proinflammatory effects of HA and HA fragments on macrophages and dendritic cells *in vitro*. Proinflammatory effects have been reported with a wide range of HA fragment sizes: from HA oligomers [2–18 mers] to HA fragments ranging from 5 to 500 kDa. Interestingly, we note that HA fragments derived from rooster comb did not generally elicit proinflammatory responses whereas human umbilical cord-derived HA did. Human umbilical cord HA is not as pure as HA purified from rooster comb (some preparations are FDA approved for injection into humans), leaving open the possibility that the effects seen with human umbilical cord HA maybe due to LPS contamination. Indeed, in our hands HA from human umbilical cord but not rooster comb, tested positive for endotoxin (unpublished data). Although endotoxin levels were checked and polymyxin B was added to bind to LPS in some cases, possible residual contamination with TLR agonists remains a concern. In some cases, DNA contamination of HA was responsible for the proinflammatory activity on monocytes (119). Alternatively, there could be something different about the HA isolated from human umbilical cord as it does appear to be of a lower average molecular mass compared to rooster comb HA. Specific sizes of HA are now available from commercial sources that are purified from bacteria and certified endotoxin-free, and so it will be of interest to see if similar data are obtained with these HA preparations. Indeed, studies monitoring endotoxin levels more closely are now emerging. One report on glomerular mesangial cells shows that hyaluronidase is contaminated with LPS and suggests that HA provides a protective barrier for cells, which when cleaved exposes the TLRs (120). Others report no proinflammatory effect of endotoxin-free HA oligomers (118) or HA fragments on immune cells [(117); see **Table 2**]. Thus, further work with endotoxin-free HA is needed to substantiate whether HA fragments induce proinflammatory cytokine production and whether they do this by directly engaging TLRs.

In vivo. Ozone-induced airway hypersensitivity is associated with increased hyaluronan in the BALF, and CD44- and I α I-deficient mice were protected from this airway hypersensitivity implying a role for CD44 and I α I in driving the hypersensitivity

(20). Interestingly, the instillation of fragmented, but not full-length HA, partially mimicked airway hyper-responsiveness (20). Ozone- and fragmented HA-induced airway hyper-responsiveness were also reduced in TLR4- and MyD88-deficient mice, leading to the conclusion that fragmented HA responses require TLR4 *in vivo* (121).

Using a mouse model of allergic contact dermatitis, Martin and colleagues found that inhibition of ROS and HA breakdown prevented sensitization and contact hypersensitivity (118). ROS stimulated hyaluronidase activity, which rapidly degraded HA in the epidermis, in a similar mechanism to that described in bronchial epithelial cells (122). Although this *in vivo* hypersensitivity reaction requires TLR2 and TLR4 (123), the authors have been unable to activate dendritic cells *in vitro* using commercially available HA oligomers of 2–12 sugars in length (118). These studies show that HA fragmentation occurs *in vivo* and is required for hypersensitivity responses, as are TLRs. If HA fragments do not act directly via the TLRs, perhaps TLRs are activated indirectly, by something that is released or produced upon their fragmentation. The involvement of I α I suggests that *in vivo* HA fragments may arise from HA–HC deposits and thus may contain other proteins and proteoglycans besides HA. This also raises the cautionary note that HA fragments and HA–HC complexes generated *in vivo* may be quite different from the purified forms of HA used *in vitro* studies and in Fl-HA labeling. Further work is thus needed to understand the HA-driven inflammatory mechanisms observed *in vitro* and *in vivo*.

HA deposits promote an allergic inflammatory response in the lung

In a mouse model of allergic asthma using Ova, a significant increase in HA deposition is observed in lung tissue (3). This was attributed to early increases in HAS 1 and HAS 2 mRNA expression and a decrease in Hyal 1 and Hyal 2 expression in the lung tissue (3). HA deposits provided sites for inflammatory cells to accumulate and supported subsequent collagen deposition (3). TSG-6 promoted HA deposition, suggesting the formation of HA–HC complexes, and eosinophilic airway inflammation and airway hyper-responsiveness (45), correlating HA deposits with the eosinophilic response. Further evidence for a role of HA in allergic airway inflammation comes from the use of a HA synthesis inhibitor, 4-methylumbelliflerone (88), which reduces Ova-induced eosinophil airway inflammation (124). Thus, these HA deposits may support inflammation by sequestering inflammatory cells and providing survival or other signals. Alternatively, it is possible that the presence of HA deposits may reflect an attempt to resolve the inflammation by promoting matrix deposition. More work is needed to fully understand the role of HA deposits in the inflammatory response.

A ROLE FOR CD44 IN RESOLVING INFLAMMATION

CD44 facilitates the clearance of HA fragments and helps resolve sterile inflammation in the lung

The mouse bleomycin model of sterile inflammation and fibrosis has been used extensively by Noble and colleagues, and has provided key data on the role of HA and CD44 in the inflammatory process [reviewed in Ref. (97, 112)]. Bleomycin induces lung injury and necrosis that triggers an inflammatory response and

Table 2 | The effect of HA and HA fragments on macrophages and dendritic cells.

Cell type	HA size (kDa)	HA source	Effect	Molecule	Steps to exclude LPS	Reference
MACROPHAGES AND MONOCYTES						
BMDM	40–80	Bovine trachea	Induced IL-1 β , TNF α mRNA	CD44	Used LPS hyporesponsive mouse	(106)
BMDM and MH-S cell line	6000 474, 267	Rooster comb Sonicated	No effect Activated NF κ β	–	Not specified	(107)
MH-S cell line and human inflammatory alveolar M ϕ	6000 470 280 35 6 mer 2 mer	Rooster comb Sonicated Human umbilical cord Rooster comb Not specified	No effect Induced MIP1 α , MIP1 β , IP-10, RANTES, MCP-1 mRNA in mouse and IL-8 in human No effect	CD44	Endotoxin tested, polymyxin added	(109)
MH-S cell line, BMDM	200	Human umbilical cord	Induced iNOS mRNA Activated NF κ β	–	Used LPS hyporesponsive mouse, polymyxin added	(108)
Elicited peritoneal M ϕ	280	Human umbilical cord	MIP1 α , MIP1 β , RANTES, IL-12 mRNA	–	Used LPS hyporesponsive mouse, endotoxin present, polymyxin added	(115)
Peritoneal M ϕ	135 200	Digested from bacterial HA Patient serum ^a	Induced MIP-2 and TNF α Induced MIP-2, MIP1 α , KC	TLR2 TLR4 Not CD44	CD44, TLR2/4 KO mice. Endotoxin tested (<40 pg/mg HA), Hyal digestion, polymyxin added	(112)
Elicited peritoneal M ϕ	6000 200	Rooster comb Human umbilical cord	No effect Induced MIP1 α , MIP1 β , KC, MCP-1, RANTES, TNF α mRNA	– TLR2	Used LPS hyporesponsive mouse, endotoxin tested solutions, polymyxin added	(111)
MH-S cell line and human THP-1 cell line	Not specified	Human umbilical cord	Induced MIP-2, TNF α , GM-csf, RANTES IL-1 α , and <i>Mmp13</i> , <i>Tgfb2</i> mRNA, and IL-8 (human)	CD44 TLR4	Endotoxin removed, DNA free	(113)
MH-S cell line and elicited peritoneal M ϕ	200	Human umbilical cord	Induced TNF α and KC secretion	–	Used LPS hyporesponsive mouse	(116)
MH-S cell line and elicited peritoneal M ϕ	500–730 Up to 500 4–18 mers	Rooster comb Human umbilical cord Not specified	No effect Induced MIP-2 and pro-IL-1 β via TLR4. Induced IL-1 β via CD44, Hyals and NLRP3 Induced MIP-2	CD44 TLR4 NLRP3	Used a LPS hyporesponsive mouse, Hyal digestion, endotoxin removal	(114)
Raw264 and MH-S cell line	Specific sizes 11–970	Acid hydrolysis of bacterial HA	No effect on NO or TNF α	–	Endotoxin tested <0.01 EU/ml	(117)
DENDRITIC CELLS						
BMDC, human blood-derived DCs	1000 80–200 4–14 mers	Rooster comb, sonicated Hyal digestion of sonicated HA	No effect Induced HLR-DR expression and IL-1 β , TNF α , IL-12. Activated NF κ β . Activated MAPK/p38 and NF κ β	– TLR4 not CD44 or RHAMM	Endotoxin tested (<0.06 EU/ml or <0.1 ng/ml LPS), polymyxin added	(29, 30)
BMDC	>570	Not specified	Upregulated CD40, CD80, CD86	Not CD44	Polymyxin added	(74)
BMDC	200	Human umbilical cord	Induced MIP1 α	–	Used LPS hyporesponsive mouse, polymyxin added	(111)
BMDC	2–12 mers	Commercial source	Unable to activate DCs <i>in vitro</i>	–	–	(118)

All cells are murine unless otherwise stated.

BMDM and BMDC, bone marrow-derived macrophages and dendritic cells, respectively; M ϕ , macrophages; Hyal, hyaluronidase. MIP-2 and KC are also known as CXCL2 and CXCL1, respectively.

^aPatient serum was from patients with acute lung injury. Items in blue indicate no proinflammatory effect was observed with HA.

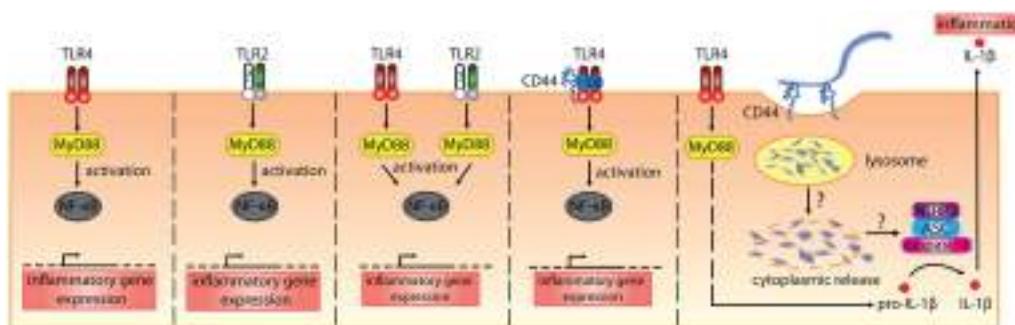


FIGURE 3 | The various receptors implicated in mediating the proinflammatory signals induced by HA fragments. From the left, HA fragments ranging from 500 kDa and below have been reported to stimulate proinflammatory cytokine production via TLR4 alone, TLR2 alone, both TLR2 and TLR4, a complex of TLR4 involving CD44, or via TLR4- and CD44-mediated uptake that leads to inflammasome activation (110–114). In the first three panels, the TLRs signal via MyD88 to activate NF- κ B and induce

proinflammatory cytokine gene expression. In the fourth panel, CD44, together with TLR4 and MD-2, is needed for HA-stimulated proinflammatory cytokine production. In the fifth panel, TLR4 signals via MyD88 to produce pro-IL-1 β and the uptake of HA via CD44 leads to the breakdown of HA which, through some unknown mechanism, leads to HA oligomers in the cytoplasm and these trigger NLRP3 inflammasome activation. This leads to cleavage of pro-IL-1 β and the generation of IL-1 β (114).

subsequent wound repair mechanisms. Inflammatory monocytes and neutrophils are recruited to the damaged site, where they are activated by inflammatory signals released from the injured and necrotic tissue. These activated cells will phagocytose cell debris but will also produce anti-microbial factors such as ROS, which cause further tissue damage. HA is normally undetectable in the BALF but increases upon inflammation to approximately 2 μ g/ml at the peak of the response. HA levels are also increased in the lung tissue upon inflammation, from 100 to 300 ng/mg of dry tissue. This increase is also accompanied by a decrease in molecular mass (500 kDa compared to the normal size of 1400 kDa) (19). Normally, the inflammatory response transitions to a healing response, where further neutrophil recruitment is halted, and debris and apoptotic neutrophils are scavenged by macrophages and/or inflammatory monocytes. Increased levels of TGF β stimulate fibroblast proliferation and ECM production, which restores the tissue back to its original state. In CD44-deficient mice, the initial inflammatory phase appears normal, except for a higher accumulation of HA fragments. However, the CD44-deficient mice cannot resolve the inflammation: TGF β activation is defective; apoptotic neutrophils are not cleared; and HA levels continue to rise in both the BALF and lung tissue (19). This points to a defect in clearance by macrophages or monocytes and a role for CD44 in this process. This defect is largely corrected by reconstitution of CD44-deficient mice with a CD44-sufficient immune system (19), further implicating CD44 on immune cells in the clearance of HA. The CD44-mediated uptake of HA and associated debris, together with CD44-assisted activation of TGF β (125), may be sufficient to tip the balance toward the resolution phase of the inflammatory response. While a protective role for CD44 has also been observed in severe hypoxia-induced lung damage by promoting HA clearance and protecting from epithelial cell death (126), this role for CD44 in the resolution phase is not universally apparent, particularly when infections occur. This highlights potential differences between pathogenic and sterile inflammation and/or suggests that additional factors drive resolution when pathogens are encountered.

SUMMARY

The key points to emerge from this review are:

1. Few immune cells bind HA during homeostasis (alveolar macrophages are a notable exception).
2. The form of HA (its size, association with other molecules, and its ability to form complexes) changes between homeostasis and inflammation.
3. Immune cell interactions with HA increase upon an inflammatory response. This may help recruit immune cells to the site of inflammation as well as keep cells at the site and may facilitate their survival and function.
4. CD44 is the only receptor that has been demonstrated to bind HA on immune cells. HA binding by CD44 can lead to HA uptake and its subsequent degradation. Macrophage CD44 is thought to play an important role in HA uptake and the clearance of HA fragments during lung inflammation.
5. Additional evidence is required to establish whether purified HA fragments and HA oligomers are proinflammatory and if so, what receptors do they interact with to mediate this effect.
6. There is a need to better understand the composition and structure of the HA fragments and complexes present *in vivo* during an inflammatory response, and to then mimic their effects *in vitro*.

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The hyaluronic acid–HDAC3–miRNA network in allergic inflammation

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We previously reported the anti-allergic effect of high molecular weight form of hyaluronic acid (HMW-HA). In doing so, HA targets CD44 and inhibits Fc ϵ RI signaling and cross-talk between epidermal growth factor receptor (EGFR) and Fc ϵ RI. We previously reported the role of histone deacetylases (HDACs) in allergic inflammation and allergic inflammation-promoted enhanced tumorigenic potential. We reported regulatory role of HA in the expression of HDAC3. In this review, we will discuss molecular mechanisms associated with anti-allergic effect of HA in relation with HDACs. The role of microRNAs (miRNAs) in allergic inflammation has been reported. We will also discuss the role of miRNAs in allergic inflammation in relation with HA-mediated anti-allergic effects.

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The Role of Hyaluronic Acid in Allergic Inflammation

Hyaluronic acid (HA), a major component of the extracellular matrix (ECM), plays a key role in regulating inflammation. HA enhances proteoglycan synthesis, reduces the production and activity of pro-inflammatory mediators and matrix metalloproteinases, and alters the behavior of immune cells (1). Inflammation is associated with accumulation and turnover of HA polymers by multiple cell types. Increased accumulation of HA has been demonstrated in joint tissue of rheumatoid arthritis (RA) patients (2); in lung disease, both in humans (3) and animal experimental models (4); in inflammatory liver disease; during vascular disease (5); in rejected kidney transplants (6) as well renal tissue of patients experiencing diabetic nephropathy (7); in the intestine of patients undergoing flares of inflammatory bowel disease (IBD) (8).

Circulating HA might be a marker of asthma control, as it correlates with airway resistance and has good sensitivity in the detection of impaired asthma control (9). The increased level of HA is correlated with asthma (10). In addition, HA appears to provide the scaffolding for inflammatory cell accumulation as well as for new collagen synthesis and deposition (10). HA deposition appears largely due to up-regulation of hyaluronan synthase 1 (HAS1) and hyaluronan synthase 2 (HAS2). HAS2 mRNA is markedly increased in asthmatic fibroblasts (11). In cases of inflammation, HA contains a variety of HA polymers with overlapping lengths and functions. HA exists as both a pro-and anti-inflammatory molecule *in vivo*, and these contradictory functions depend upon polymer length. High molecular weight form of hyaluronic acid (HMW-HA) elicits protective anti-inflammatory effects that protect lung epithelial cells from apoptosis and is protective against liver injury, acting to reduce pro-inflammatory cytokines in a T-cell-mediated injury model (12). HMW-HA inhibits macrophage proliferation and cytokine release, leading to decreased inflammation in the early wound of a preclinical post laminectomy rat model (13). HMW-HA exerts a negative effect on the activation of mitogen-activated protein kinase (MAPK) by allergic inflammation (14). HA with an average molecular mass <500 kDa can be considered a fragment. HA fragments with an average molecular weight of 200 kDa have been shown to stimulate chemokines, cytokines, growth

factors, proteases, and by macrophages (15–20). Organic contact sensitizers induce production of reactive oxygen species (ROS) and a concomitant breakdown of HA to pro-inflammatory low molecular weight fragments in the skin (21). Importantly, inhibition of either ROS-mediated or enzymatic HA breakdown prevents sensitization as well as elicitation of Chediak–Higashi Syndrome (CHS) (21). Mucus hyper secretion with elevated MUC5B mucin production is a pathologic feature in many airway diseases associated with oxidative stress (22). ROS-induced MUC5AC expression in normal human bronchial epithelial cells (NHBE) is dependent on HA depolymerization and epidermal growth factor receptor (EGFR)/MAPK activation (22). Although most of the work on low molecular weight HA (LMW-HA) fragments initially illustrated a pro-inflammatory response, a number of studies have shown that HA fragments can also be protective. In a murine model of colitis, intraperitoneal injection of HA <750 kDa protects colonic epithelium in a Toll-like receptor (TLR) 4-dependent manner (23). This functional difference between HAs of varying sizes is a matter of controversy since many studies have reported opposing results in regard to which type of HA can bring about cellular changes (24). These contradictory functions of HA, depending on the polymer length, may result from differential effects of these HA on HA receptors such as CD44 and receptor for HA-mediated motility (RHAMM). Exogenous HAs used in many studies are not homogenous with respect to size. Therefore, it is difficult to conclude that size alone determines the function of HAs of various sizes. These discrepancies may also be due to differences in experimental settings, purity of HA (25), and the possibility of diverse responses to HA depending on the cell type. Although many reports suggest anti-allergic effect of exogenous HA, the effect of endogenous HMW-HA on the allergic inflammation needs further investigation.

Hyaluronic acid levels are elevated in allergic animals and the increase correlates with the influx of inflammatory cells. This increase in HA levels is largely due to up-regulation of hyaluronidase-1 (HYAL-1) and hyaluronidase-2 (HYAL-2) (26). HYAL-1, -2, and -3 are expressed in airway epithelium and may operate in a coordinated fashion to depolymerize HA during allergen-induced asthmatic responses associated with up-regulation of tumor necrosis factor-alpha (TNF-alpha) and interleukin-1 beta (IL-1beta) (27). Degradation of HA by HYAL-1 primarily depends upon CD44 or other HA receptors to internalize HA fragments. Patients deficient in HYAL-1 have been reported with plasma HA levels at 40 times normal (28). The finding of HYAL-2 in complex with CD44 at the plasma membrane suggests that HA-binding proteins may enhance the activity of HA degrading enzymes, and CD44 binding may provide HYAL-2 with a preferable conformation of HA. IL-1beta exerts inflammatory activity via CD44 by the mediation of HA fragments derived from HA depolymerization (29).

CD44, a receptor for HA, expressed on CD4(+) T cells plays a critical role in the accumulation of antigen-specific Th2 cells, but not Th1 cells, in the airway and in the development of airway hyper-responsiveness (AHR) induced by antigen challenge (30). Airway fibroblasts from patients with asthma produced significantly increased concentrations of LMW-HA compared with those of normal fibroblasts (30). CD44, but not CD62L, is required

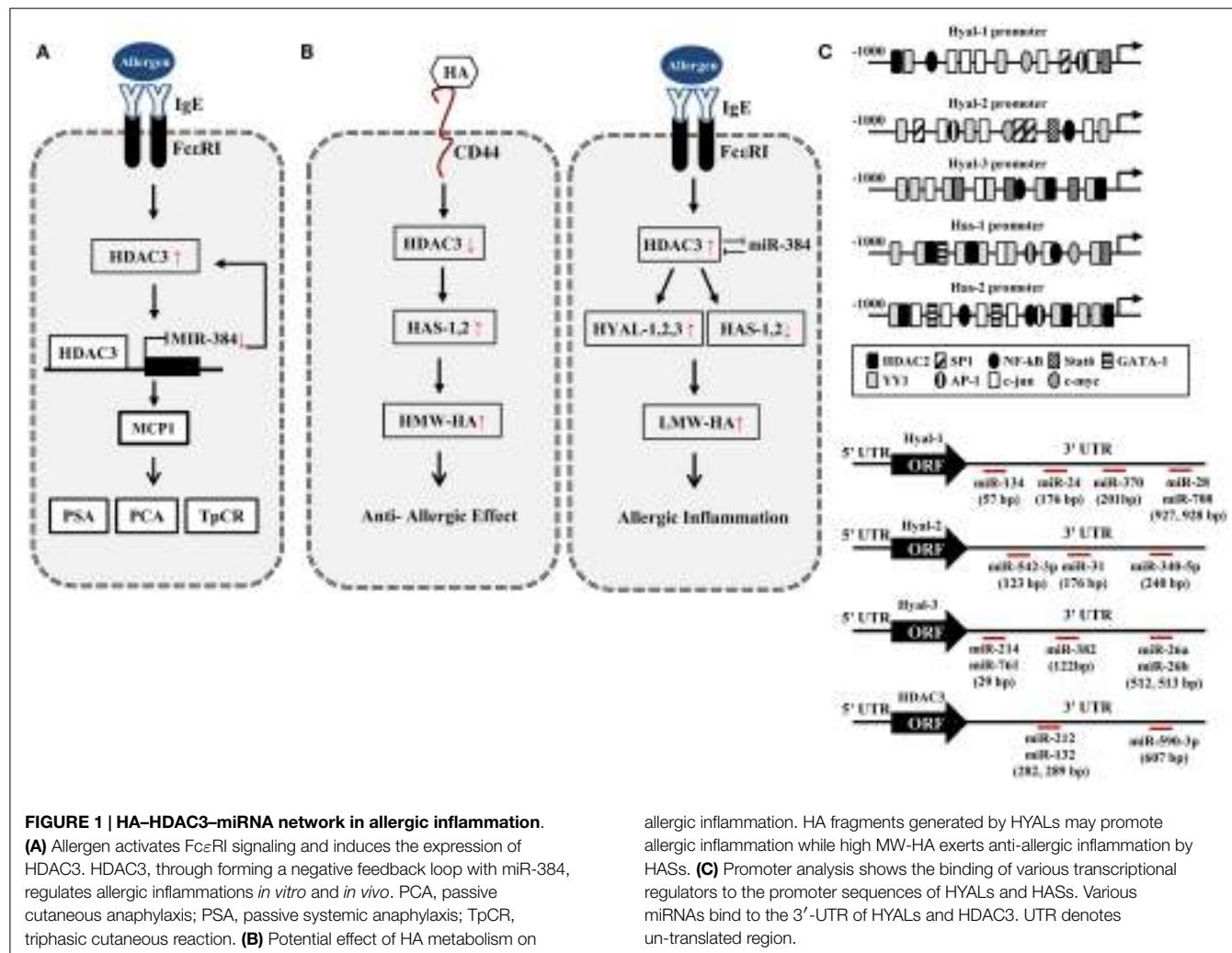
for leukocyte extravasations during a Th2-type inflammatory response such as allergic dermatitis (31). HMW-HA inhibits interaction between IgE and Fc ϵ RI and between Fc ϵ RI and protein kinase C δ (PKC δ) during allergic inflammation (14). A role for CD44 in the regulation of allergic inflammation *in vivo* has been shown by studies in which anti-CD44 treatment inhibited the development of optimal contact allergic responses (32). CD44 has been shown to be responsible for the development of pulmonary eosinophilia (33). CD44-hyaluronan interaction is necessary for allergic asthma (34). The serum-derived hyaluronan-associated protein (SHAP)-HA complex has an inhibitory role in the development of airway hyper responsiveness and allergic airway inflammation which may be attributed, at least in part, to negative feedback mechanisms exerted by SHAP (35). It will be necessary to examine effects of HAs of various sizes on the expression and/or activity of CD44.

The Role of HDAC3 in Allergic Inflammation

Histone acetylation/deacetylation plays an important role in the regulation of inflammatory genes associated with allergic inflammation (36). Histone deacetylase-3 (HDAC3)-deficient macrophages are unable to activate almost half of the inflammatory gene expression program when stimulated with lipopolysaccharide (LPS) (37). Pulmonary inflammation is ameliorated in mice lacking HDAC3 in macrophages (38). The induction of cyclooxygenase (COX)-2, which occurs during allergic inflammation, is accompanied by degradation of HDAC1 (39). HDAC2 expression and activity are decreased in asthmatic subjects, smokers, and smoking asthmatic subjects (40). HDAC3, induced by antigen stimulation, interacts with Fc ϵ RI and is necessary for allergic inflammation both *in vitro* and *in vivo* (41). DNA methyl transferase I (DNMT1) acts as a negative regulator of allergic inflammation and the down-regulation of DNMT1 induces the expression of HDAC3 (42). HDAC3 is necessary for the induction of TNF- α , a cytokine increased during allergic inflammation, in cardiomyocytes during LPS stimulation (43). HDAC3 mediates allergic inflammation by regulating the expression of monocyte chemoattractant protein-1 (MCP1) (41). HMW-HA, but not LMW-HAs, decreases the expression of HDAC3 in human vascular endothelial cells to promote angiogenesis which is accompanied by allergic inflammation (44).

Role of miRNAs in Allergic Inflammation

microRNAs (miRNAs) are small (20–23 nucleotides), single-stranded non-coding RNAs that play important roles in the post-transcriptional regulation of gene expression in mammalian cells by regulating translation. Upon binding of their 5' extremity (seed sequence encompassing nucleotides 2–7 or 2–8) with a complementary site located most of the time in the 3' un-translated region (3'UTR) of target mRNAs, miRNAs alter gene expression by translational repression or RNA degradation (45). Because miRNAs regulate the expression of transcription factors that regulate the expression of miRNAs themselves, miRNAs form feedback loops. miR-384 and HDAC3 form a negative feedback loop to regulate allergic inflammation [(46), **Figure 1A**]. This suggests the involvement of miR-384 in the anti-allergic effect of HA. Several



reports suggest role of HDACs in the expression regulation of miRNAs (47–50). miRNA let-7a regulates the expression of IL-13, a cytokine necessary for allergic lung disease (51). The down-regulation of miR-145 inhibits Th2 cytokine production and AHR (52). HA-CD44 interaction enhances the expression of miR-10b (53). miR-199a-3p and miR-34a miR-590-3p target CD44 (54, 55). Polymorphisms of CD44 3'UTR weaken the binding of miRNAs (55), suggesting that miRNAs regulate the expression of CD44. Given the fact that CD44 is involved in allergic inflammation, miRNAs may regulate HA-mediated anti-allergic inflammation.

The Regulation of HA Metabolism by miRNAs and HDAC3

In silico screening of expression data with predicted miR-23 target sites combined with *in vivo* testing, predicts HAS2 as novel direct target of miR-23 (56). miR-23a-3p in non-senescent fibroblasts leads to the decreased HAS2-mediated HA synthesis (57). This implies that miR-23 may regulate the production of HA during allergic inflammation. Based on our previous report (44), HA-CD44 may decrease the expression of HDAC3 (**Figure 1B**). Promoter analysis shows that HAS1 and HAS2

contain the binding sites for YY1, STAT6, NF-kB, and HDAC2 (**Figure 1C**), suggesting that the production of HA is under epigenetic regulation. Because HDAC3 shows an inverse relationship with HDAC2 (41), HDAC3 may regulate the expression of HASs to mediate allergic inflammation. Many reports suggest that HASs may increase the production of HMW-HA to exert anti-allergic effects (**Figure 1B**). Thus, the decreased expression of HDAC3 by HA-CD44 interaction may increase the expression of HAS1 and HAS2 to exert anti-allergic effect (**Figure 1B**). HDAC3, increased during allergic inflammation, may regulate the expression of HYALs and HASs differentially to increase the production of LMW-HA. This may result in allergic inflammation (**Figure 1B**).

Promoter analysis shows that HYAL-1, -2, and -3 contain binding sites for various transcriptional regulators including HDAC2 (**Figure 1C**), suggesting the role of HDAC3 in the expression regulation of HYALs. TargetScan analysis predicts the binding of miRNAs, such as miR-24, -28, -134, and -370, to the 3'-UTR sequences of HYAL-1 (**Figure 1C**). TargetScan analysis predicts the binding of various miRNAs to the 3'-UTR sequences of HYAL-2 and HYAL-3 (**Figure 1C**). These miRNAs may prevent the production of HA fragments by negatively regulating the expression of these HYALs. Thus, these miRNAs may mediate

allergic inflammation. TargetScan analysis predicts the binding of miR-212, -132, and -590 to the 3'-UTR of HDAC3 (**Figure 1C**). These miRNAs may exert anti-allergic effects by decreasing the expression of HDAC3. Taken together, miRNAs and HDAC3 may regulate allergic inflammation through their effects on HA metabolism.

Concluding Remarks and Perspectives

In this study, we show the possible involvement of miRNAs and HDAC3 in the regulation of HA metabolism. HA-HDAC3-miRNA network described in this review may offer valuable mechanism for HA-mediated anti-allergic effects. For better understanding of HA-mediated anti-allergic effect, it will be

necessary to identify downstream targets of HA. The downstream targets of HA would be valuable for the development of anti-allergic drugs. Identification of more miRNAs that regulate allergic inflammation in relation to HA and HDAC3 will be necessary for better understanding of HA-mediated anti-allergic inflammation.

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Interactions between CD44 and hyaluronan in leukocyte trafficking

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Recruitment of leukocytes from the bloodstream to inflamed tissues requires a carefully regulated cascade of binding interactions between adhesion molecules on leukocytes and endothelial cells. Adhesive interactions between CD44 and hyaluronan (HA) have been implicated in the regulation of immune cell trafficking within various tissues. In this review, the biology of CD44–HA interactions in cell trafficking is summarized, with special attention to neutrophil recruitment within the liver microcirculation. We describe the molecular mechanisms that regulate adhesion between neutrophil CD44 and endothelial HA, including recent evidence implicating serum-derived hyaluronan-associated protein as an important co-factor in the binding of HA to CD44 under flow conditions. CD44–HA-mediated neutrophil recruitment has been shown to contribute to innate immune responses to invading microbes, as well as to the pathogenesis of many inflammatory diseases, including various liver pathologies. As a result, blockade of neutrophil recruitment by targeting CD44–HA interactions has proven beneficial as an anti-inflammatory treatment strategy in a number of animal models of inflammatory diseases.

Keywords: inflammation, leukocyte recruitment, leukocyte trafficking, CD44, hyaluronan

INTRODUCTION

The transit of leukocytes from the bloodstream into inflamed tissues involves a series of sequential events, each mediated by the engagement of adhesion molecules on leukocytes and their ligands on vascular endothelium. First, cells flowing in the bloodstream tether to the vessel wall, and begin rolling along the luminal surface. Recent advances in live-cell imaging have revealed that rolling cells form surface appendages called “long tethers” and “slings” that stabilize the rolling phase to enable this dynamic cell–cell interaction to occur at high shear stress (1, 2). Subsequently, cells firmly adhere to the vascular endothelium, and begin crawling along the vessel wall until they arrive at a favorable site for trans-endothelial migration out of the vasculature (3). Upon arrival within the inflamed tissue, leukocytes are guided by gradients of chemoattractants to shepherd them toward their final target where they carry out their effector functions (4).

Each phase of the multi-step cascade of leukocyte recruitment involves binding interactions between adhesion molecules on leukocytes and their ligands on endothelium. Selectins and selectin ligands are the prototypic adhesion molecules that mediate tethering and rolling, through their ability to form transient adhesive “catch” bonds at high shear stress (5). Firm adhesion is classically ascribed to binding between integrins on immune cells (Mac-1, LFA-1, VLA-4, etc.) and receptors of the immunoglobulin-superfamily on endothelium (ICAM-1, VCAM-1, etc.). The ability of leukocytes such as neutrophils to locomote across the luminal surface of blood vessels via intra-luminal crawling has also been shown to involve integrins, while transmigration requires the coordinated interplay of multiple molecular binding partners (6).

An explosion of research in the field of leukocyte trafficking in the past 25 years has identified many additional “non-classical”

adhesion molecules that can support cell recruitment in the myriad of cell, organ, and disease-specific contexts that are required for a functional immune system (7). Among these, CD44 and its ligand, hyaluronan (HA), have emerged as important adhesion molecules required for cell trafficking in multiple organs, and contribute to the pathogenesis of a variety of inflammatory diseases. CD44 is a type I transmembrane glycoprotein expressed by multiple hematopoietic and non-hematopoietic cells. CD44 is encoded by a single gene, but can be expressed in over 20 isoforms as a result of alternative splicing, and can undergo an array of post-translational modifications, enabling nuanced regulation and diverse functions. The key ligand for CD44, HA, is a member of the glycosaminoglycan family of extracellular matrix molecules, formed from a basic structural unit of repeating disaccharides of *N*-acetylglucosamine and *N*-glucuronic acid. HA is a nearly ubiquitous component of the extracellular matrix, and can be produced by multiple cell types, including endothelium [for a comprehensive review of the regulation of HA synthesis and metabolism, see Ref. (8)]. Below, we review the biology of CD44–HA interactions in immune cell trafficking, explore the molecular mechanisms that regulate the binding between these molecules, and highlight the critical role of CD44–HA-mediated leukocyte recruitment in the pathogenesis of inflammatory diseases.

CD44 AND HYALURONAN IN THE LEUKOCYTE RECRUITMENT CASCADE

ROLLING

Seminal experiments using a parallel-plate flow-chamber system to simulate the hemodynamic environment of post-capillary venules were the first to demonstrate a role for CD44 and HA in leukocyte trafficking. Lymphocytes were observed to roll on

HA-coated plates, and this rolling could be inhibited by incubating the lymphocytes with a function-blocking antibody against CD44 (9). Subsequently, similar *in vitro* systems were used to demonstrate that CD44 on lymphocytes could support rolling on a monolayer of cultured endothelial cells in an HA-dependent manner (9, 10). These findings were confirmed *in vivo* in a model of peritonitis, in which T-cells utilized CD44–HA interactions to home to the inflamed peritoneal cavity (10). Recruitment of superantigen-activated T-cells into the inflamed peritoneum could be inhibited by CD44-blocking antibodies, as well as intravenous infusion of hyaluronidase to degrade endothelial HA (10). However, unlike lymphocytes, neutrophils are unable to roll on a purified HA substrate *in vitro*, and neutrophil rolling on cultured endothelial cells as well as the endothelium of post-capillary venules *in vivo* is not dependent on CD44–HA interactions.

In addition, CD44 can also contribute to leukocyte rolling through interactions with non-HA ligands. Teder and colleagues identified CD44 as a novel E-selectin ligand, and showed that binding between these molecules can support neutrophil rolling in mice and humans (11). Specifically, engagement between neutrophil CD44 and endothelial E-selectin was required for slow rolling, whereas P-selectin-PSGL-1 interactions were required for initial tethering and fast rolling. Furthermore, neutrophil CD44 from patients with leukocyte adhesion deficiency (LAD) syndrome type II (due to mutations of the GDP-fucose transporter gene, resulting in complete deficiency of fucosylated selectin-ligand moieties) was unable to bind E-selectin, implicating CD44–E-selectin interactions in the pathogenesis of this disease (11). In addition to neutrophils, T-lymphocytes also utilize CD44–E-selectin interactions for slow rolling *in vivo*, in a manner that is independent of the contribution of CD44–HA interactions (12).

FIRM ADHESION

Intravital microscopy of the inflamed cremaster muscle of mice has revealed that unlike rolling, firm adhesion of neutrophils can be supported by interactions between CD44 and HA. Blocking antibodies against CD44, genetic deletion of CD44, and removal of HA from the vascular endothelium have each been shown to attenuate neutrophil adhesion (but not rolling), within post-capillary venules (13). Similarly, as described below, CD44 can support neutrophil adhesion within low-shear microvascular beds such as the liver sinusoids, in which neutrophils undergo primary adhesion without a pre-requisite rolling step (14).

A role for CD44 in mediating firm adhesion of lymphocytes has been suggested, but the exact functional contribution of CD44 is less clear. Both *in vitro* and *in vivo*, disruption of CD44–HA interactions limits the adhesion of activated lymphocytes to endothelium (10, 15). However, given that CD44–HA binding also mediates the pre-requisite rolling step, the reduction of firm adhesion may be a secondary effect. This question has been addressed directly by Nandi and colleagues, who investigated the contribution of CD44 versus integrins (the prototypic molecules supporting firm adhesion) to adhesion of activated T-cells to endothelium under flow conditions (16). Interestingly, it was found that CD44 was required for firm adhesion, although not through binding interactions with HA. Co-immunoprecipitation experiments revealed bi-molecular complex formation between

CD44 (which supports rolling) and the integrin VLA-4 (which supports adhesion) (16). Expression of truncated forms of CD44 that lacked the cytoplasmic tail prevented physical coupling of CD44 with VLA-4, resulting in cells that could roll (using CD44) but were unable to adhere. These experiments revealed that while CD44–HA interactions support lymphocyte rolling, CD44 is also required for adhesion by directly collaborating with integrins to enable the formation of high affinity binding to VCAM-1.

TRANS-ENDOTHELIAL MIGRATION AND CHEMOTAXIS

Conflicting data exist regarding the function of CD44 and HA in trans-endothelial migration and chemotaxis through interstitial tissues. With respect to transmigration, neutrophil transit across epithelial monolayers *in vitro* was found to be attenuated when neutrophil CD44 was activated, but was unaffected by functional blockade of the HA-binding domain (17). Importantly, these experiments tested neutrophil migration across intestinal epithelial monolayers under static conditions, and therefore their applicability to trans-endothelial migration under flow is unknown. In contrast, visualization of neutrophil recruitment *in vivo* within the inflamed cremaster muscle of mice demonstrated reduced transmigration in CD44 knockout animals (13). The molecular mechanism by which CD44 contributes to trans-endothelial migration has not been studied. Similarly, the role of CD44–HA interaction in chemotaxis is incompletely understood. Khan et al. found conflicting results between the ability of CD44-deficient neutrophils to migrate toward chemoattractants *in vivo* versus *in vitro* (13). In an under-agarose migration assay, neutrophils from CD44-deficient mice had markedly impaired migration toward MIP-2/CXCL2 compared to wild-type neutrophils. However, neutrophil chemotaxis within cremaster muscle *in vivo* was independent of CD44–HA interactions. The differences observed between *in vitro* and *in vivo* conditions is similar to that observed for the role of integrins in neutrophil chemotaxis, and may be the result of differences in the matrix composition through which cells must migrate in the various models (18, 19). Overall, these studies reveal a paucity of data on the contribution of CD44–HA interactions to the final phases of leukocyte recruitment, and highlight a need for further research in this area.

CD44–HA-MEDIATED LEUKOCYTE RECRUITMENT IN INFLAMMATORY DISEASE

Numerous studies have confirmed the importance of CD44 and HA in leukocyte recruitment within a variety of organs *in vivo*. Antibody blockade of CD44, CD44 deficiency, or enzymatic depletion of endothelial HA (by administration of hyaluronidase) have been shown to decrease neutrophil, monocyte, and/or lymphocyte recruitment and attenuate disease activity in models of arthritis (20, 21), dermatitis (22), peritonitis (10), myositis (13), experimental autoimmune encephalomyelitis (23), orchitis (24), retinitis (25), allergic asthma (26), and graft-versus-host disease (27). However, perhaps one of the best-characterized roles for CD44–HA interactions in pathological leukocyte recruitment is seen in inflammatory liver disease.

The liver vasculature represents a unique structural and hemodynamic environment for leukocyte recruitment, as the majority of infiltrating cells are recruited within the dense labyrinth of

low-flow, low-shear sinusoids. As a result, the mechanisms of leukocyte trafficking within these vessels deviate from the classic multi-step paradigm, in that recruitment does not require a rolling phase (28). Instead, cells are observed to undergo primary adhesion within these low-flow vessels. Therefore, the pleiotropic abilities of CD44–HA interactions to support all steps from tethering to firm adhesion make this binding interaction well suited to the biology of the liver sinusoids. Furthermore, multiple studies using a variety of imaging techniques have demonstrated that the luminal surface of liver sinusoidal endothelium is densely coated with HA (14, 29, 30). Unlike other vascular beds, in which endothelial cell CD44 is required to anchor HA to the vessel wall, liver sinusoidal endothelial cells (LSEC) do not use CD44 to anchor HA, but instead express a variety of scavenger receptors that capture circulating HA on the cell surface, and present it to passing leukocytes before finally promoting its endocytosis and clearance from the bloodstream (31–33). However, no studies to date have investigated the functional role of these scavenger receptors in leukocyte adhesion to HA within liver sinusoids.

A prominent role for CD44–HA interactions in immune cell recruitment to the liver was first observed in a model of acute hepatic inflammation due to sepsis/endotoxemia. Direct visualization of neutrophil trafficking within liver sinusoids revealed that blockade of CD44–HA interactions led to a 50–70% reduction in the number of adherent neutrophils (14). While CD44 can engage with other ligands to support leukocyte–endothelial interactions within other vascular beds (E-selectin and VLA-4, as described above), this promiscuous activity is not observed in liver sinusoids, as multiple studies have confirmed that selectins and α 4-integrins do not contribute to neutrophil recruitment within the liver (14, 28, 34). Menezes and colleagues performed a more in-depth functional analysis of CD44–HA interaction using confocal intravital microscopy in mouse models of endotoxemia and Gram-negative bacterial sepsis (35). It was found that in addition to mediating initial adhesion, CD44 was also required for subsequent stages of recruitment including cell spreading and the initiation of intravascular crawling.

Subsequently, the role of CD44–HA interactions in cell recruitment to the liver has been observed for other cell types in a variety of inflammatory contexts. First, Shi et al. revealed that monocyte recruitment to foci of *Listeria monocytogenes* infection in the liver was reduced by more than 50% by anti-CD44 antibody treatment (36). Interestingly, this study observed a roughly equivalent reduction in monocyte infiltration when Mac-1–ICAM-1 interactions were blocked, but it is not known how each receptor–ligand pair contributes to cell recruitment. One possibility is that CD44 and Mac-1 function cooperatively to promote initial monocyte adhesion. Alternatively, these molecules may function in a sequential manner, with CD44 mediating initial tethering and adhesion, while Mac-1 supports subsequent intravascular crawling toward the bacterial targets. Similar to monocytes and neutrophils, an important role for CD44–HA interactions has also been observed in cytotoxic T-lymphocyte (CTL) recruitment to the inflamed liver in a mouse model of viral hepatitis (37). Hepatitis B transgenic mice develop substantial lymphocytic infiltrates within the liver, whereas transgenic

mice that are also CD44 deficient have markedly attenuated CTL recruitment. Interestingly, adoptive transfer experiments revealed that CD44 was required on endothelium, but not on CTLs. This stands in stark contrast to the role of CD44 in neutrophil and monocyte recruitment, in which CD44 is required on leukocytes but not endothelium (14, 36). The mechanism underlying this observation is not known, but possible explanations include interactions with non-HA ligands on CTLs, or possibly a role for CD44 in upstream inflammatory signaling rather than as an adhesion molecule in CTL trafficking.

A number of recent studies have described a critical role for CD44–HA interactions in the pathogenesis of fatty-liver disease, the leading cause of chronic liver disease in United States and a growing problem worldwide (38). CD44-deficient mice have markedly attenuated hepatitis in a mouse model of non-alcoholic steatohepatitis (NASH), induced by administration of a lithogenic diet (39, 40). Leukocytes harvested from lithogenic diet-fed mice display significant upregulation of their HA-binding capacity (39). This correlated with diminished leukocyte infiltration into the livers of CD44-deficient animals. Using a similar model of lithogenic diet-induced hepatic steatosis, Kang et al. observed that CD44-deficient mice displayed reduced leukocyte infiltration into the liver, in addition to broader defects in steatosis development, adipose tissue inflammation, and insulin resistance (40). Therefore, in addition to supporting leukocyte infiltration into the liver and the development of NASH, CD44 may be a keystone to the systemic pathogenesis of obesity-associated diseases and the metabolic syndrome.

Lastly, CD44–HA interactions have been implicated in the trafficking of hematopoietic stem cells (HSC) to the liver. *In vitro* experiments studying adhesive interactions between HSC and cultured LSEC found that binding was partially dependent on CD44–HA interactions (41). Similarly, CD44 blockade reduced adhesion of HSC to frozen liver sections from patients with primary biliary cirrhosis and alcoholic liver disease (41). While these findings await confirmation *in vivo*, a similar role for CD44–HA interactions has been observed in mesenchymal stem cell recruitment to the kidney in an animal model of acute tubular necrosis (42). Together, these findings suggest that CD44–HA interaction are important not only for the generation of inflammatory responses, but may also be critical to the resolution phase and tissue regeneration by facilitating stem cell homing to injured tissues. This would add an additional role to the growing list of contributions made by CD44 to the resolution of inflammatory responses (43).

REGULATION OF CD44–HA INTERACTIONS IN CELL TRAFFICKING

Despite the fact that CD44 is constitutively expressed on most leukocytes, and HA is a ubiquitous component of the extracellular matrix, trafficking leukocytes only adhere to HA under inflammatory conditions (44). Engagement between CD44 and HA can be regulated at a number of levels including the quantity of surface expression, as well as their functional states of activation.

REGULATION OF CD44 FUNCTION

CD44 expression on the surface of leukocytes can be upregulated in response to a variety of stimuli including antigen receptor

cross-linking, mitogens, cytokines, chemokines, and bacterial products (44). Numerous studies have demonstrated that upregulation of CD44 expression can lead to augmented recruitment of various leukocyte subsets to sites of inflammation. Conversely, defects that result in impaired CD44 surface expression can result in attenuation of leukocyte trafficking kinetics. For example, neutrophils from Rab27a-deficient mice display reduced surface expression of CD44, and as such were found to have impaired adhesion within liver sinusoids in response to endotoxemia (45). Although the precise pathway through which Rab27a controls CD44 expression is not fully elucidated, *in vitro* observations suggest that this molecule may regulate the intracellular trafficking and surface presentation of CD44 on the plasma membrane within neutrophils. Interestingly, genetic defects in the *Rab27a* gene in humans results in a rare immunodeficiency syndrome (Griscelli syndrome 2) characterized by defective neutrophil, NK cell, and CTL function (46). Given the defects in CD44 expression on neutrophils observed in Rab27a-deficient mice, it will be of interest in future studies to determine if the impairments in CTLs and NK cells are also linked to defective CD44 expression and function.

It is not only the absolute quantity of CD44 expression on the cell surface that regulates binding to HA but also the relative expression compared to other adhesion molecules. Studies of neutrophil adhesion in the liver have revealed that the balance between CD44 and integrin (Mac-1) expression can dramatically alter the role of CD44 in leukocyte recruitment. Menezes and colleagues found that CD44-HA interactions are the dominant mechanisms of adhesion in response to endotoxemia/sepsis, whereas CD44 is dispensable when hepatic inflammation was induced by a single neutrophil chemoattractant (fMLP), wherein adhesion required Mac-1 (35). Analysis of surface expression levels revealed equivalent quantities of CD44 expression regardless of the stimulus, whereas Mac-1 expression was significantly reduced during endotoxemia as a consequence of high circulating IL-10. The authors postulated that during endotoxemia, downregulation of Mac-1 allows for CD44 to dominate, resulting in preferential adhesion via CD44-HA interactions, highlighting the functional importance of relative CD44 expression levels in the control of neutrophil trafficking.

In addition to regulation of surface expression levels, activation of leukocytes can also induce a multitude of post-translational modifications (phosphorylation, glycosylation, sulfation, and others) that yield a structurally activated, HA-avid form of CD44 [reviewed by Puré and Cuff (44)]. The functional significance of such post-translational modifications has been demonstrated *in vitro* using flow-chamber experiments, in which lymphocyte activation, and the resultant modifications of CD44, are required to enable rolling and adhesion upon HA-coated coverslips (47). Similarly, activated lymphocytes that are recovered from inflamed tissues *in vivo* possess functionally activated forms of CD44, and demonstrate enhanced binding to HA compared to naïve lymphocytes (10, 15). Perhaps the most direct evidence for conformational activation of CD44 in the control of CD44-HA interaction comes from studies using the IRAWB14.4 antibody, which binds CD44 and alters its conformation directly to generate high HA-avidity. As a result, this antibody commonly serves as a positive control in HA-binding experiments (14, 15).

REGULATION OF HA FUNCTION

While expression and activation of leukocyte CD44 are clearly important for the regulation of cell trafficking, there is emerging evidence that leukocyte recruitment is also modulated by changes in HA expression and/or function at the level of vascular endothelium. First, endothelial cells have the ability to augment their surface expression of HA under inflammatory conditions. Using cultured endothelial cell lines as well as primary endothelial cells, Siegelman and colleagues found that stimulation with TNF α , IL-1 β , or LPS resulted in upregulated surface expression of HA (48). Interestingly, this phenotype was only observed in endothelial cells derived from microvascular beds (but not larger vessels) in which the majority of leukocyte recruitment occurs (48). Using a parallel-plate flow-chamber assay, the authors confirmed that CD44-dependent lymphocyte rolling was increased fourfold upon activated endothelial monolayers as a result of upregulated surface expression of HA (48).

In addition to quantitative regulation of HA expression, leukocyte trafficking can also be modulated by functional changes to the structure of HA polymers. First, HA polymer length has received a great deal of attention in recent years as an important rheostat for the inflammatory response, as a result of its multi-functional inflammatory/anti-inflammatory signaling properties mediated by low versus high molecular weight HA (49). Polymer length may also regulate the function of HA as an adhesion molecule, as evidenced by the fact that digestion with hyaluronidase impairs leukocyte–endothelial interactions. In addition, a growing body of research suggests that a major regulator of HA-CD44 interactions involves structural modification of HA induced by a variety of HA-binding proteins. Serum-derived hyaluronan-associated proteins (SHAPs), is an HA-binding protein that has been shown to modulate leukocyte trafficking through functional alterations of the adhesion properties of HA. Structurally, SHAP is composed of the heavy chains (HC) of inter- α -trypsin inhibitor, a circulating proteoglycan consisting of a single chondroitin sulfate molecule bound to bikunin (a serine protease inhibitor) and the aforementioned HC (HC1, HC2, HC3) (50). At sites of inflammation, I α I HC (SHAP) are transferred onto HA through a trans-esterification reaction catalyzed by tumor necrosis factor-stimulated gene 6 (TSG-6), yielding a covalently bound SHAP-HA complex (50). SHAP-HA complexes purified from the synovial fluid of patients with rheumatoid arthritis bind with greater avidity to lymphocyte CD44 than native HA alone (51). Furthermore, lymphocyte rolling and adhesion *in vitro* upon a substratum of SHAP-HA is increased more than fourfold compared to HA alone (51). Some studies have suggested that SHAP-HA complexes may enable adhesion regardless of the activation state of leukocyte CD44 (52). Systematic inhibition studies have confirmed that adhesion to SHAP-HA still occurs exclusively between HA and CD44, and that the role of SHAP is indirect through functional alterations of HA macromolecular structure (51). Immunofluorescence imaging has revealed that binding of SHAP induces profound architectural changes to the macro-structure of HA polymers, resulting in coalescence of HA polymers into multi-cell-length cables (52). This concentration of HA polymers may augment binding to leukocytes by enabling high avidity interactions with leukocyte CD44, potentially providing favorable clustering

of CD44 within plasma membrane microdomains. Alternatively, formation of SHAP–HA complexes may protect HA from degradation or endocytosis, thereby stabilizing the surface landscape of the vascular endothelium to promote interactions with passing leukocytes.

Serum-derived hyaluronan-associated protein–HA complexes are observed in multiple inflamed organs. High concentrations have been demonstrated in the synovial fluid of patients with rheumatoid arthritis (51), and colon tissue from patients with active inflammatory bowel disease (52). Within the colon of patients with IBD, SHAP–HA complexes were observed primarily around blood vessels, as well as within the hyperplastic muscularis layer (52). Within the liver microvasculature, neutrophil adhesion in response to bacterial LPS is preceded by marked induction of SHAP–HA complex formation on the endothelial surface (14, 53). Unlike HA, which is present in liver sinusoids constitutively at high levels, SHAP–HA complexes are only seen after exposure to bacterial products or other stimuli, and CD44-bearing neutrophils adhere within regions that are richly decorated with SHAP–HA complexes (14, 54). Experiments in mice that lack the SHAP precursor ($\text{I}\alpha\text{I}$), and are therefore SHAP-deficient, have revealed that neutrophil adhesion is reduced by ~50% compared to wild-type animals (53). Together, these studies implicate functional “activation” of HA in response to SHAP (and perhaps other HA-binding proteins) in the regulation of leukocyte adhesion. Further studies are needed to clarify the mechanisms responsible for the enhanced avidity of HA for CD44 following binding by SHAP, and whether the macro-structural alteration (such as cable formation) that are seen *in vitro* also occur within the vasculature *in vivo*.

CONCLUSION

As illustrated in this issue of *Frontiers*, CD44 and HA contribute to a remarkably diverse spectrum of biologic processes. As leukocyte adhesion molecules, CD44 and HA display a variety of functional roles in cell trafficking, and contribute broadly to the initiation, propagation, and resolution of inflammatory responses. Within many domains, CD44 and HA are being investigated as potential therapeutic targets to modulate disease pathology, and leukocyte recruitment is no exception. Through our expanded knowledge of the mechanisms regulating CD44 and HA interactions, and how these influence inflammatory responses, we will gain further insight into potential therapeutic targets to treat inflammatory diseases.

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Selective hyaluronan–CD44 signaling promotes miRNA-21 expression and interacts with vitamin D function during cutaneous squamous cell carcinomas progression following UV irradiation

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Hyaluronan (HA), the major extracellular matrix component, is often anchored to CD44, a family of structurally/functionally important cell surface receptors. Recent results indicate that UV irradiation (UVR)-induced cutaneous squamous cell carcinomas (SCC) overexpress a variety of CD44 variant isoforms (CD44v), with different CD44v isoforms appear to confer malignant SCC properties. UVR also stimulates HA degradation in epidermal keratinocytes. Both large HA polymers and their UVR-induced catabolic products (small HA) selectively activate CD44-mediated cellular signaling in normal keratinocytes and SCC cells, with all of the downstream processes being mediated by RhoGTPases (e.g., Rac1 and Rho). Importantly, we found that the hormonally active form of vitamin D 1,25(OH)₂D₃ not only prevents the UVR-induced small HA activation of abnormal keratinocyte behavior and SCC progression, but also enhances large HA stimulation of normal keratinocyte activities and epidermal function(s). The aim of this hypothesis and theory article is to question whether matrix HA and its UVR-induced catabolic products (e.g., large and small HA) can selectively activate CD44-mediated cellular signaling such as GTPase (Rac and RhA) activation. We suggested that large HA–CD44 interaction promotes Rac-signaling and normal keratinocyte differentiation (lipid synthesis), DNA repair, and keratinocyte survival function. Conversely, small HA–CD44 interaction stimulates RhoA activation, NFκB/Stat-3 signaling, and miR-21 production, resulting in inflammation and proliferation as well as SCC progression. We also question whether vitamin D treatment displays any effect on small HA–CD44v-mediated RhoA signaling, inflammation, and SCC progression, as well as large HA–CD44-mediated differentiation, DNA repair, keratinocyte survival, and normal keratinocyte function. In addition, we discussed that the topical application of signaling perturbation agents (e.g., Y27623, a ROK inhibitor) may be used to treat certain skin diseases displaying upregulation of keratinocyte proliferation such as psoriasis and actinic keratoses in order to correct the imbalance between Rac and RhoA signaling during various UV irradiation-induced skin diseases in patients. Finally, we proposed that matrix HA/CD44-signaling strategies and matrix HA (HA_S vs. HA_L or HA_S → HA_L)-based

therapeutic approaches (together with vitamin D) may be used for the treatment of patients suffering a number of UV irradiation-induced skin diseases (e.g., inflammation, skin cancer, and chronic non-healing wounds).

Keywords: hyaluronan, CD44, RhoGTPase, vitamin D, UVR, miR21, vitamin D, skin cancer

Introduction

The incidence of skin cancer has been increasing rapidly over recent decades. UVB radiation (UVR)-induced squamous cell carcinomas (SCC) is the second most common cancer among Caucasians in the United States, contributing substantially to morbidity among elderly people. Recent studies report that the age-adjusted incidence of SCC has grown by 50–200% over the past 10–30 years. Invasive SCC has the potential to recur locally, tending to invade deeply into subcutaneous structures, fascia, and muscle; and to metastasize, most commonly to regional lymph nodes (1, 2). Several lines of evidence indicate that matrix hyaluronan (HA) plays an important role in regulating inflammation, proliferation, and migration/invasion in the progression of a variety of tumors (3–7). Because little is known about the molecular basis underlying HA effects on influencing skin cancer development, there is currently a need to investigate some key aspects of HA signaling in regulating UVR-induced human SCC progression.

Normal keratinocytes and healthy skin tissues express predominantly one large species of the transmembrane glycoprotein, CD44 (Epican), required for many keratinocyte functions (3, 4). However, UVR-induced cutaneous SCC cells and tissues overexpress a variety of variant isoforms of CD44 (3–9). Different CD44 variant (CD44v) isoforms appear to confer the malignant properties of increased tumor cell growth and cancer progression (5–10). CD44v isoforms bind a number of extracellular matrix (ECM) ligands (e.g., HA), and are known to participate in a variety of both normal keratinocyte and SCC functions (3–10). Our recent results indicate that UVB stimulates HA degradation in normal keratinocytes. Both large HA polymers and their smaller catabolic products selectively activate CD44 isoform-mediated cellular signaling that regulates inflammation, anti-apoptosis, and tumor cell growth, as well as differentiation, DNA repair, and keratinocyte survival function.

Skin Cancer

Recent studies report that the age-adjusted incidence of skin cancer has grown by 50–200% over the past 10–30 years. Exposure to (UVR) has well-recognized clinical effects on the skin, including sunburn (inflammation) and keratinocyte transformation, leading to neoplasia and SCC progression. In particular, skin type determines sensitivity to the acute and chronic effects of UVR on the skin. Today, highly aggressive variants of SCC are frequently seen in organ-transplant recipients, as well as in patients who are on immunosuppressive medications or have immunocompromised status for other reasons (1, 2). Human papillomavirus infection has also been associated with some types of cutaneous SCC. Invasive SCC has the potential to recur locally, tending to invade deeply into subcutaneous structures, fascia, and muscle; and to metastasize, primarily to the regional lymph nodes (1, 2). Because little is known about the molecular basis underlying the

progression to the invasive phenotype, it is very difficult to predict individual tumor aggressiveness and design effective treatment plans. Thus, there is currently a real need to clarify aspects of tumor biology underlying the clinical behavior of SCC. It is well-known that the tumor-specific phenotype (characteristics such as inflammation, anti-apoptosis, and tumor cell proliferation) is linked to oncogenic signaling. Dissection of the transmembrane pathways controlling cellular signaling and tumor functions should significantly aid in understanding the intracellular events underlying SCC progression.

Hyaluronan and CD44 in Normal Keratinocytes and Squamous Cell Carcinomas

Hyaluronan, the major glycosaminoglycan of ECM component, serves not only as a primary constituent of connective tissue extracellular matrices but also as a bio-regulatory molecule (1, 2). Many studies indicate that HA is also abundant in stratified squamous epithelia, including mammalian epidermis, and that it influences epidermal functions such as skin integrity (10, 11). However, the mechanisms by which HA stimulates keratinocyte functions and regulates tissue integrity are not well understood. HA is synthesized by several HA synthases (12), and its size further modified by hyaluronidases (13). Generally, small size-HA (1×10^5 – 1×10^4 Da) induces the expression of proinflammatory cytokine/chemokine and proliferative genes as well as cell proliferation and migration; whereas large size-HA ($>1 \times 10^6$ Da) promotes transcriptional activation and differentiation (14–20). UVR-induced changes in HA production and degradation/fragmentation have also been reported (19, 20). Specifically, while large HA appears to predominate in normal mouse skin, small HA becomes prevalent in tumor tissues (19) and UVR-induced keratinocytes (20). All of these observations are consistent with our hypothesis that both HA production and HA size modifications underline UVR-induced changes associated with onset of keratinocyte transformation.

Both large and small HAs are capable of binding to CD44 (21), which is an ubiquitous, abundant, and functionally important receptor expressed on the surface of many cells, including normal and transformed keratinocytes (23). CD44 is encoded by a single gene which contains 19 exons (22). The most common form, CD44s (CD44 standard form), contains exons 1–5 (N-terminal 150 a.a.), exons 15 and 16 (membrane proximal 85 a.a.), exon 17 (transmembrane domain), and a portion of exons 17 and 19 (cytoplasmic tail, 70 a.a.). Out of the 19 exons, 12 exons can be alternatively spliced. Most often, the alternative splicing occurs between exons 5 and 15 leading to an insertion in tandem of one or more variant exons (exon 6–exon 14; v1–v10) within the membrane proximal region of the extracellular domain (31). For example, in keratinocytes, additional exons v3–v10 are inserted into the CD44s transcripts, and this

isoform has been designated as CD44v3-10 (23). Various skin cancer cells and tissues express different CD44 variant (CD44v) isoforms (e.g., CD44v3 and CD44v10) in addition to CD44s and CD44v3-10 (23). These CD44 isoforms have the same amino acid sequences at the two ends of the molecule, but differ in the middle region (v3–v10) within the CD44 membrane proximal domain located at the external side of the membrane (23). Several lines of evidence clearly indicate that CD44 selects unique downstream effectors and coordinates certain downstream, intracellular signaling pathways that influence multiple cellular functions (23).

Hypothesis

In this article, we described the hypothesis that HA and its UVR-induced catabolic products (e.g., large and small HA) selectively activate CD44-mediated Rac and RhoA signaling. Specifically, large HA–CD44 interaction promotes Rac/PKN γ and p38MAPK-dependent normal keratinocyte differentiation (lipid synthesis), DNA repair, and keratinocyte survival function. Conversely, small HA–CD44v isoform interaction stimulates RhoA/ROK-dependent NF κ B/Stat-3 signaling and miR-21 production, resulting in inflammation and proliferation as well as SCC progression. Vitamin D treatment inhibits small HA–CD44v-mediated RhoA/ROK signaling, inflammation, and SCC progression, and it also enhances large HA–CD44-mediated differentiation, DNA repair, keratinocyte survival, and normal keratinocyte function. The results of this study will definitely provide a better understanding of the cellular and molecular mechanisms involved in normal keratinocyte-mediated epidermal function and UVR-induced human SCC progression, as well as vitamin D effects

on reducing UVR-induced keratinocyte transformation and skin cancer.

Experimental Evidence

Detection of CD44v Isoform Expression in Normal Keratinocytes, SCC Cells, and Skin SCC Tissues

Invasive SCC induced by UVR has the potential to recur locally, tending to invade deeply into subcutaneous structures, fascia, and muscle, and to metastasize, preferably to the regional lymph nodes (1, 2). At the present time, very limited information is available regarding the factors responsible for the onset and progression of skin SCC. One promising candidate in this regard is the CD44 molecule. Our data using anti-CD44 antibody (recognizing the common domain of all CD44 isoforms) confirm that undifferentiated keratinocytes express various CD44 isoforms in abundance (Figure 1—low calcium-treated sample). However, following high Ca²⁺-induced keratinocyte differentiation, the lower molecular weight forms cease to be expressed leaving Epican as the dominant species (Figure 1). CD44v isoforms are overexpressed in cutaneous SCC cell lines as compared to normal keratinocytes (Figure 1). Furthermore, immunohistochemical staining using anti-CD44v3 antibody and anti-CD44v6 antibody confirms that both CD44v3 and CD44v6 isoforms are overexpressed in human cutaneous SCC tissue (Figure 2). Only a very small amount of CD44v isoforms is detected in normal skin tissue (Figure 2). CD44v3 overexpression can also be detected in mouse skin following chronic (to a lesser extent acute) UVB exposure (Figure 2). These findings clearly indicate a strong correlation between CD44v isoform expression and UVB-induced skin SCC progression.

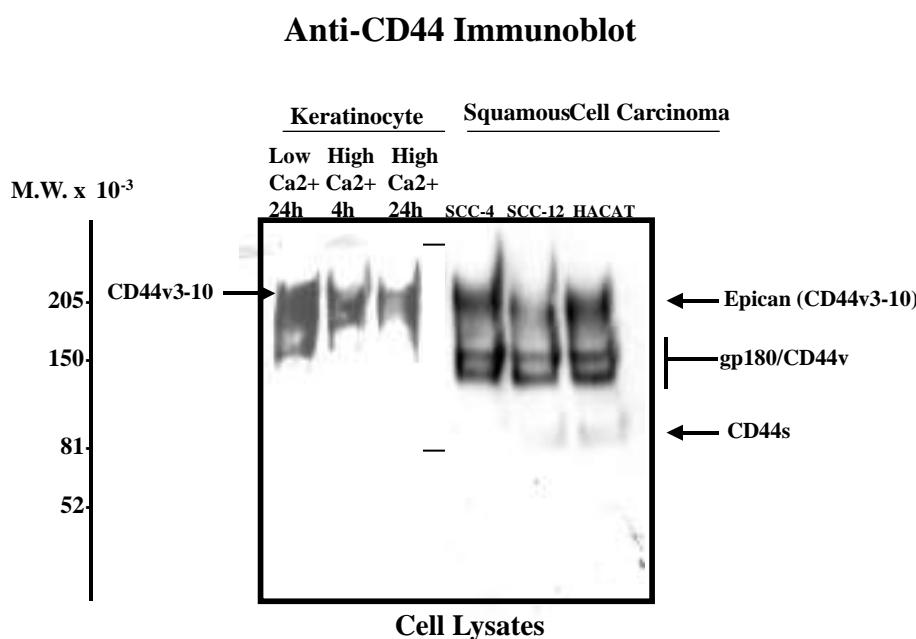


FIGURE 1 | Detection of CD44 isoform expression in normal keratinocytes and SCC cell lines using anti-CD44-mediated immunoblotting techniques [SCC-4, SCC-12 cell lines, and HACAT (a

transformed cell line) Epican is designated as a CD44v3-10 form; anti-CD44-mediated immunoblot was used to detect CD44 signal as indicated].

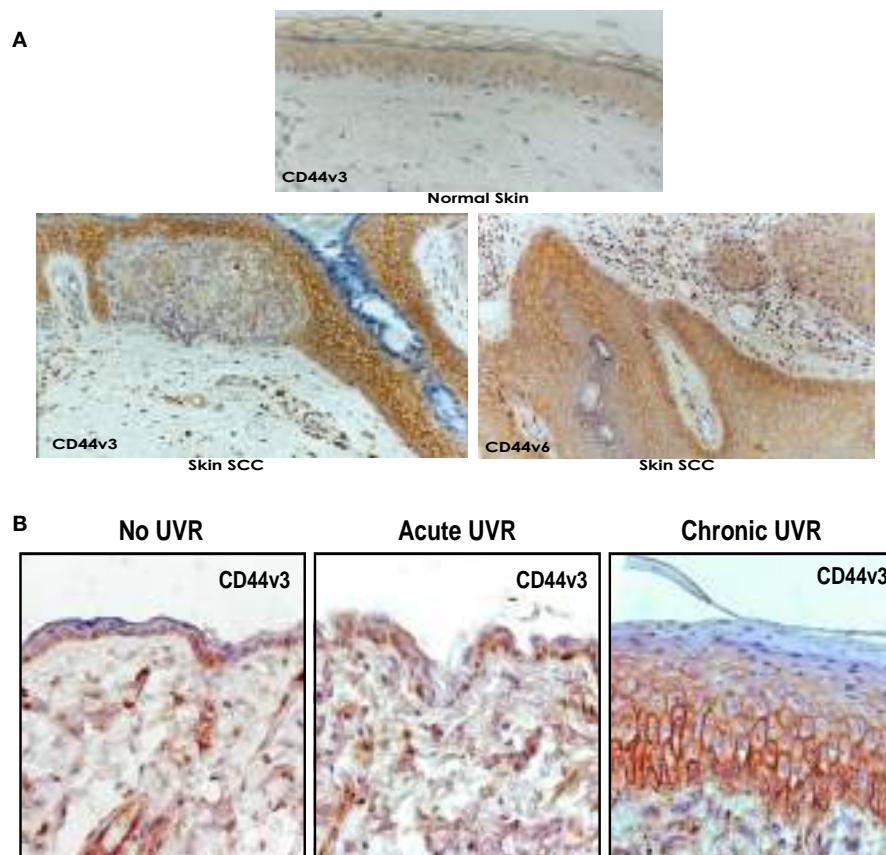


FIGURE 2 | (A) Immunoperoxidase staining of CD44v3 and CD44v6 isoforms in normal and SCC tissues of human skin; and **(B)** immunoperoxidase staining of CD44v3 isoform in mouse skin following acute or chronic UVR exposure.

UVB-Induced Hyaluronan Fragmentation and Abnormal Keratinocyte Functions

Hyaluronan belongs to one of the major glycosaminoglycan polysaccharide families and plays an important role in the formation/remodeling of ECM, specifically in maintaining the integrity of skin tissues (17, 18). Our data show that HA in normal epidermis (without UVB treatment) exists as a large polymer of approximately $1-2 \times 10^6$ Da molecular weight (“large HA”) (Figure 3). These large HA polymers are degraded into smaller HA units (1×10^5 – 1×10^3 Da; “small HA”) following UVB treatment (Figure 3).

Both large and small HAs are capable of binding to CD44 – a ubiquitous, abundant, and functionally important receptor expressed on the surface of many cells including normal keratinocytes and SCC (Figure 2). Generally, small HA-CD44 interaction activates pro-inflammatory cytokine/chemokine gene expression/production, proliferation, and migration, whereas large HA-CD44 binding promotes certain transcriptional activation and differentiation (19, 20). HA-CD44 interaction induces intracellular RhoGTPase (RhoA and Rac) signaling cascades that regulate cytoskeletal reorganization and cell migration (18). Whether HA/CD44 and RhoGTPase signaling actually cause keratinocyte functions and SCC activation is not presently known, and is addressed below.

HA/CD44-Mediated RhoA and Rac1 Signaling in Cytoskeleton Activation and Keratinocyte and SCC Functions

Members of the Rho subclass of the Ras superfamily [small molecular weight GTPases (e.g., RhoA, Rac1, and Cdc42)] act as molecular switches that alternate between GTP- and GDP-bound states. The “activated” GTP-bound enzymes preferentially interact with downstream effector molecules that modulate other effector activities (24). For example, activation of RhoA and Rac1 signaling has been shown to regulate cytoskeleton-associated functions in normal and transformed keratinocytes (SCC cells) (19, 20).

RhoA-Activated Rho-Kinase Signaling Events

Previous work indicated that HA (mixed sizes) promotes the interaction between CD44 and several Rho-specific guanine nucleotide exchange factors [e.g., p115RhoGEF (25) and LARG (26)], thereby up-regulating RhoA (a member of the Rho subclass of the Ras superfamily), leading to altered cytoskeleton-mediated cell functions (25, 26). For example, activation of RhoA signaling has been shown to be involved in cytoskeleton-associated functions. Several enzymes have been identified as possible downstream targets for RhoGTPases (e.g., RhoA) in regulating cytoskeleton-mediated cell motility (27–38). One such enzyme is Rho-Kinase (ROK, also called Rho-binding kinase), which is a serine-threonine kinase

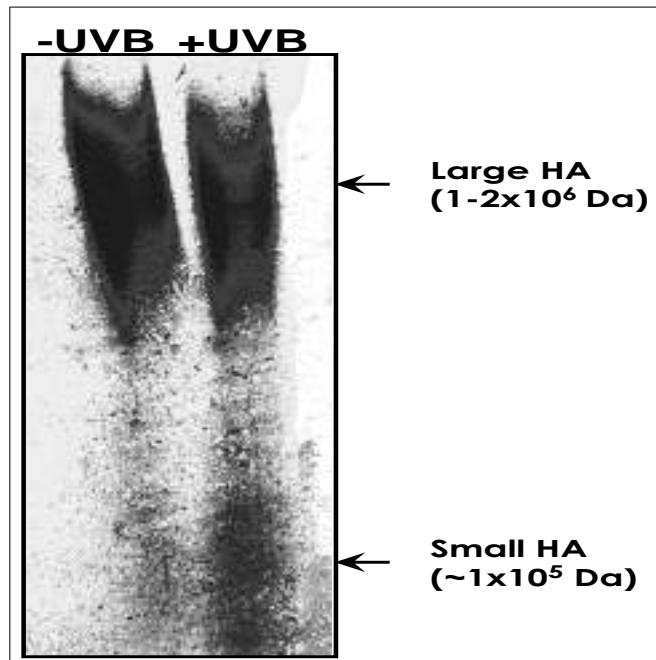


FIGURE 3 | Measurement of HA-size distribution in untreated mouse epidermis vs. acute UVB-treated mouse epidermis. HA from untreated mouse epidermis vs. acute UVB-treated mouse epidermis was isolated and purified using gel filtration column chromatography, and then analyzed by an enzyme-linked binding protein assay, that uses microwells coated with a highly specific HA binding protein (HABP) to capture HA, and an enzyme-conjugated HABP using a commercially available enzyme-linked immunosorbent assay (ELISA)-type test kit. HA was also be analyzed by 0.5% agarose gel followed by Alcian blue and silver staining.

(27–38). ROK interacts with RhoA in a GTP-dependent manner, and phosphorylates a number of cellular substrates including myosin light chain phosphatase, adducin, and LIM kinase (27–38). HA-CD44 interaction promotes RhoA-ROK activation in a number of tumor cells (27–38). Structurally, ROK is composed of a catalytic (CAT), a coiled-coil, a Rho-binding (RB), and a pleckstrin-homology (PH) domain (27–38). Overexpression of either the RB domain or the PH domains (dominant-negative forms) of ROK by transfecting cells with RB cDNA or PH cDNA blocks HA/CD44-specific phenotypic changes (27–38). Also, inhibition of RhoA-activated ROK by Y27632 treatment effectively blocks the HA/CD44-induced cellular signaling and functions (31). These findings suggest that selective activation of CD44 signaling (via small size- vs. large size-HA) induces different RhoA-ROK pathway-specific effects in normal keratinocytes and/or SCC.

Rac1-Activated PKN γ Signaling Events

HA (mixed sizes) also promotes the interaction between CD44 and several Rac1-specific guanine nucleotide exchange factors [e.g., Tiam1 (32) and Vav2 (33)], thereby up-regulating Rac1 (another member of the Rho subclass of the Ras superfamily), leading to altered cytoskeleton-mediated cell functions (32, 33). Rac1 signaling has been shown to play an important role in promoting epidermal stem cells to undergo self-renewal and subsequent terminal differentiation (34). A number of enzymes

have been identified as possible downstream effectors for Rac1 signaling. One such enzyme is protein kinase N- γ (PKN γ) (also called PRK2), which belongs to a family of serine-threonine kinases known to interact with Rac1 in a GTP-dependent manner. It also shares a great deal of sequence homology with protein kinase C in the C-terminal region (35–38). The N-terminal region of PKN contains three homologous sequences of approximately 70 aa (relatively rich in charged residues), which form an antiparallel coiled-coil fold (ACC domain) (35–38). This ACC domain has been shown to interact with RhoGTPases such as RhoA and Rac1 (and to a lesser extent with Cdc42) (35–38). The C-terminal region contains the C2-like region, which functions as an auto-inhibitory domain (35–38). Both the ACC and the C2-like domains, together with the catalytic domain, are conserved among the PKN family members (35–38). In keratinocytes, RhoA-activated PKN γ has been found to be involved in Fyn/Src kinase-regulated cell-cell adhesion during Ca²⁺-induced differentiation (39). Our results indicate that large HA specifically promotes CD44-mediated Rac1-PKN γ kinase signaling (3) and the activation of downstream effectors, including p38MAPK, AP-1 protein (c-Jun), and p63 (Figure 4). This pathway, in turn, regulates keratinocyte differentiation.

Hyaluronan, CD44, and RhoGTPases in Normal Keratinocytes and SCC

HA-mediated CD44 signaling has been shown to play an important role in various cellular functions. Our preliminary data indicate that small HA and large HA binding to cultured keratinocytes and SCC-12 cells selectively activates RhoA and Rac1 (Table 1). In particular, small HA (but not large HA) promotes RhoA signaling in cultured SCC-12 cells (to a lesser extent normal keratinocytes) (Table 1). Further analyses indicate that small HA-activated RhoA-ROK is capable of phosphorylating NF κ B-p65 (Table 2), PKC ϵ (Table 3), and Stat-3 (Table 3), leading to NF κ B-specific (Table 2) and Stat-3-specific transcriptional activation (Table 3), cytokine (IL-6)/chemokine [monocyte-chemoattractant protein-1 (MCP-1)] gene expression (Table 2), and cell proliferation (Table 2). The fact that the ROK inhibitor, Y27632, significantly blocks small HA-induced RhoA-ROK signaling and SCC functions (Tables 2 and 3) suggests that small HA-mediated RhoA-ROK activation plays an important role in regulating oncogenic signaling events and SCC functions.

We also observed that the addition of large HA stimulates Rac1 activation in normal keratinocytes and to a lesser extent SCC (Table 1). In contrast, only a low level of Rac1 activation was detected in keratinocytes treated with small HA or no HA (Table 1). These findings suggest that large HA directly promotes Rac1 activation in normal keratinocytes. It is noted that the level of p38MAPK phosphorylation and AP-1 protein (c-Jun) phosphorylation is greatly enhanced in keratinocytes treated with large HA (Figure 4). Transfection of keratinocytes with PKN γ -ACCcDNA (but not vector-transfected keratinocytes) (Figure 4) or treatment of keratinocytes with Vitamin D not only inhibits large HA-mediated p38MAPK phosphorylation but also blocks AP-1 protein (c-Jun) phosphorylation

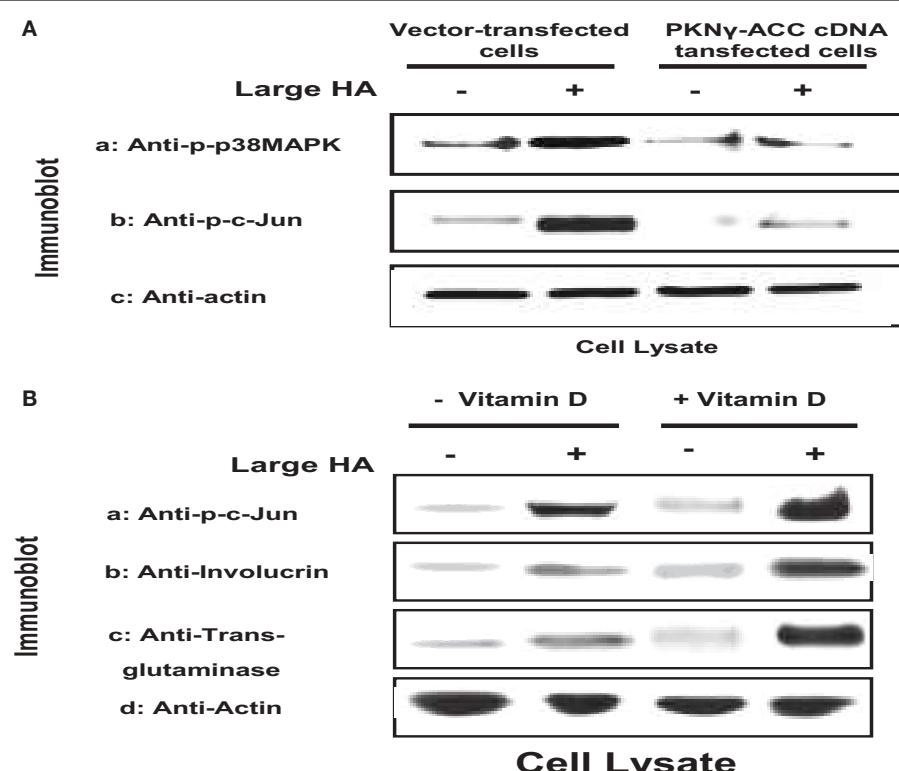


FIGURE 4 | (A) Immunoblot analyses of large HA and PKN-dependent phosphorylation of p38MAPK and AP-1 proteins (e.g., c-Jun) [the PKN-dependent phosphorylation is proven by the effect of PKN γ -ACC cDNA

dominant negative mutant], and **(B)** immunoblot analyses of large HA and vitamin D-induced c-Jun phosphorylation and differentiation marker (involucrin and transglutaminase) expression in cultured keratinocytes.

in cultured keratinocytes (**Figure 4**). These results imply that the ACC fragment of PKN γ (similar as Vitamin D) acts as a dominant-negative mutant that downregulates large HA/CD44-induced Rac1-PKN γ activation and p38MAPK/c-Jun signaling required for normal keratinocyte functions. In addition, we have found that large HA promotes the expression of differentiation markers such as involucrin and transglutaminase in normal keratinocytes, and overexpression of PKN γ -ACC domain (or treatment of Vitamin D) also blocks differentiation marker expression as described previously (3). These findings suggest that large HA-mediated Rac1-PKN γ plays an important role in regulating p38MAPK-AP1 signaling and keratinocyte differentiation.

Selective Small HA-Mediated CD44 Isoform Signaling that Modulates Acute/Chronic UVR-Induced Inflammation in SCC, and Determine the Role of Large HA in Inhibiting Small HA/CD44-Mediated Signaling and Thereby Preventing UVR-Induced SCC Progression

There is compelling evidence that RhoA-activated ROK is involved in the regulation of oncogenesis (18). UVR has acute clinical effects on skin including inflammation. Pro-inflammatory cytokines and chemokines such as IL-6, IL-1 β , RANTES, and MCP-1 contribute to inflammatory response in UVR-treated skin (40, 41). Our results indicate that upregulation of cytokine (IL-6)

TABLE 1 | HA-induced RhoA and Rac1 activation in cultured keratinocytes and SCC12 cells.

Samples	[³⁵ S]GTP γ .RhoA (cpm) (% of control)		[³⁵ S]GTP γ .Rac1 (cpm) (% of control)	
	Normal keratinocytes	SCC-12 cells	Normal keratinocytes	SCC-12 cells
No HA (control)	100 ± 2	100 ± 5	100 ± 3	100 ± 4
Small HA treatment	155 ± 5	320 ± 10	153 ± 2	127 ± 3
Large HA treatment	113 ± 3	110 ± 4	313 ± 8	129 ± 5

and chemokine (MCP-1) can be detected in mouse skin following acute or chronic UVR exposure (**Figure 5**). These findings clearly indicate a strong correlation between cytokine/chemokine expression and UVR-induced skin cancer.

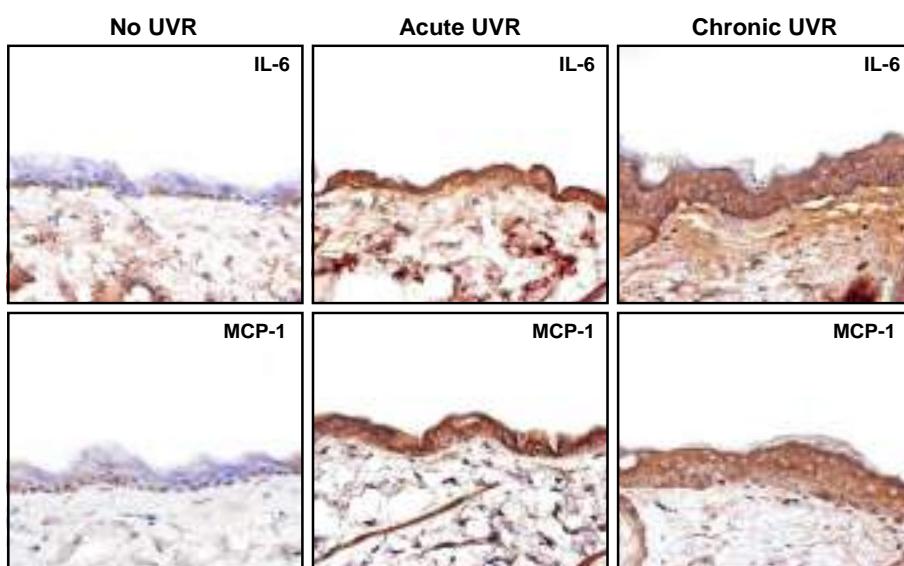
The transcription factor, NF κ B, is known to regulate genes involved in inflammatory responses (42). NF κ B also controls cell cycle regulatory genes such as cyclin D1 and promotes cellular transformation (43, 44). Small HA (but not large HA)-mediated CD44 isoform interaction has been shown to play an important role in stimulating NF κ B-inducing kinase (NIK). Activation of the inhibitor of κ B (IkB) kinase (IKK) complex (IKK α and IKK β) by NIK causes phosphorylation of the IkBs, targeting them for ubiquitylation and degradation by proteasomes, which liberate NF κ B-p65 for nuclear translocation and transactivation of a variety of pro-inflammatory gene expression (21). Small

TABLE 2 | Small HA-mediated RhoA-ROK activation of NF κ B-p65 and in cultured SCC-12 cells.

Samples	NF κ B-p65 phosphorylation (% of control)	NF κ B-p65-specific transcriptional activity (% of control)	IL-6/MCP-1 gene expression relative abundance (%)	Cell proliferation (% of control)
Untreated ROK (control)	100 ± 2	100 ± 2	0.74 ± 0.03/0.63 ± 0.01	100 ± 3
Large HA-treated RhoA-ROK	104 ± 3	106 ± 4	0.66 ± 0.02/0.69 ± 0.01	105 ± 5
Small HA-treated RhoA-ROK	311 ± 14	315 ± 13	3.08 ± 0.10/2.88 ± 0.05	306 ± 10
Small HA-treated RhoA-ROK + Y27632	153 ± 8	120 ± 3	0.60 ± 0.03/0.65 ± 0.04	127 ± 7
Small HA-treated RhoA-ROK + Vitamin D	140 ± 6	134 ± 5	0.63 ± 0.02/0.67 ± 0.03	105 ± 4

TABLE 3 | HA-mediated RhoA-ROK phosphorylation of PKC ϵ and Stat-3 in cultured SCC-12 cells.

Samples	PKC ϵ phosphorylation (mol of Pi/mol of protein)	Stat-3-Ser (727) phosphorylation (% of control)	Stat-3-specific transcriptional activity (% of control)
Unactivated ROK	0.191 ± 0.02	100 ± 2	100 ± 3
Large HA-activated RhoA-ROK	0.193 ± 0.03	106 ± 4	104 ± 5
Small HA-activated RhoA-ROK	1.254 ± 0.10	265 ± 10	270 ± 10
Small HA-activated RhoA-ROK + Y27632	0.136 ± 0.03	124 ± 7	136 ± 4
Small HA-treated RhoA-ROK + Vitamin D	0.158 ± 0.02	135 ± 8	128 ± 5

**FIGURE 5 | Immunoperoxidase staining of cytokine (IL-6) and chemokine (MCP-1) in mouse skin following acute or chronic UVR exposure.**

HA also stimulates NF κ B, which controls proinflammatory genes (e.g., IL-6, IL-1 β , RANTES, or MCP-1 for inducing inflammatory responses) and cell cycle regulatory genes (e.g., cyclin D1 or c-Myc for activating cell proliferation) (42–44). A recent study indicates that RhoA signaling plays a role in activating NF κ B signaling (45).

UVR is also known to promote PKC ϵ overexpression (46) and Stat-3 phosphorylation, leading to skin SCC development (47). Our preliminary data indicate that small HA promotes ROK phosphorylation of PKC ϵ and Stat-3, which in turn stimulates Stat-3-specific transcriptional activities (Figure 6; Table 3) in SCC-12 cells. Large HA exerts direct anti-inflammatory and anti-proliferation effects on UVR-induced skin cancers. Whether large HA prevents small HA-mediated RhoA-ROK activation and NF κ B-p65 signaling, as well as PKC ϵ -Stat-3 function in

transformed keratinocytes/SCC, and reverses small HA/CD44v-mediated tumor-specific behaviors (e.g., pro-inflammatory gene expression, cytokine/chemokine production, and proliferation) in transformed keratinocytes and SCC will be investigated in the future.

MicroRNA-21 (miR-21) Production and Vitamin D 1,25(OH)₂D₃ Effect on Mouse Skin Following Acute and/or Chronic UVR Exposure

Dysregulation of microRNAs (miRNA) is observed in many cancers including skin cancer. In particular, miR-21 appears to play an important role in tumor cell growth and SCC progression. Mature miRNA (e.g., miR-21) are detected in UVB-treated cells

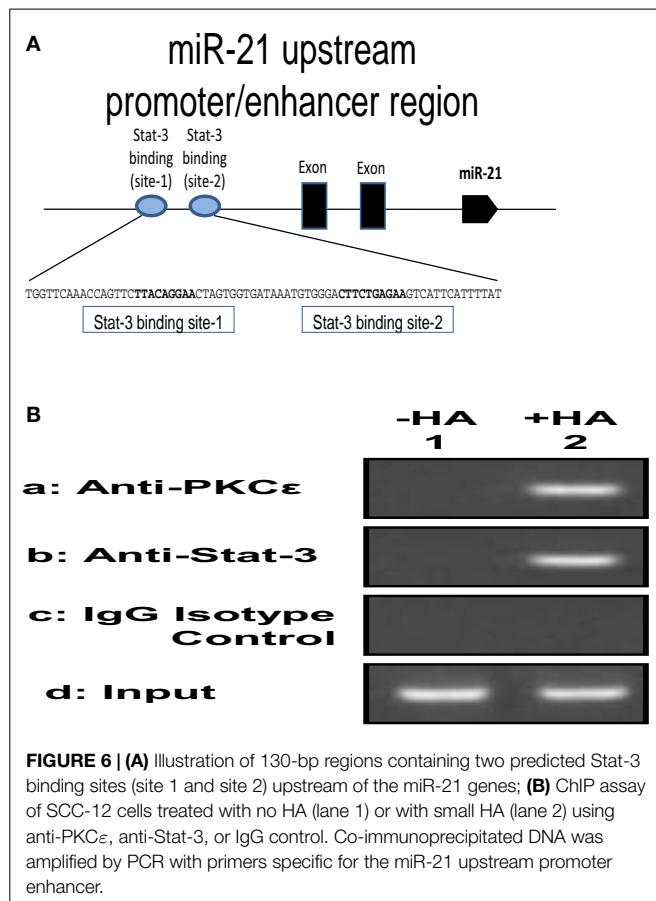


FIGURE 6 | (A) Illustration of 130-bp regions containing two predicted Stat-3 binding sites (site 1 and site 2) upstream of the miR-21 genes; **(B)** ChIP assay of SCC-12 cells treated with no HA (lane 1) or with small HA (lane 2) using anti-PKC ϵ , anti-Stat-3, or IgG control. Co-immunoprecipitated DNA was amplified by PCR with primers specific for the miR-21 upstream promoter enhancer.

and skin carcinogenesis. In this study, we investigated the regulation and expression of miR-21 in mouse skin following acute and/or chronic UVR exposure as described below:

Identify HA/CD44-Mediated PKC ϵ -Stat-3-Specific Target Genes

The mechanism of cellular transformation in keratinocytes and SCC is likely to be the results of several genes that are transcriptionally controlled by the PKC ϵ -Stat-3 interaction. Previous studies indicate that activated Stat-3 up-regulates the mRNA levels of many genes, including miR-21. The expression of mature miR-21 is detected in various SCC cell lines and patient specimens. Many studies indicate that miR-21 may function as an oncogene and play a role in anti-apoptosis and tumorigenesis. A recent study indicates that the gene encoding oncogenic miR-21 is regulated by an upstream promoter/enhancer containing two Stat-3 binding sites (as diagrammed in Figure 6). In order to investigate whether HA-CD44-induced PKC ϵ -Stat-3 complex is involved in the regulation of miR-21 expression in normal/transformed human keratinocytes (SCC 12F2 and SCC12B2) or UVB-treated murine keratinocytes (from epidermal sheets), we conducted chromatin immunoprecipitation (ChIP) reporter gene assays as described below.

Chromatin Immunoprecipitation Assays

To examine whether the PKC ϵ -Stat-3 complex directly interacts with the upstream promoter/enhancer region of miR-21,

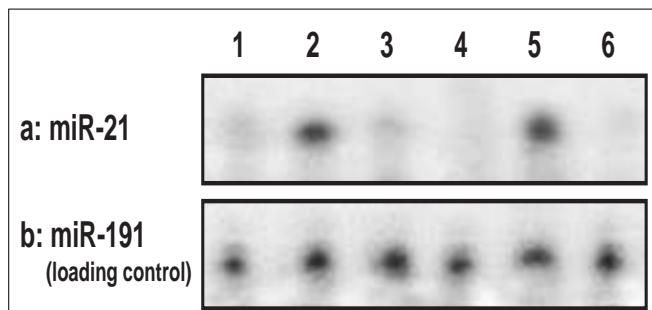


FIGURE 7 | UVB-induced miR-21 production using RNase protection assay in cultured keratinocytes [treated with no UVB (lane 1) or with UVB (lane 2) or treated with ROK inhibitor (Y27632) plus UVB (lane 3) or treated with UVB and vitamin D (lane 4)], and in UVB-induced SCC tumor tissues (lane 5) or normal skin tissues (lane 6).

ChIP assays were performed in normal/transformed human keratinocytes (SCC12F2 and SCC12B2) or UVB-treated murine keratinocytes (from epidermal sheets) in the presence or absence of small/large HA (50 μ g/ml) for 2 h at 37°C using a kit (EZ ChIP) from Millipore Corp., according to the manufacturer's instructions. Cross-linked chromatin lysates were sonicated and diluted with ChIP sonication buffer plus protease inhibitors, divided, and incubated with normal rabbit IgG or rabbit anti-PKC ϵ antibody or rabbit anti-Stat-3 antibody at 4°C overnight, then precipitated with protein G-agarose. Cross-linking will be reversed by overnight 65°C incubation; DNA fragments then will be extracted with PCR purification kit, analyzed by PCR, and quantitated by real-time PCR using primer pair specific for the miR-21 upstream promoter/enhancer region containing the Stat-3 binding sites: forward primer: 5'-CTGGGAGAACCAAGAGCTG-3' and reverse primer: AGGGGACAAGTCAGAGAGAGG-3' on an agarose gel as described previously (48). Results from these proposed experiments will allow us to verify the direct involvement of PKC ϵ and/or Stat-3 in regulating the expression of specific target genes such as miR-21 in UVB-treated normal and transformed keratinocytes. Preliminary data indicate that PKC ϵ and Stat-3 are recruited to the miR-21 upstream promoter enhancer region in SCC-12 cells treated with small HA (Figure 6).

Effect of MicroRNA-21 (miR-21) Production on Mouse Skin Following Acute and/or Chronic UVR Exposure

Our results using RNase protection assays (Figure 7) show that the level of miR-21 production is significantly elevated in cultured keratinocytes treated with UVB (Figure 7, lane 2) as compared to those cells without UVB treatment (Figure 7, lane 1). The expression of Programmed Cell Death 4 (PDCD4) gene is strongly induced during apoptosis in a number of cell types (49–51). PDCD4 encodes a tumor suppressor protein whose expression is lost in progressed carcinomas of many solid tumors (49–51). PDCD4 has been identified as one of the tumor suppressor genes regulated by miR-21 (48, 52, 53). It inhibits translation of PDCD4 by forming a complex with the translation initiation factor eIF4A (an RNA helicase) (54–56).

The “inhibitors of apoptosis family of proteins” (IAPs) constitute a family of at least nine proteins including survivin that block apoptosis by direct binding to caspases (57). Overexpression

of IAPs (e.g., survivin) is thought to be linked to chemoresistance by suppressing apoptosis (57, 58). Our data indicate that HA-CD44-mediated Stat-3 activation induces up-regulation of survival proteins (surviving) expression leading to anti-apoptosis and multidrug resistance in epithelial tumor cells. The fact that downregulation of either PKC ϵ or Stat-3 by treating SCC-12 cells with PKC ϵ siRNA or Stat-3 siRNA blocks HA/CD44-mediated cyclin D1/survivin expression suggests that both PKC ϵ and Stat-3 play important roles in the expression of all these proteins.

Vitamin D 1,25(OH)₂D₃ Effect on Mouse Skin Following Acute and/or Chronic UVR Exposure

Vitamin D 1,25(OH)₂D₃ and its analogs exert direct anti-inflammatory and anti-proliferation effects on UVR-induced skin cancers (59–62). A previous study indicated that one of the hormonally active vitamin D analogs (BXL-628) inhibits ROK membrane localization and activation in tumor cells (63). Recently, a study also indicates that vitamin D [via vitamin D receptor (VDR) action] was shown to abrogate the ability of NF κ B-p65 to transactivate gene transcription and to block cytokine/chemokine gene expression as well as subsequent inflammatory responses and drug resistance in epithelial cells (64, 65). Our data indicate that 1,25(OH)₂D₃ (similar as large HA) (65) prevents small HA-mediated RhoA-ROK activation and NF κ B-p65 signaling, as well as PKC ϵ -Stat-3 function in transformed keratinocytes/SCC, and reverses small HA/CD44v-mediated tumor-specific behaviors (e.g., pro-inflammatory gene expression, cytokine/chemokine production, and proliferation) in transformed keratinocytes and SCC (**Tables 2 and 3**).

In addition, we found that cultured keratinocytes treated with either ROK inhibitor (Y27632) (**Figure 7**, lane 3) or vitamin D (**Figure 7**, lane 4) or normal skin tissues (**Figure 7**, lane 6) contain significantly less UVB-induced miR-21 [as compared

with UVB-induced SCC tumor tissues (**Figure 7**, lane 5)]. The expression of miR-21 occurs in SCC tumor tissues (**Figure 8**) (but not in normal skin tissues) (**Figure 8**). Using digoxigenin (DIG)-labeled miR-21 probe (LNA probe) (and scrambled probe) and *in situ* hybridization [incubated with an anti-DIG-AP (alkaline phosphatase)-FAB fragment plus NBT/BCIP], we have detected an upregulation of miR-21 in VDR-null mouse skin (to a lesser extent wild-type mouse skin) following chronic UVR exposure. In contrast, very little miR-21 is detected in either VDR-null mice or wild-type mice following acute UVR or no UVR treatment (**Figure 8**). These findings suggest that VDR has a protective role to attenuate miR-21 production, and downregulation of VDR enhances miR-21 expression leading to tumor cell growth and skin SCC progression. These observations strongly suggest that UVB promotes miR-21 production in both cultured keratinocytes and SCC tumors. Our observations also support our hypothesis that vitamin D (together with ROK inhibition) may serve as a protective agent to impair UVB-induced miR-21 production and skin cancer progression.

Determine How Large HA/CD44 Epican Interactions with Rac1-Activated PKN γ Lead to Modulations in Epidermal Functions, DNA Repair, and Keratinocyte Survival in Mouse Epidermis Following Acute or Chronic UVR-Induced Skin Cancer Development

A previous study showed that HA/CD44-mediated Rac1-PKN γ activation plays an important role in regulating keratinocyte differentiation (8). Our recent work indicates that large HA (but not small HA) stimulates keratinocyte signaling (**Figure 4**). The question of how large HA/CD44-induced Rac1-PKN γ signaling pathway mediates keratinocyte differentiation is not well-understood. In mammalian cells, at least three distinct

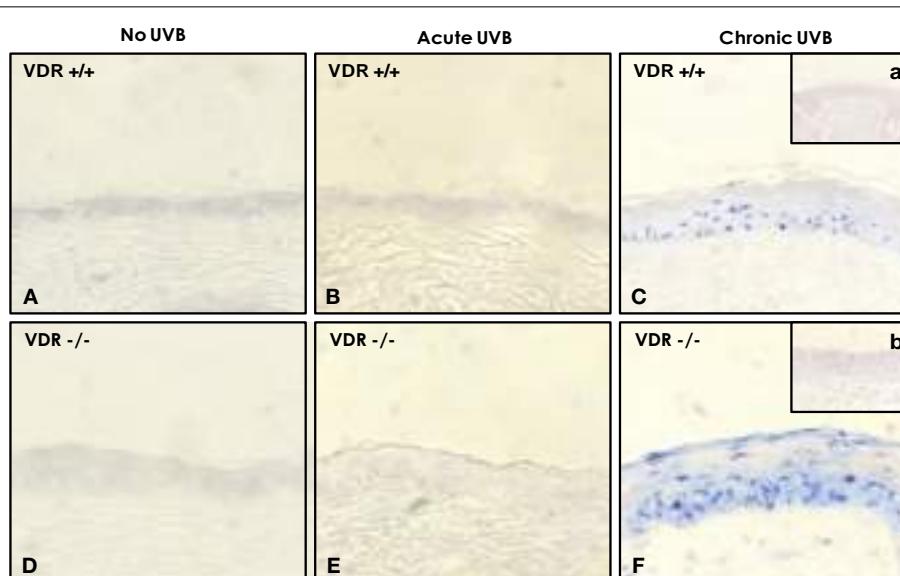


FIGURE 8 | Detection of UVB-induced miR-21 expression in wild-type (VDR^{+/+}) and VDR-null (VDR^{-/-}) mouse skin using DIG-labeled miR-21 probe (LNA probe) (A–F) (and scrambled probe) (a & b) and *in situ* hybridization [incubated with an anti-DIG-AP (alkaline phosphatase)-FAB fragment plus NBT/BCIP].

mitogen-activated kinases (MAPKs) [e.g., ERKs (extracellular signal regulated kinases), the stress-responsive JNK/SAPKs, and p38MAP kinases (p38MAPK)] have been characterized (66, 67). There is compelling evidence that activation of p38MAPK occurs in the membrane (68, 69). Activated p38MAPK has been shown to phosphorylate specific transcription factors and proteins including the transcription factor activator proteins (AP-1) (consisting of members of Jun family of nuclear proteins such as c-Jun, JunB, JunD, Fra-1, Fra-2, c-Fos, and FosB) (70–74). Subsequently, phosphorylated AP-1 proteins bind to DNA elements and induce target gene expression (72, 73). In keratinocytes, the promoter activity of several differentiation-related markers (e.g., involucrin and transglutaminase) and cholesterol synthesis-related proteins (e.g., HMG-CoA synthase and HMG-CoA reductase). Both cholesterol synthesis-related proteins (e.g., HMG-CoA synthase and HMG-CoA reductase) known to be involved in keratinocyte lipid synthesis and differentiation appear to be closely associated with p38MAPK and AP-1 phosphorylation (activated forms) (71–73). Our data indicate that large HA stimulates p38MAPK and c-Jun phosphorylation (Figure 4) as well as keratinocyte differentiation (Figure 4).

UV-induced DNA lesions are known to be repaired by nucleotide excision repair (NER) (74), which operates via either global genomic repair (GGR) or transcription-coupled repair (TCR) (75, 76). Loss of certain NER proteins such as xeroderma pigmentosum group C (XPC) results in a selective impairment of GGR (75, 76). The p38MAPK appears to be involved in activating p53 and p63 – both of which are key regulators of NER (75–80) and have been shown to play an important role in maintenance and induction of VDR and certain key DNA damage recognition proteins (e.g., XPC). Whether large HA/CD44-activated Rac1-PKN γ and p38MAPK are involved in regulating p63/p53

signaling leading to DNA repair and keratinocyte survival following UVR exposure is investigated in this study. Our results indicate that pretreatment of cultured keratinocytes with exogenously added large HA exhibited a 1.7-fold enhancement of cell survival ($P < 0.0004$) following UVB 708 (J/m²) treatment.

These findings reveal selective activation of Rac1-PKN activation by large HA with pathway-specific effects on normal keratinocyte functions. In particular, we have discovered a novel signaling mechanism showing large HA stimulation of Rac1-PKN γ and phosphorylation of p38MAPK in cultured keratinocytes. It is possible that Rac1-PKN γ phosphorylated p38MAPK promotes AP-1 (c-Jun) (Figure 4) and p63/p53-mediated transcriptional upregulation (Figure 9), resulting in target gene expression and epidermal functions. We have found that the protein level of certain regulatory molecules such as p38MAPK, p63, VDR, and XPC is greatly enhanced in both cultured keratinocytes/epidermal sheets treated with large HA (but not small HA) (Figure 9). In contrast, the expression level of these key regulatory proteins (e.g., p38MAPK, p63, XPC, or VDR) is relatively low in keratinocytes treated with HA or p38MAPK inhibitor (anti-CD44 antibody and SB203580, respectively) plus large HA (Figure 9). These findings suggest that large HA/PKN-induced p38MAPK signaling plays an important role in both maintenance and induction of tumor suppressor proteins (e.g., p63) and VDR as well as certain key DNA damage recognition proteins (e.g., XPC). These findings support the notion that large HA/CD44 signaling events may interact with DNA repair pathways required for epidermal protection against UVB-induced skin damage.

In summary, we would like to propose the following model (Figure 10): UVR induced HA degradation and selective HA (small HA vs. large HA)-CD44 interaction with RhoA-ROK and Rac1-PKN γ in regulating transformed and normal keratinocyte

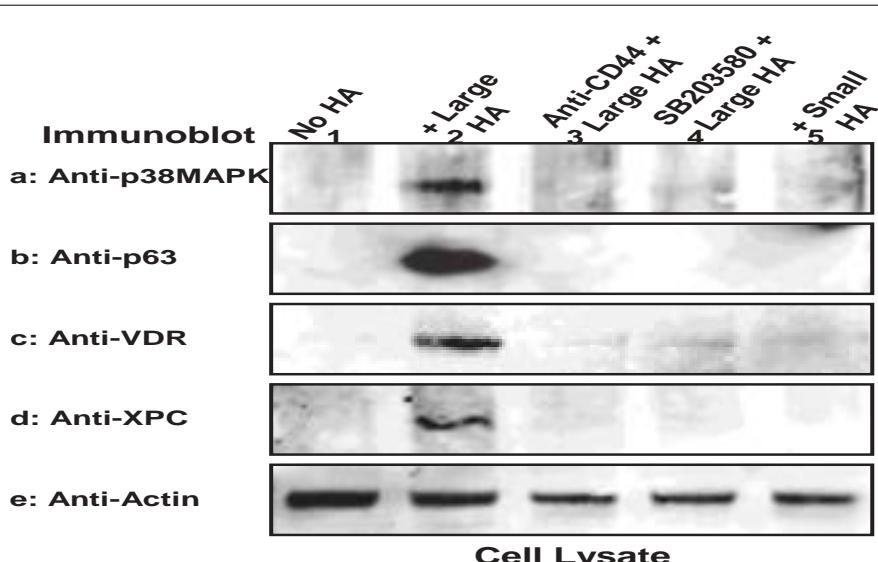


FIGURE 9 | Large HA-mediated upregulation of signaling regulators in keratinocytes. Murine keratinocytes isolated from epidermal sheets were pretreated with HA (50 μ g/ml) (lane 2) in the presence or absence of p38MAPK inhibitor (SB203580) for 24 h followed by irradiation with UVB (708 J/m²), harvested and analyzed by immunoblotting using anti-p38MAPK (a) or anti-p63

antibody (b) or anti-VDR antibody (c) or anti-XPC antibody (d) respectively. Actin was probed by anti-actin antibody as a loading control. (Cells were treated with no HA (lane 1) or with large HA (lane 2) or with anti-CD44 plus large HA (lane 3) or with SB203580 plus large HA (lane 4) or with small HA (lane 5) [p63, a p53-like transcription factor; VDR, vitamin D receptor; XPC, DNA repair protein].

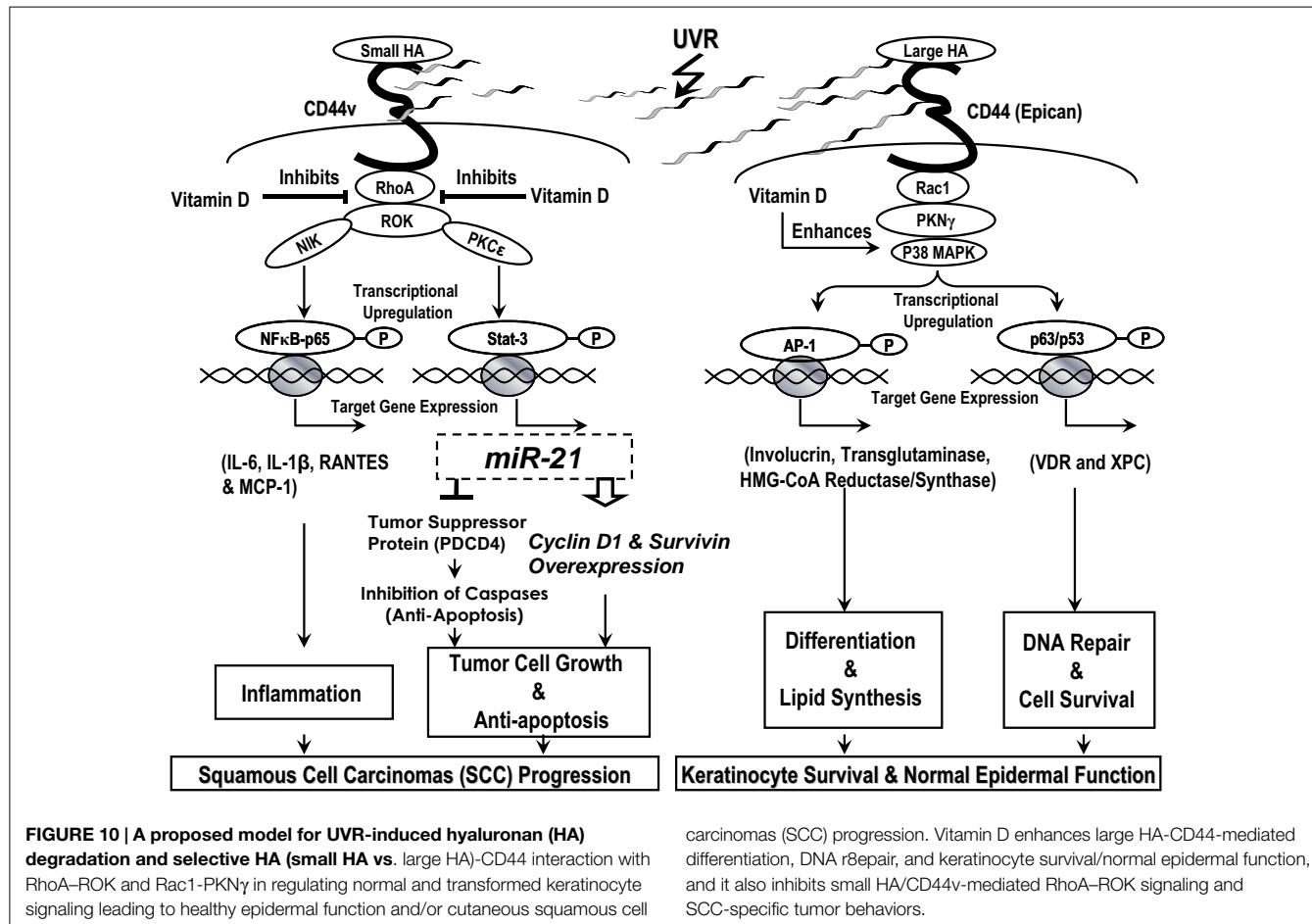


FIGURE 10 | A proposed model for UVR-induced hyaluronan (HA) degradation and selective HA (small HA vs. large HA)-CD44 interaction with RhoA-ROK and Rac1-PKN γ in regulating normal and transformed keratinocyte signaling leading to healthy epidermal function and/or cutaneous squamous cell carcinomas (SCC) progression.

carcinomas (SCC) progression. Vitamin D enhances large HA-CD44-mediated differentiation, DNA repair, and keratinocyte survival/normal epidermal function, and it also inhibits small HA/CD44v-mediated RhoA-ROK signaling and SCC-specific tumor behaviors.

signaling leading to cutaneous SCC progression and/or healthy epidermal function. Vitamin D enhances large HA-CD44-mediated differentiation, DNA repair, and keratinocyte survival/normal epidermal function, and it also inhibits small HA/CD44v-mediated RhoA-ROK signaling and SCC-specific tumor behaviors.

Summary and Outlook

To test our hypothesis, we provided new experimental evidence showing that administration of large HA or vitamin D-related treatments can selectively downregulate the CD44-RhoA/ROK-mediated inflammatory pathways and upregulate CD44-Rac/PKN γ -mediated cell differentiation, DNA repair, and keratinocyte survival function in mouse skin exposed to acute or chronic UVR. The ability for us to identify key HA (small vs. large HA)/CD44-mediated Rho/Rac signaling mechanism(s) by acute or chronic UVR in the regulation of keratinocyte functions will provide valuable insights regarding the possible HA/CD44 involvement of acute or chronic UVR-induced changes in influencing abnormal keratinocyte function, epidermal dysfunction, and tumor formation/SCC progression. In addition, we suggested that signaling perturbation agents (e.g., Y27623, a ROK inhibitor) can be applied to acute or chronic UVR-induced tumor formation/SCC progression displaying upregulation of keratinocyte

inflammation and proliferation in order to correct the imbalance between RhoA-ROK signaling and Rac1-PKN γ activation during epidermal dysfunction.

In conclusion, we believe that the information obtained from these studies described in this article will allow us to delineate UVR-induced HA (small vs. large HA)-mediated CD44 signaling mechanisms and subsequent keratinocyte activities, as well as to provide important HA-based and vitamin D-related therapeutic approach regarding the treatment of UVR-induced keratinocyte transformation and skin cancer progression. The overall idea here is to develop innovative approaches to affect skin cancer growth and SCC progression that may be useful for future clinical studies. The new knowledge obtained from our HA/CD44-signaling strategies and HA-based/vitamin D therapeutic approaches should reveal new avenues for possible treatment of UVR-induced skin cancers.

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Interactions between hyaluronan and its receptors (CD44, RHAMM) regulate the activities of inflammation and cancer

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The glycosaminoglycan hyaluronan (HA), a major component of extracellular matrices, and cell surface receptors of HA have been proposed to have pivotal roles in cell proliferation, migration, and invasion, which are necessary for inflammation and cancer progression. CD44 and receptor for HA-mediated motility (RHAMM) are the two main HA-receptors whose biological functions in human and murine inflammations and tumor cells have been investigated comprehensively. HA was initially considered to be only an inert component of connective tissues, but is now known as a "dynamic" molecule with a constant turnover in many tissues through rapid metabolism that involves HA molecules of various sizes: high molecular weight HA (HMW HA), low molecular weight HA, and oligosaccharides. The intracellular signaling pathways initiated by HA interactions with CD44 and RHAMM that lead to inflammatory and tumorigenic responses are complex. Interestingly, these molecules have dual functions in inflammations and tumorigenesis. For example, the presence of CD44 is involved in initiation of arthritis, while the absence of CD44 by genetic deletion in an arthritis mouse model increases rather than decreases disease severity. Similar dual functions of CD44 exist in initiation and progression of cancer. RHAMM overexpression is most commonly linked to cancer progression, whereas loss of RHAMM is associated with malignant peripheral nerve sheath tumor growth. HA may similarly perform dual functions. An abundance of HMW HA can promote malignant cell proliferation and development of cancer, whereas antagonists to HA-CD44 signaling inhibit tumor cell growth *in vitro* and *in vivo* by interfering with HMW HA-CD44 interaction. This review describes the roles of HA interactions with CD44 and RHAMM in inflammatory responses and tumor development/progression, and how therapeutic strategies that block these key inflammatory/tumorigenic processes may be developed in rodent and human diseases.

Keywords: hyaluronan, CD44, RHAMM, inflammation, cancer

Abbreviations: CD44v, variant isoform of CD44; GAG, glycosaminoglycan; HA, hyaluronic; HAS, hyaluronan synthase; HMW HA, high molecular weight HA; HYAL, hyaluronidase; LMW HA, low molecular weight HA; oHAs, HA oligosaccharides; PEG, polyethylene glycol; PEI, polyethyleneimine; PTX, paclitaxel; RHAMM, receptor of HA-mediated motility; RTK, receptor tyrosine kinase; shRNA, short hairpin RNA; siRNA, short interfering RNA; Tf, transferrin.

Introduction

The interplay between cells and with components in the extracellular matrix (ECM) of the microenvironment is tightly regulated during normal physiological processes of tissues and organs (1–3). During inflammation and tumorigenesis, cellular communications are dramatically lost with subsequent extensive remodeling of cellular and molecular composition of the tumor microenvironment, which supports inflammation, cancer cell proliferation and migration/motility, invasion, and metastasis. Carcinogenesis is characterized by an inflammatory response where biologically active ECM fragments regulate tissue injury/remodeling. Notably, the biosynthesis and degradation of the glycosaminoglycan (GAG) hyaluronan (HA), a major component in ECMs, is associated with the rapid matrix remodeling that occurs during embryonic morphogenesis, inflammation, and tumorigenesis (4–7).

Hyaluronan is a non-sulfated, linear GAG composed of repeating disaccharides of (β , 1–4)-glucuronic acid (GlcUA) and (β , 1–3)-N-acetyl glucosamine (GlcNAc) (MW ~ 400 Da) (Figure 1). Native HA in most tissues has a high molecular mass of 1–10

million Da with extended molecular lengths of 2–20 μ m (8–11). HA has crucial roles in structuring tissue architecture, in cell motility, in cell adhesion, and in proliferation processes (12, 13). These cellular events are mediated mainly through two major signal-transducing cell surface HA-receptors, CD44 (14–19) and the receptor for HA-mediated motility (RHAMM) designated as CD168 (20) (Figure 2), which was first described by Turley (21, 22) as a soluble HA-binding protein released by sub-confluent migrating cells (23). HA is the principal ligand of CD44 (16), and alternative splicing and differential glycosylation produce multiple structural and functional versions of CD44 that are responsible for proinflammatory activities, including cell–cell and cell–matrix interactions (6, 24–26). The CD44 ectodomain includes an amino-terminal domain that contains a HA-binding “link module” motif related to those in the HA-binding proteoglycans and the link proteins (27). Like CD44, RHAMM is alternatively spliced, and variant forms of RHAMM are found both on cell surfaces and inside the cells (28, 29). However, they do not have the link module domain. They have a BX7B motif that also can bind to HA, where “B” represents arginine or lysine, and “X” represents any non-acidic amino acid (30). Studies indicate that CD44-mediated cell

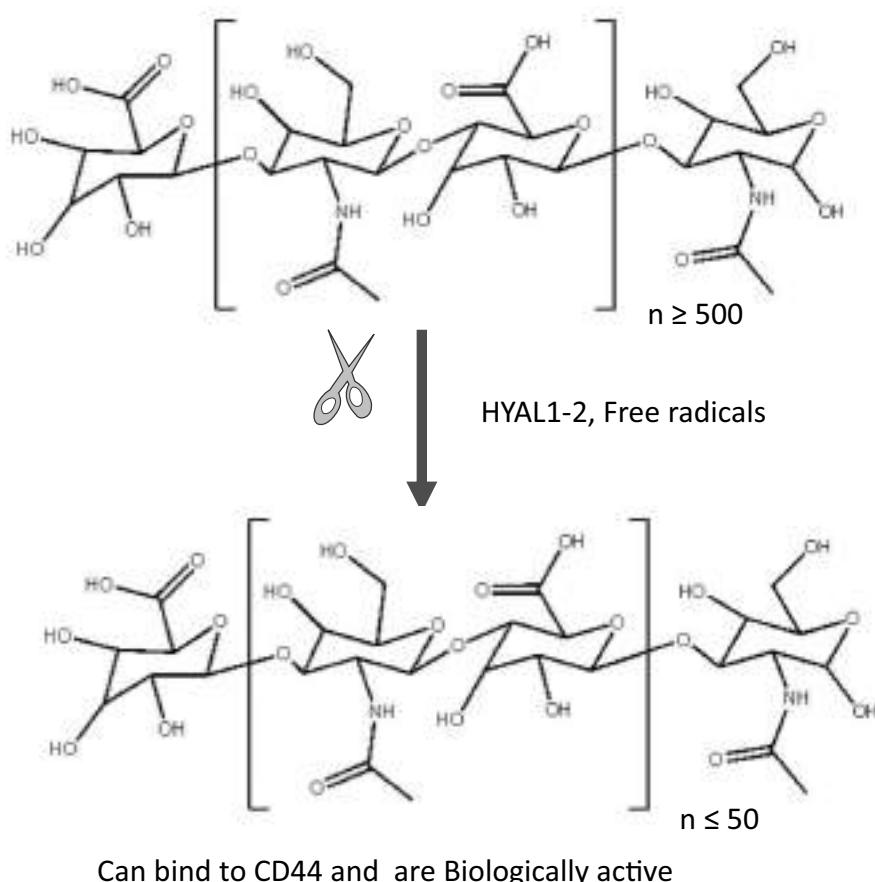


FIGURE 1 | Native polymeric HA and degraded HA fragments. High molecular weight (HMW ≥ 500 disaccharide units; β -1, 4-GlcUA- β -1, 3-GlcNAcJn synthesized in the normal homeostatic condition is degraded by free radicals and hyaluronidases (HYAL1-2) during inflammation/tumorigenesis when tissue injury occurs. These fragments are ≤ 50 disaccharide units. As a

result, the fragments of different molecular weights have different biological functions. For example, intermediate fragments (30–500 kDa) can stimulate cell proliferation while smaller fragments <50 kDa promote cell migration. HA oligosaccharides down to three disaccharides can still bind to CD44.

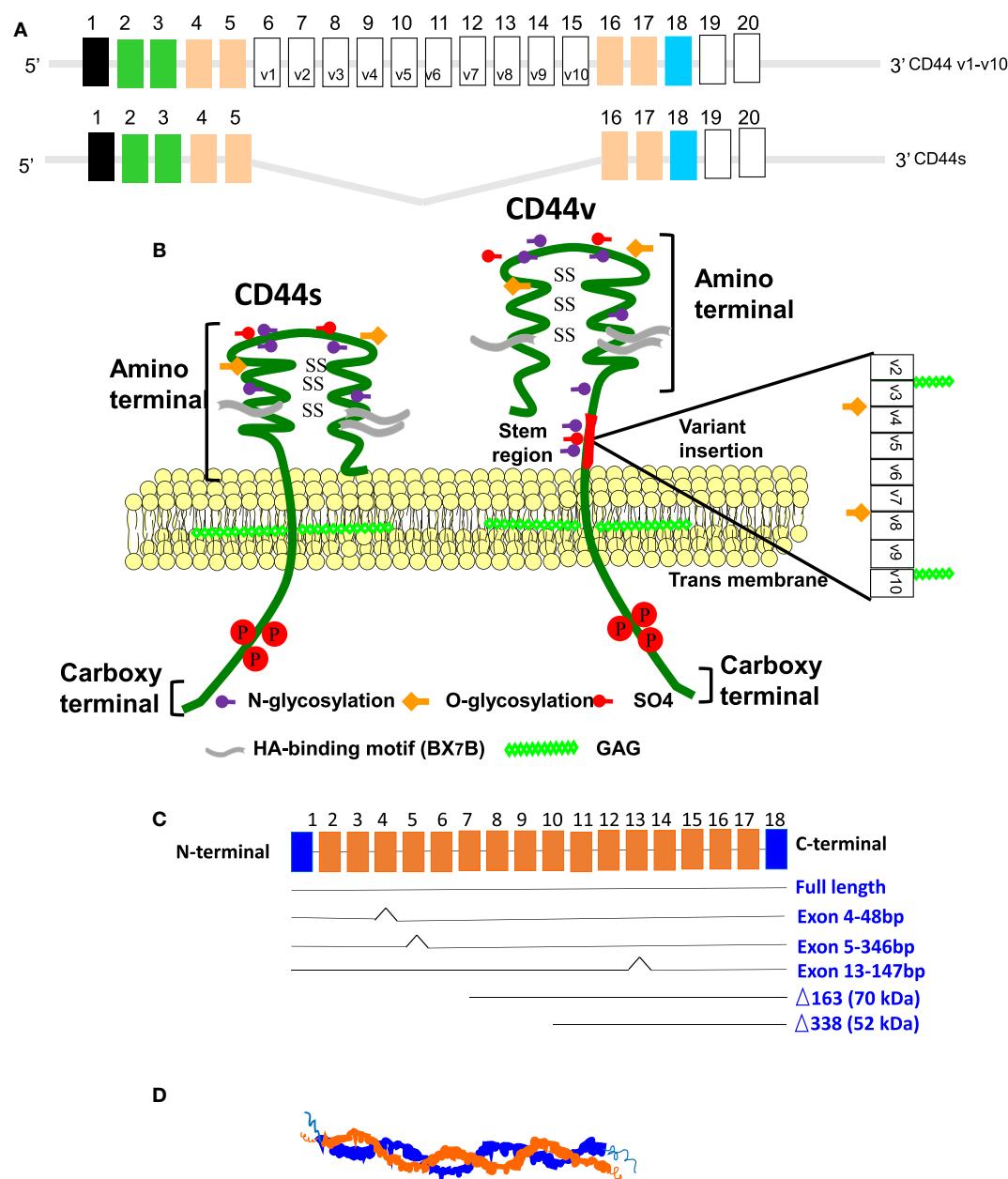


FIGURE 2 | (A) Mouse CD44 and RHAMM gene/protein structures in mice. Model structure of alternative splicing in CD44. CD44 pre-mRNA is encoded by 20 exons in mouse and 19 exons in human being. The common standard CD44s (hematopoietic) form contains no extra exons, and the protein has a serine motif encoded in exon 5 that can initiate synthesis of a chondroitin sulfate or dermatan sulfate chain. Alternative splicing of CD44 predominantly involves variable insertion of 10 extra exons with combinations of exons 6–15 and spliced in v1–v10 into the stem region, of which v3 encodes a substitution site for a heparan sulfate chain. Variable numbers of the v exons can be spliced in epithelial cells, endothelial cells, and inflammatory monocytes and also are upregulated commonly on neoplastic transformation depending on the tissue. **(B)** Model structure of alternatively spliced CD44 proteins. The CD44 protein is composed of an extracellular N-terminal domain, a stem region in the extracellular domain close to the transmembrane region, where the variant exon products (red/violet circles) are inserted, the transmembrane region, and the carboxyl terminal cytoplasmic tail. There are multiple sites for N-glycosylation

(purple circles) and O-glycosylation (orange circles), and a sulfation domain. The N-terminal portion contains highly conserved disulfide bonds as well as 2 BX7B motifs, both of which are essential for HA binding. CD44 is subjected to extensive glycosylation, sulfation, and attachment of GAGs that contribute to regulation of the HA-binding activity. The C-terminal cytoplasmic tail contains several phosphorylation sites that regulate the interaction of CD44 with the cytoskeletal linker proteins, as well as with SRC kinases. **(C)** RHAMM exon structure. The full-length protein (85 kDa in human beings) is largely associated with microtubule formation during the cell cycle progression. Three isoforms are generated by alternative splicing of exon 4, 5, or 13. Loss of exon 4 disrupts association with microtubules and results in the appearance of RHAMM in the cell nucleus. N-terminal truncations that may be generated by a posttranslational mechanism are constitutively present in some aggressive breast cancer cell lines and tumors. These accumulate in the nucleus and on the cell surface. **(D)** The secondary structure of RHAMM. RHAMM can self-associate to form random coiled coils (132).

migration during inflammation, wound healing, and tumorigenesis can require surface expression of RHAMM. However, the mechanism of cooperativity between RHAMM and CD44 is not clearly understood. In this review, we discuss the nature of such interactions and the important therapeutics that can target CD44 and RHAMM in inflammation and cancer.

HA in Inflammation and Cancer

Biology of HA

Hyaluronan is a ubiquitous component of tissue ECM found in a native homeostatic form within hydrated tissues such as the vitreous of the eye, articular cartilage, synovial fluid, lymphatics, and skin. HA is present in interstitial collagenous matrices and increases viscosity and hydration. This impedes fluid flow through matrices by forming a barrier (31). HA is found in pericellular matrices attached to the HA-synthesizing enzymes or its receptors and is also present in intracellular degradation compartments (6, 7, 32–37). HA differs from the other GAGs: it is not synthesized on a core protein as a proteoglycan in the Golgi. Instead it is synthesized by single transmembrane HA synthases (HAS1, HAS2, or HAS3) at the inner face of the plasma membrane. The cytoplasmic active HAS sites alternately add the UDP-GlcUA and UDP-GlcNAc substrates to the reducing, UDP-anchored elongating HA molecule that is being systematically extruded through the plasma membrane into the ECM to form the very long, unmodified HA macromolecules without any sulfated or epimerized uronic acid residues (23, 38). The HAS isozymes (HAS1, HAS2, HAS3) differ from each other in their catalytic activities as well as in the sizes of the synthesized HA. HAS1 and HAS2 polymerize long high molecular weight (HMW) chains while HAS3 produces predominantly shorter low molecular weight (LMW) chains (<300 kDa), and HAS3 appears to be more active than HAS1 and HAS2 (38–40).

While its structure is simple, HA is an extraordinarily versatile macromolecule. Its biophysical properties provide HA with functions that influence the hydration and biomechanical properties of different tissues, especially those of the vitreous humor in the eye, the synovial joint fluid, and the dermis (41). In addition, HA also interacts with extracellular macromolecules and HA-binding proteoglycans, including versican and aggrecan, which are important in the assembly of ECMs and of pericellular glycocalyxes that can act as protective cellular barriers and are essential for the assembly and structure of many tissues (41–44). For example, increased levels of aggrecan immobilized on HA in collagen networks resists the variable compressive loads essential for the physical properties of cartilages (45).

Successful morphogenesis also relies on physical properties of HA as well as on signaling events triggered by HA-CD44 and/or HA-RHAMM interactions. During embryogenesis, HA promotes proliferation and migration of undifferentiated stem cells to sites of organ development (26). Importantly, Has2 null mice fail to synthesize HA during cardiac cushion development, and the endothelial cushion cells do not undergo mesenchymal transformation (EMT) and cannot form the underlying connective tissue, which leads to midgestational death (5, 46). Recent studies also indicate that the matricellular protein periostin binding to the

integrins activates the HA synthesis and HA-mediated Akt/PKB and focal adhesion kinase (FAK)/Erk signaling pathways, which by feedback loop, further sustains Has2 expression for cell survival, and importantly, differentiation of embryonic cardiac mitral valve cells (47) (**Figure 3B**).

Hyaluronan turnover is important for the maintenance of tissue homeostasis, and ~30% of HA is replaced by newly formed HA per 24 h (48). Removal of HA occurs by endocytic uptake within the tissue, especially in lymph nodes and liver. Importantly, the presence of reactive oxygen species (ROS) enhances HA turnover (49–51). Internalization and degradation of HA is triggered by its binding to CD44 (52) and/or lymphatic vessel endothelial receptor-1 (LYVE-1), which is expressed in lymphatic endothelium (53). HA is degraded into monosaccharides by three enzymatic reactions. Hyaluronidases (HYALs) degrade HA to oligosaccharides, which are then digested into GlcUA and GlcNAc by β -D-glucuronidase and β -N-acetyl-D-hexosaminidase (54, 55). In human beings, there are six HYAL genes, HYAL1–4, HYAL-P1, and sperm-specific PH-20 (54, 56–58). These enzymes can have different catalytic profiles. For example; HYAL1 degrades HA into oligosaccharides while HYAL2 degrades HMW HA into fragments of intermediate size (~20 kDa). PH-20 is anchored to the sperm acrosomal membrane through a glycosyl-phosphatidylinositol (GPI) moiety, which promotes penetration of spermatozoa through the HA matrix in cumulus-oocyte complexes for fertilization (54, 59). In recent years, recombinant human PH20 devoid of the GPI anchor has been prepared (60) and tested for increasing drug penetration in skin and pancreatic tumors in an animal model (61).

As mentioned earlier, under physiological conditions, HA exists as a HMW polymer $>10^6$ Da providing structural frameworks for cells. Such HMW HA functions as an extracellular molecule transmitting signals and regulates a variety of cell behaviors, including cell adhesion, motility, and growth (6, 8–11, 37). HA chains up to 20 MDa are involved in ovulation, embryogenesis, wound repair, and tissue regeneration (62). In malignant cells, HA polysaccharides ($>10^6$ Da) are abundant. HA levels can be increased around tumor cells themselves or within the tumor stroma to maintain normal cellular functions of the tumor cells. Because of the close association of high HA levels with malignancy in many tumor types (37) considerable experimental evidence implicates HA and its main receptor CD44 variants in tumor progression both in cell and animal models (6, 25, 37, 63–69). HMW HA induce activation of tyrosine kinase in endothelial cells and Ras-transformed fibroblasts at lower level (70, 71) compared to fragmented HA (71). However, the ability of the naked mole rat to synthesize high molecular mass HA (five times larger than human HA) is correlated to the cancer resistance and longevity of naked mole rat (72), but this animal is a rare species. A recent study established that reducing HAS levels using antisense techniques suppresses tumor growth, but showed that extremely high HA levels also inhibit tumor growth, possibly by suppressing cell-cell interactions, or ligand access to cell surface receptors (73). Remarkably, following tissue injury, HA and its degraded fragments $<5 \times 10^5$ Da size accumulate. Such smaller size HA was shown to be crucial for the production of cytokine and chemokine for differentiation/activation of the macrophage (74–76). HA of

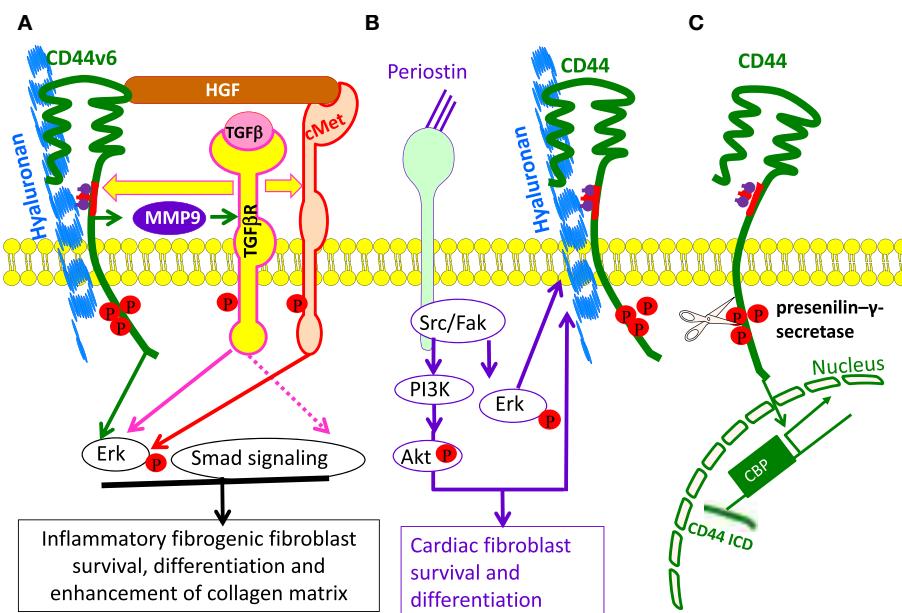


FIGURE 3 | The involvement of HA and CD44 in cell survival and differentiation. **(A)** Model for the involvement of CD44v6 and Met due to autocrine TGF β 1 signaling in lung fibrogenic fibroblasts. The repetitive lung injury in pulmonary fibrosis results in overexpression of TGF β 1 and TGF β 1-induced autocrine signaling that induces a sustained expression of CD44v6 and its co-receptor c-Met. This activates fibrogenic lung fibroblasts with subsequent increased collagen matrix synthesis. Therefore, TGF β 1-induced CD44v6 and Met can have a crucial role for the sustained fibrogenic activation of lung fibroblasts. The CD44-phosphorylated ERM complex initiates activation of transforming growth factor- β receptor 1 and 2 (TGF β RI and II) and the downstream SMAD signaling complex, which contribute to fibrosis. **(B)** Model for involvement of periostin in

HA-CD44-mediated cell survival and differentiation. Matricellular protein [periostin (PN)] binding to β 1 or β 3-integrin activates FAK, which activates downstream MAPK/Erk and PI3K/Akt to regulate cardiac valve cell growth, survival, differentiation into fibroblasts, and matrix organization (maturation). PN binding to β 3-integrin also activates Has2 mRNA expression, Has2 phosphorylation, and HA synthesis. The interaction of HA with CD44, may, in turn, amplify the downstream effects of PN on heart valve cushion cell differentiation/maturation processes. **(C)** Cleavage of the extracellular domain is accompanied by the cleavage of the intracellular domain (ICD) by the presenilin- γ -secretase complex. The CD44 ICD acts together with CBP or p300 as a transcription factor and promotes CD44 transcription and extracellular matrix production.

size $<5 \times 10^5$ Da ($\sim 250,000$ Da) induce inflammatory genes with renal tubular epithelial cells (77), T-24 carcinoma cells (78), and eosinophils (79). Similarly, HA fragments of size 8–16 disaccharides induce angiogenesis in a chick corneal assay, whereas the native HA molecules do not (80–82). Similarly, smaller HA fragments in the 6–20 kDa size range induce inflammatory gene expression in dendritic cells (83). Studies have shown that HA of 40–400 kDa interact with HA-receptors to activate the NF κ B-mediated gene expression for endocytosis (84). Under certain conditions, HA fragments (20–200 kDa size) function as endogenous danger signals, while even smaller HA fragments ($\sim 2,500$ kDa) can ameliorate these effects in cancer (37, 85, 86). Thus, generation of HA fragments by HYALs (87) or by free radicals (88) during inflammation/tumorigenesis/tissue injury send the signals to the injured host that normal HA homeostasis is disturbed due to activation of different signal transduction pathways. The biological function of various sizes of HA is presented in Table 1.

Both physiological and pathological functions of HA are mediated by molecular interactions with CD44 and RHAMM and other HA-binding proteins (42). HA-rich glycan chains anchored on cell surfaces by CD44 and RHAMM can activate intracellular signaling pathways (6, 42, 89), which can induce gene expression related to cell-cell adhesion, cell spatial orientation and trafficking, cell growth and differentiation, inflammation (11), wound healing

and tissue remodeling (11, 80, 90), tissue morphogenesis (11, 91) matrix organization, and many inflammatory pathologies (6, 7, 19, 33–37, 92–100).

HA in Inflammation

The cell microenvironment has an important role in controlling inflammation. Prolonged inflammation leads to an influx of inflammatory cells, and it is increasingly clear that ECM degradation products are not only the result of inflammation but also can perpetuate inflammatory processes. LMW HA interactions with CD44 are associated with certain pathological conditions, including inflammation, cancer, and induction of angiogenesis (23, 33, 101–103).

Hyaluronan chains can be organized into ECM structures by association with binding proteins. For example, HA cross-linking is essential to stabilize the cumulus-oocyte complex and is needed for successful ovulation and fertilization. The same mechanisms are likely to occur at sites of inflammation, where HA synthesis is upregulated (104, 105). Importantly, HA complexes of indeterminate sizes originate from the surface of multiple cells and can be coalesced into cable-like structures or HA fibrils at sites of tissue inflammation, viral infection, endoplasmic reticulum (ER) stress, and hyperglycemia. Monocytes/macrophages adhere to these HA inflammatory matrices and fragment them by a mechanism that

TABLE 1 | Various sizes of HA and their function in health and disease.

Sizes (kDa, or disaccharide)	Function of HA	References
HA size > 10 ⁶ Da	Space-filling, providing structural frameworks for cells, functions as an extracellular molecule transmitting signals, and regulates a variety of cell behaviors, including cell adhesion, motility, and growth	(6, 8–11, 37)
HA size > 10 ⁶ Da	Rapid matrix remodeling that occurs during embryonic morphogenesis, as well as tumorigenesis	(4–7, 27, 37, 63–69)
HA size > ~20 MDa	Ovulation, embryogenesis, wound repair, and tissue regeneration	(62)
HA size > 10 ⁶ Da	Tyrosine kinase in endothelial cells and Ras-transformed fibroblasts at lower level	(70, 71)
Extremely HMW HA (five times larger than human HA)	Cancer resistance and longevity of naked mole rat	(72, 73)
Excessive HMW HA production	Inhibit tumor growth, possibly by suppressing cell–cell interactions, or ligand access to cell surface receptors	
HA size < 5 × 10 ⁵ Da	Cytokine and chemokine production for differentiation/activation of the macrophage	(74–76)
HA size ~250,000	Induces inflammatory genes in renal tubular epithelial cells, T-24 carcinoma cells, and in eosinophils	(77–79)
HA size 8–16 disaccharides (~3200–6400 Da)	Induce angiogenesis in a chick corneal assay whereas the native HA molecules do not	(80–82)
HA size ~6–20 kDa	Induce inflammatory gene expression in dendritic cells	(83)
HA of 40–400 kDa	NFKB-mediated gene expression for endocytosis	(84)
HA size ~2,500 Da	Upregulation of PTEN in tumor cells, and inhibit anchorage-independent growth as well as xenograft tumor growth	(37, 86)

involves cell surface HYAL2 and CD44, which forms a cap on the surface while a portion of the HA matrix is internalized. Therefore, HA cross-linking represents an important new pathway in the regulation of inflammatory processes (33, 105–110).

One of the functions of HMW HA is to maintain water homeostasis and matrix structure (111). However, during inflammation, there is increased breakdown of HMW HA by HYALs (62), resulting in the accumulation of LMW forms that have different functions than their HMW precursors (101, 112) (see Figure 1). LMW HA is a potent activator of macrophages and airway epithelial cells (113–115). Specifically, LMW HA (~200 kDa) can induce the expression of proinflammatory genes, such as macrophage inflammatory protein (MIP), keratinocyte chemoattractant (KC), macrophage chemoattractant protein-1 (MCP-1), and IFN induced protein-10, as well as cytokines, such as IL-8, IL-12, and TNF- α (76, 101, 112, 115–117). HA-stimulation of macrophages is associated with matrix-modifying enzymes (MMEs), inducible nitric oxide synthase (iNOS), and plasminogen activator inhibitor (74, 116, 118). In addition, HA is also depolymerized by ROS, and the HA fragments are implicated in chronic inflammation (119). These HA-induced inflammatory mediators can enhance the inflammatory response that has already been set in motion, sending the system into a positive-feedback loop where inflammation promotes further inflammation, which, if unchecked, may eventually lead to fibrosis.

HA in Cancer

There is a complex cross-talk between cancer cells and their microenvironment. Strong evidence indicates that the tumor microenvironment can regulate the capacity of tumors to grow and metastasize (120). HA not only provides a cellular support and hydrophilic matrix but also regulates cell–cell adhesion, cell migration, growth, and differentiation (11). These properties make it a suitable candidate for involvement in pathological processes such as cancer. Furthermore, by forming pericellular coats, HA can protect tumor cells from immune attack (43,

44). Several tumor cells produce increased amounts of HA or induce the production of HA by the release of growth factors and cytokines. Such signals can regulate both HAS1, 3 and HYAL1. Similarly, ROS-induced fragmented HA also contributes to overproduction of HA (54). Likewise, both tumor cells and stromal cells express HAS isoforms and produce HA ECM, which then accumulates in tumor parenchyma and in the peritumor stromal tissues, which contributes to metastatic spreading (121–125). Moreover, HA overproduction in tumor cells may induce EMT-like epithelial changes of cancer cells toward a migratory fibroblastic phenotype (126). HA-rich ECM may also mediate the recruitment of mesenchymal stem cells, which are progenitors of tumor-associated fibroblasts (127).

In addition, HAS1–3 synthesize HA of dissimilar sizes, which are linked to specific HA functions in wound injury (37, 125, 128–131) as seen in keratinocyte wound repairs, and during mechanical injury in mesothelial cells (73, 132–136). Increased production of HA in non-malignant cells impairs contact inhibition of cell growth and migration (137). Likewise, forced expression of HAS2 and HAS3 genes results in HA overproduction, which enhances the tumor forming ability of fibrosarcomas and melanoma cells (67, 68) while abrogation of HAS2 blocks xenograft prostate tumor growth (69). Transgenic mouse models have shown that tumor-derived HA induces stromal reactions with subsequent promotion of tumor angiogenesis and lymphatic penetration within intratumoral stromal compartments (126). In addition, HA-rich tumor-associated micro-environments also accelerate the recruitment of inflammatory cells and the production of cytokines and chemokines, and the HA fragments generated by the degradation of HA can stimulate tumor growth and angiogenesis (82). Thus, targeting HA-tumor cell receptor interactions may identify promising therapeutic approaches in cancer treatment. In addition to interaction with cell surface receptors that initiate signaling pathways that impair vascular function (138), HA can increase interstitial fluid pressure and form a physical barrier against movement of small molecule

therapeutics especially in pancreatic ductal adenocarcinomas (61). Thus, depolymerizing HA with HYAL may improve vascular function and ease movement of therapeutics.

Interaction of CD44 and RHAMM with HA

Biology of CD44

CD44 is a broadly distributed cell surface glycoprotein found on hematopoietic cells, fibroblasts, and numerous tumor cells. It was first identified as gp85 (14) and was then shown to be a HA-receptor in placenta cells when their adherence to immobilized HA was inhibited by an anti-CD44 monoclonal antibody, by soluble HA, and by HYAL (16, 139–142). However, the presence of the amino-terminal HA-binding region of CD44 does not guarantee that cells expressing CD44 will bind HA. Indeed, most CD44-expressing cells taken from normal animals, as well as from CD44+ cell lines, do not bind HA. Binding of CD44 to HA is cell specific and depends on the activation state of CD44 (143). CD44 has seven extracellular domains, a transmembrane domain, and a cytoplasmic domain (Figure 2) (144). The extracellular structure has two regions (amino acids 21–45 and amino acids 144–167) that contain clusters of conserved basic residues that have been implicated in HA binding, the BX7B motif. This motif, which is found in other HA-binding proteins, including RHAMM, is present as a single copy in the first of these regions, and as an overlapping pair in the second region (Figure 2). The pairs of intramolecular disulfide bonds are also crucial for HA-binding activity. The HA-binding domain located in the amino-terminal region is present in all isoforms (145). The membrane proximal region is less well conserved and contains the insertion site for the variant exon domains. The transmembrane and C-terminal cytoplasmic domains are highly conserved (142, 146, 147) (Figure 2).

CD44 is encoded by a single gene. Due to alternative splicing, multiple forms of CD44v are generated that are further modified by N- and O-linked glycosylation. The smallest CD44 standard isoform (CD44s) lacks variant exons, contains an N-terminal signal sequence (exon 1), a link module that binds to HA (exons 2 and 3), a stem region (exons 4, 5, 16, and 17), a single-pass transmembrane domain (exon 18), and a cytoplasmic domain (exon 20). In all forms of CD44 cDNAs, exon 19 is spliced out so that the transmembrane domain encoded by exon 18 is followed by the cytoplasmic domain encoded by exon 20, producing the 73 amino acid cytoplasmic domain. CD44s is found in most cells (6), whereas the isoforms that contain a variable number of exon insertions (v1–v10) at the proximal plasma membrane external region are expressed primarily on cells during inflammation and on tumor cells (24, 28, 65, 94) (Figure 2). More importantly, variants of CD44, specifically CD44v6, promote tumor progression and metastatic potential in lung, breast, and colon cancer (6, 24, 94, 95, 148, 149). Subsequently, several tumors, including colon cancer (150–154), Hodgkins lymphoma (155), gastric cancer (156), and melanoma (157), have been screened for CD44 isoforms, indicating that certain CD44 variants have crucial roles for tumor progression. HA and CD44s are present on the membrane of most vertebrate cells (65).

CD44 is a multifunctional receptor having diverse roles in cell-cell and cell-matrix interactions such as cell traffic, lymph node homing, prothymocyte homing, lymphocyte activation, cell

aggregation, releasing chemokines and growth factors, and presenting them to traveling cells (158, 159). CD44 can be a proteoglycan with a potential chondroitin sulfate (CS) or dermatan sulfate (DS) substitution. Insertion of the v3 exon also includes the potential for heparan sulfate (HS) chain substitution (24), which can influence ligand binding and cell behavior by allowing CD44 to be a co-receptor for hepatocyte growth factor (HGF) with c-Met (160). The affinity of CD44 for these GAG substitutions depends on posttranslational modifications, such as oligosaccharide and GAG addition (161–163), and their subsequent functions depend on cell types and growth conditions (6, 24). These modifications can be altered by physiological stimuli, resulting in the induction of HA binding. In the immune system, HA binding can be induced in T cells upon antigen recognition (164) and upon monocyte activation by inflammatory stimuli (51, 52, 147). Unlike HAS2 deficient mice (19), CD44-null mice develop normally, indicating that CD44 is dispensable for development (53).

In addition to binding to HA, CD44 can interact with several ECM proteins, including fibronectin, collagens, growth factors, cytokines and chemokines, and matrix metalloproteinases (MMPs) (6, 24, 26). To contribute to pericellular proteolysis, CD44 facilitates activation of MMP-9 on the surface of carcinoma cells (165). CD44-mediated localization of MMP-9 on tumor cells can regulate tumor cell motility, growth factor activation, and survival mechanisms. It can also activate latent TGF β and promote tumor invasion and angiogenesis (166) (Figure 3A). Tumor growth and metastasis is associated with increased levels of soluble CD44 (sol-CD44), which is detected in plasma from cancer patients (167), indicating increased proteolytic activity and matrix remodeling by CD44. Sol-CD44 is likely to affect cellular behavior by perturbing HA-CD44 interactions by acting as a sink for HA and may regulate cell migration by disrupting CD44-dependent cell-cell and cell-matrix adhesion. Cleavage of the extracellular domain of CD44 can be followed by subsequent proteolysis within the transmembrane domain and subsequent liberation of the CD44 intracellular domain (ICD) (168). The ICD can then translocate to the nucleus and promote transcription of CD44, providing a feedback mechanism for regulating CD44 expression (169) (Figure 3C).

Decades of research have shown that CD44 participates in major oncogenic signaling networks and in complexes with oncogenes that promote every aspect of tumor progression (6, 24). CD44 is extremely sensitive to changes in the microenvironment. For example, CD44 in breast cancer cells may act as a metastatic suppressor gene when influenced by ROS, as seen by decreased CD44 protein expression in the malignant and tumorigenic breast cancer alpha 5 cell line in a compensatory response to increased manganese superoxide dismutase (MnSOD) protein expression (170). Studies by Stoop et al. (171) showed that the cancer-initiating function in CD44-null mice was less severe, whereas the inflammatory functions were persistent in these mice suggesting again the possibility of a molecular redundancy in this model. Many of the contradictory findings published to date may be due to experimental and technical differences among studies. However, a picture has emerged suggesting that CD44 may function differently at different stages of cancer progression (172, 173). For example, mice with germline disruptions of CD44 display relatively mild phenotypes compared with mice in

which tissue-specific CD44 function is disrupted at adult phases of development, or in later phases. This suggests that the absence of CD44 in early development and a loss of CD44 function late in development are tolerated differently (24). As CD44 is the major HA-receptor and a co-receptor for EGF, it was surprising to find that CD44-null mice had a mild phenotype. However, roles for this molecule in the immune system were revealed by a bacterial pneumonia model in which the null mice had enhanced edema and lung neutrophil accumulation (174). Therefore, CD44 appears to have a role in limiting inflammatory responses, which has also been shown in inflammation models (24).

Unlike CD44s, CD44v variants are only expressed on some epithelial cells during embryonic development, during lymphocyte maturation and activation, and in several types of carcinomas (175). In particular, upregulation of CD44v6 is an early event in carcinogenesis and requires adenomatous polyposis gene inactivation (153, 176, 177). We found that overexpression of HAS2 in pre-neoplastic Apc10.1 cells induces CD44v6 expression, and the intestinal/colon tumors of Apc1 Min/+ mice express CD44v6 at substantially higher levels compared to expression of CD44s (94). A considerable number of studies indicate that CD44 variant isoforms correlate with bad prognosis in patients with most human cancers (151, 178–184) except in neuroblastomas and prostate cancer (185, 186). CD44v6 is quite likely to be a suitable target for anticancer therapy because it is: (a) causally involved in metastasis of a rat pancreatic carcinoma (187); (b) redundantly correlates with the human tumors mentioned above; and (c) correlates with oncogenic functions in colorectal cancer (CRC) both *in vitro* and *in vivo* (6, 94, 178, 180, 181, 188).

CD44 in Inflammation

The role of CD44 in the immune system was first found when immune responses were examined using monoclonal CD44 antibodies (mAbs) in wild type mice. KM201 blocked HA-CD44 interaction, whereas IRAWB14 enhanced HA binding. IM7 induced the shedding of CD44 from the cell surface and induced neutrophil depletion (189–192), indicating that in addition to blocking HA-CD44 interaction, CD44 mAbs can also alter HA-independent functions, such as interactions of CD44 and E- or L-selectin. These approaches support a proinflammatory role for CD44 (193, 194).

Other studies show that leukocyte rolling on inflamed endothelium is not only mediated by the selectin molecules, but can also be mediated by the interaction of T cell CD44 with HA on activated microvascular endothelial cells (195, 196). Moreover, CD44 and HA can facilitate the recruitment of neutrophils to sites of inflammation in some instances (197–199). Reduced recruitment of CD44-null macrophages to atherosclerotic lesions (200) indicates the contribution of CD44 to monocyte/macrophage recruitment to inflammation sites. CD44-null mice also experienced reduced levels of cerebral ischemia injury, further supporting a proinflammatory role for CD44 (201, 202). Studies also revealed that treatment with anti-CD44 mAbs reduced the severity of arthritis in a collagen-induced mouse model for human rheumatoid arthritis (RA) (203–205) and reduced the diabetic activity in NOD mice (206). The decrease in disease severity was associated with the delayed access of donor lymphocytes into the RA joints of

recipient animals (171, 207). In human RA, CD44v5, CD44v6, and CD44v10 have been detected in synovial fluid and serum of patients (208, 209). In an inflammatory bowel disease (IBD) model, expression of CD44v7 is crucial for colonic inflammation (210, 211). Furthermore, CD44v6 expression is associated with IBD severity in patients (212–214). Extensive HA matrix accumulates in bleomycin-induced lung fibrosis in CD44-null mice with persistent lung inflammation, extended chemokine production, impaired clearance of apoptotic lymphocytes, and death (215).

Our recent study showed that a feedback loop between CD44v6 and TGF β 1 augments the fibrogenic functions of lung fibroblasts in interstitial lung disease (92). In this study, we showed that TGF β promotes c-Met expression and CD44v6 expression that is accompanied by the CD44v6-induced formation of α -SMA, increased cell proliferation and collagen production (Figure 3A). The CD44v6 signaling complex with TGF β RI and TGF β RII stimulates downstream SMAD signaling (Figure 3A). These findings provide clear evidence that TGF β I initiates the signaling cascade through CD44v6 toward differentiation of fibroblasts to myofibroblasts (92). They do not exclude a further contribution of CD44v6 by activating the TGF β 1 proform through associated MMPs (166, 216). Overall, these studies indicate the critical involvement of CD44 and its variants in a number of inflammatory situations. However, the specific role of CD44 depends on the model system and the disease.

CD44 in Cancer

Although studies *in vitro* indicate that the tumor promoting function of HA partly depends on its molecular weight (37, 86, 99, 217), and on its capacity to interact with other proteins (26, 218), many of the tumor promoting activities of HA could be explained by its interaction with CD44. There are three ways how CD44 can interact with HA.

CD44 Binds to Soluble Extracellular HA Molecules and ECM

CD44 proteins exist in three states with respect to HA binding: non-binding, non-binding unless activated by physiological stimuli, and constitutive binding (140, 219, 220). CD44 is endogenously expressed at low levels on various cell types in normal tissues (169), but it requires activation before it can bind to HA. Importantly, the minimal size of HA fragments binding to CD44 are six monosaccharide units (HA6). Thus, HMW HA in the ECM degraded by HYALs into smaller fragments can still bind to CD44 (221). Activated CD44 is overexpressed in solid tumors, but much less, or not at all on their non-tumorigenic counterparts. Adhesion of CD44 to HA induces upregulation of integrins that strengthen stem cell adhesion (222). Cross-talk between CD44 and CXCR4 signaling is a key role for HA and CD44 in CXCL12-dependent trans endothelial migration of stem cells (223). Tumor-derived cells express CD44 in a high-affinity state that is capable of binding and internalizing HA. Transitions from the inactive, low-affinity state to the active, high-affinity state by CD44 require posttranslational modifications, i.e., glycosylation in the extracellular domain and/or phosphorylation of specific serine residues in the cytoplasmic domain (26, 161). Such modulation of binding affinity of CD44 with HA is important for cellular migration that

enables CD44 to be incorporated into the leading edge of the cells and lamellipodia (224).

CD44 can also react with other molecules, including collagen, fibronectin, osteopontin, growth factors (24), and MMPs in tumor cells (167, 225), but the functional roles of such interactions are less well known (24). Inhibiting cleavage of CD44 inhibits tumor cell migration on a HA substrate, suggesting that CD44 cleavage could release cells bound to a HA ECM (24). CD44 can also influence adhesion and de-adhesion to the ECM by regulating the pericellular HA matrix metabolism (226).

CD44 Interacts with Receptor Tyrosine Kinases for Anti-Apoptosis and Drug Resistance

Receptor tyrosine kinases (RTKs) are a subclass of cell surface growth factor receptors (GFRs) with an intrinsic, ligand-controlled tyrosine kinase activity (227). The cytoplasmic domains of RTKs contain catalytic kinase activity and phosphorylation motifs that on activated RTKs assemble many intracellular signaling molecules. Apart from their activation by the auto phosphorylation of cytoplasmic subunits of RTKs, they are also activated by their association with several proteins, which are known as co-receptors of RTKs. These co-receptors do not have kinase activity, but they modulate the kinase activity of RTKs.

HA-CD44 or HA-CD44v interaction has a general effect on activation of cell survival anti-apoptotic proteins, which is initiated through the association with RTK activation. In malignant colon, prostate, and breast carcinoma cells, HA-CD44 interaction activates multiple RTKs, including ERBB2, ErbB3, EGFR, IGF1R- β , PDGFR- β , and c-MET, as well as assembly of lipid-raft-integrated signaling complexes containing these activated RTKs, CD44, ezrin, PI3-kinase (PI3K) and the chaperone molecules HSP90 and CDC37, which strongly promotes apoptosis resistance in cancer cells (94, 96, 149, 228–231). Increased HA production, however, induces RTK activation and signaling complex assembly in phenotypically normal epithelial cells (96). These macromolecular signaling complexes of CD44 also contain RhoA-specific guanine nucleotide exchange factor (p115RhoGEF), which is upstream of Grb2-associated Ras and PI3-kinase (98) and VAV2, which regulates cytoskeletal reorganization through RAC1 activation (232) (Figure 4A). A blockade of the HA-CD44 interaction causes macromolecular lipid-raft-integrated complex disassembly and inactivation of RTKs in various cancer cells including breast, colon, and prostate cancer (6, 19, 93, 96, 188, 228–231). CD44 also associates with non-RTKs, such as SRC, which has a central role by linking various extracellular signals to crucial intracellular signaling pathways (233). Thus, the lipid-raft location of CD44 is of particular importance for the involvement of CD44 in cell motility and signal transduction and accounts for the CD44-HA-binding-initiated cross-talk between RTKs, non-RTKs, and linker proteins (234, 235). In colon cancer, the HA-CD44v6 interaction and recruitment of ERBB2 also induces the transcription of COX2 initiated downstream of CD44 through PI3K-Akt and β -catenin (93, 188). COX2 further strengthens apoptosis resistance and HA-CD44 interaction through prostaglandin E₂ expression (93, 188) (Figure 4B). CD44v6 also initiates MET activation through HGF binding. This requires the cytoplasmic tail of CD44 and the

interaction with ezrin, radixin, and moesin (ERM) proteins for activation of the Ras-MAPK pathway (236). In addition, CD44v6 binding to the ECM also activates the PI3K-Akt pathway (237, 238) (Figure 4B) and regulates *Met* transcription (239).

Hyaluronan-CD44 cross-linking regulates drug transporter expression (228, 240–242). In addition, a positive-feedback loop, involving HA, PI3K, and ErbB2, augments MDR1 expression and regulates drug resistance in breast cancer cells (228) (Figure 4A). MDR1 is associated with CD44 in lipid microdomain and can be linked via CD44 with the actin cytoskeleton so that expression of both CD44 and MDR1 are concomitantly regulated (240) (Figure 4A). HA-CD44 binding promotes protein kinase C ϵ (PKC ϵ) activation, and this increases NANOG phosphorylation and translocation to the nucleus (243). These events initiate the upregulation of MDR1, and then CD44 can associate with and stabilize MDR1 gene expression (244). In contrast, pro-metastatic miR-373-520c downregulates CD44 (245) indicating that oncogenic miRNAs can regulate CD44 cell behavior in a specific way.

CD44 Can Act as Co-receptor

CD44v6 can function as co-receptor for RTKs and alter the activation of GFR kinases. The CD44v segments contain some specific posttranslational modifications that include HS on a site in exon v3, which can bind proteins such as fibroblast growth factor 2 (FGF2). Such a function is not confined to HS-modified CD44v3 isoforms. It can also be provided by CD44v6 isoforms, which act as co-receptors, i.e., CD44v6 contains a binding site for RTKs, HGF receptor c-Met, vascular endothelial growth factor (VEGF) receptor VEGFR-2, proteins involved in cancer, and in fibrosis (92, 103, 236, 246, 247). Thus, HA interactions with CD44v can have a central role in RTK activated cell pathways that promote tumorigenic functions, including cell survival, through the RTK activation and consequent MDR1 gene activation. Importantly, activation of signaling pathways initiated by HA-CD44v interactions in the tumor matrix can be inhibited by HA degradation, by inhibiting HA binding to CD44v with small HA fragments, by blocking the CD44v HA-binding site, or by CD44v knockdown (6, 94, 95, 248). Further, blockade of an individual RTK does not recapitulate all of the effects observed when HA-CD44v interaction is inhibited (149). HYAL, as well as low molecular mass HA oligosaccharides (oHAs), also improves drug efficacy (228, 241) and drug transporter expression (228, 242, 249) (Figure 8).

Binding of CD44 to Actin Cytoskeleton

CD44 and its associations with partner molecules, such as ERM proteins, participate in CD44-induced cell survival signaling, altered cell shape, and protein localization to the plasma membrane subdomains during cell migration (250). Ankyrin is involved in HA-CD44 dependent cell adhesion and motility. This involves binding of the N terminus of activated ERM to a motif between the transmembrane region and the ankyrin-binding site in the cytoplasmic domain of CD44, and subsequent binding of their carboxyl termini to filamentous actin (F-actin). This binds CD44 to cytoskeletal linker proteins, and this interaction is modulated by HA-CD44 interaction (251, 252). Overexpression of merlin, another member of the ERM family, inhibits subcutaneous growth of Tr6BC1 cells in immunocompromised Rag1

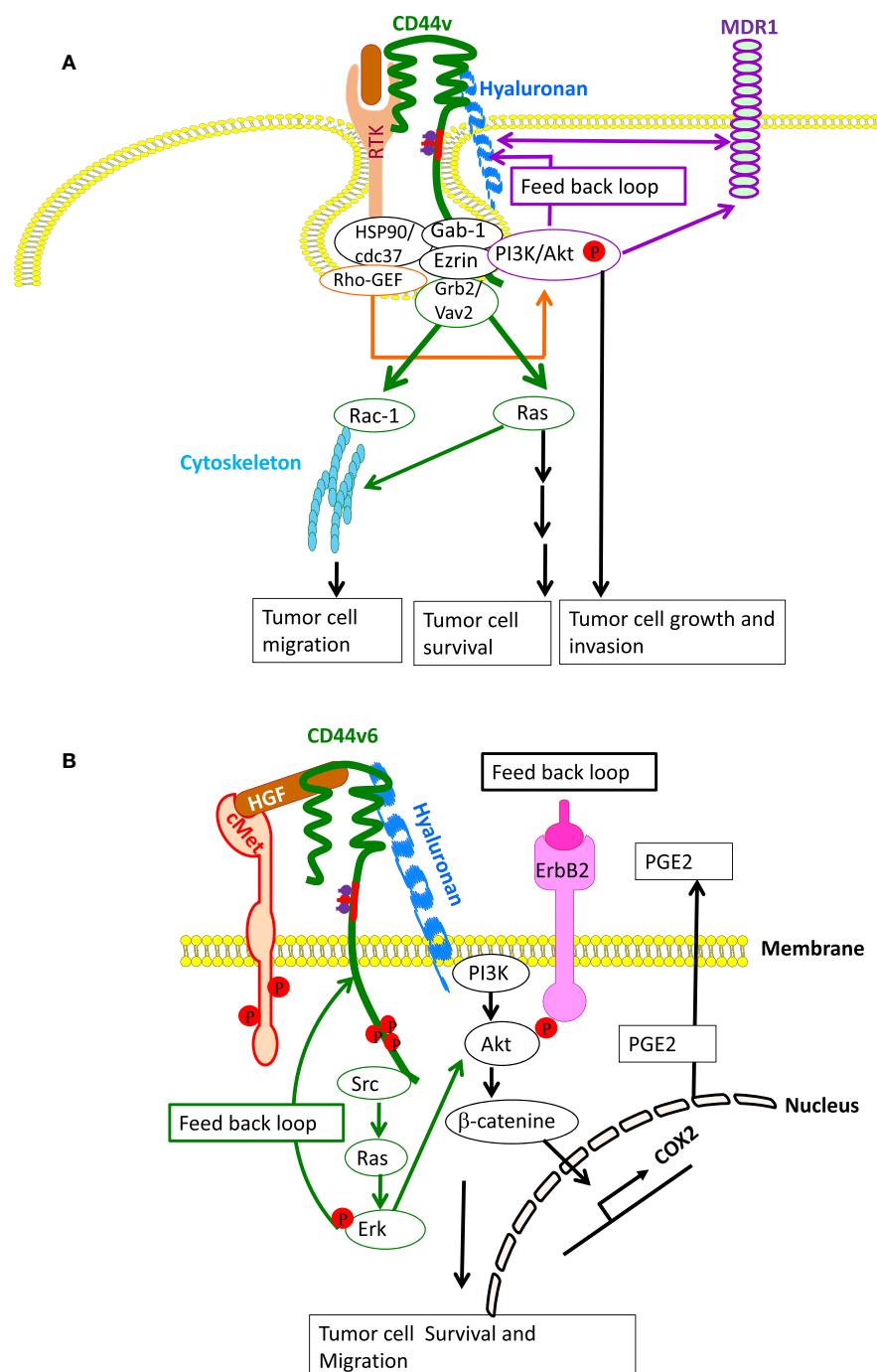


FIGURE 4 | CD44- and CD44v-induced RTK for apoptosis resistance.
(A) CD44-HA binding, accompanied by activation of CD44-associated SRC, ezrin phosphorylation, and PI3K activation leads to the lipid-raft-integrated assembly of a complex that includes heat shock protein 70 (HSP70), the co-chaperone CDC37, Rho-GEF, Grb2/VAV2, and Gab-1/PI3-kinase (PI3K), which promotes phosphorylation and activation of the receptor tyrosine kinases (RTKs), including ERBB2, ErbB3, EGFR, IGF1R- β , PDGFR- β , and c-MET. CD44-HA binding initiates cross-talk between RTKs, non-RTKs [SRC (Src)] and linker proteins. Studies have indicated that ErbB2 most likely complexes with CD44 via interactions with Grb2 and Vav2, whereas the interaction of PI3K and CD44 is mediated by Gab-1. PI3K activates Akt and downstream anti-apoptotic events, which contribute to drug resistance. However, HA and PI3K stimulate MDR1 expression, and the stimulatory effects of PI3K would be mainly due to its

feedback stimulation of HA production by a positive feedback loop. Studies indicate that MDR1 is associated with CD44 in lipid microdomain and can be linked via CD44 with the actin cytoskeleton so that expression of both CD44 and MDR1 are concomitantly regulated. **(B)** Particularly, in colorectal cancer, the CD44-ERBB2 complex provides a strong apoptotic resistance through stimulation of cyclooxygenase 2 (COX2) transcription via PI3K and β -catenin. COX2-induced PGE2 stimulates HA synthesis and HA-CD44 signaling. CD44v6 also binds hepatocyte growth factor (HGF) and presents it to c-MET. Activation of MET and the downstream signaling cascades require sustained activation of CD44-associated phosphorylated ezrin, radixin, and moesin (ERM) and SRC (Src) signaling via the Ras-MAPK and the PI3K-Akt pathway. Ras-Erk pathway can augment CD44v6 synthesis through a feedback loop between CD44v6 and c-Met/Ras-Erk pathway.

mice by negatively regulating CD44 function. In contrast, knocking down expression of endogenous merlin promoted tumor cell growth (253, 254).

Biology of RHAMM

Receptor for HA-mediated motility, an acidic and coiled-coil protein designated as CD168 (20), was first identified as a component of the HA-receptor complex in the conditioned media of murine fibroblasts (21, 22). It is located intracellularly in the cytoplasm, in the nucleus, and on the cell surface (255). There is no link module domain in RHAMM, but it includes a HA-binding region through the BX7B motif on its COOH terminus (27). RHAMM lacks a transmembrane domain but is GPI-anchored to the cell membrane, where it can interact with CD44 and participate in many cell functions, including cell motility, wound healing, and modification of signal transduction of the Ras signaling cascade (28, 256–259). Surprisingly, RHAMM contains no signal peptide and is thought to be transported to the cell surface via unconventional transport mechanisms, where it associates with the cell surface via docking with HA synthase (260), and like CD44, it transduces signals that influence cell motility (28, 30).

RHAMM Signaling in Inflammation and Cancer

Extracellular RHAMM interacts with protein tyrosine kinase receptors (RTKs) and non-protein-TK receptors, including PDGFR (29), TGF β receptor-1 (261), CD44 (259, 262), CD44-EGFR complexes (263, 264), bFGFR (265), and RON (266). These extracellular interactions can mediate motility necessary for inflammation through activation of ERK1/2/MAPK in the absence of intracellular RHAMM. Extracellular RHAMM can regulate cellular transformation and migration in an HA-dependent manner. It can bind to CD44 on mesenchymal cells in wounds, which is necessary for the sustained activation and nuclear translocation of activated ERK1, 2, and for cell migration with increased mesenchymal differentiation within wound sites (259, 267).

Intracellular RHAMM binds to both actin filaments and microtubules in the cytoskeleton, in addition to interacting with ERK and SRC kinases (30, 268). Moreover, intracellular RHAMM also binds to a number of proteins, which can regulate microtubule dynamics and centrosome structure/function through ERK1/2/MAP kinase activation that contributes to microtubule-mediated cell polarity and cell migration. Nuclear RHAMM also binds to ERK1/2/MAP kinase, which mediates activation of PAI-1 and MMP-9 that are involved in cell motility and inflammation (132) (Figure 5). Secreted RHAMM can bind HA and, in concert with CD44, augment invasiveness in breast cancer. These interactions suggest that RHAMM may be necessary for CD44-mediated migration during inflammation, wound healing, tumorigenesis, and regulation of stemness/EMT phenotypes within tumor-initiating populations (256, 269, 270). Intracellular RHAMM, both cytoplasmic and nuclear, interacts with several signaling proteins and cytoskeletal components, including SRC, ERK1, actin, and microtubules (28, 29, 271). RHAMM regulates mitotic organization of microtubules through cytoskeletal elements aurora kinase A (AURKA), and BRCA1 (267, 272, 273), which is crucial for cellular fates, such as luminal differentiation

and EMT. In addition, disruption of AURKA (274), BRCA1 (275), or RHAMM (276) modifies neurite extension, an alternate differentiation program dependent on microtubule nucleation (267) (Figure 5).

Biology of HA-CD44/RHAMM Interaction

Given the crucial role that HA has in cancer cell survival, proliferation, and invasion, it is clear that HA interactions with both CD44 and RHAMM can induce numerous cell behaviors, including activation of tyrosine kinases, protein kinase C, FAK, and PI3K, MAPK, NF κ B, and RAS, as well as cytoskeletal components required for inflammation and cancer (6, 24, 28, 132, 277) (model in Figure 5). While CD44 and RHAMM can interact independently with HA to induce cell behavior, in some cases their relative contributions result in redundant and overlapping functions. For example, in a collagen-induced model of arthritis, inhibiting CD44 function attenuates the disease, indicating the involvement of CD44 (193). RHAMM is significantly elevated in the knee synovial tissue of patients with advanced osteoarthritis compared to those without (278). These results are consistent with animal model studies demonstrating an isoform-specific role for RHAMM in collagen-induced arthritis (193). The findings from Nedvetzki et al. (193) indicate that arthritis in WT mice is CD44 dependent, whereas in CD44-knockout mice, it is RHAMM-dependent, as determined by antibody blocking and soluble peptide competition studies. This suggests that CD44 and RHAMM coexist in WT mice, but cell surface CD44 functions are dominant. Cell surface CD44 may primarily influence the joint inflammatory cascade in WT mice by its ability to quantitatively compete with cell surface RHAMM for HA and/or by its ability to regulate the potency of RHAMM-mediated signaling that may or may not be HA dependent. Absence of CD44 exacerbates bleomycin-induced lung inflammation in CD44-null mice due to accumulation of extensive HA ECM (215). In light of Nedvetzki's findings (193), the augmented lung inflammation/fibrosis in bleomycin-induced CD44-null mice (215) may be due to RHAMM (193). Further, HA accumulation was greater in the arthritic joints of CD44-deficient mice than in those of wild type mice where CD44 (but not RHAMM) promotes endocytosis of HA and its subsequent lysosomal digestion. The excess HA in CD44-null mice may contribute to prolonged signaling through RHAMM (28), which might lead to aggravation of the inflammatory lung fibrosis. Thus, the loss of the CD44 gene could be compensated with enhanced expression of RHAMM. Moreover, the loss of CD44 allows enhanced accumulation of the HA in ECM, with which both CD44 and RHAMM interact (193). In other words, this study indicates dual functions of CD44 in arthritis, one that causes disease initiation and another that limits disease severity, at least in part by reducing pathology promoting effects of RHAMM (193).

Similarly, in RHAMM null fibroblasts, migration is impaired due to reduced CD44 at the cell surface, and to impaired ERK1 activation and impaired wound healing (259). Interestingly, the dual functions of CD44 exist in cancer where tissue-specific expression of specific variant isoforms of CD44v appears to control progression of some cancers (6, 94, 95), and these isoforms regulate tumor-initiating cells in sub populations of cancer cells

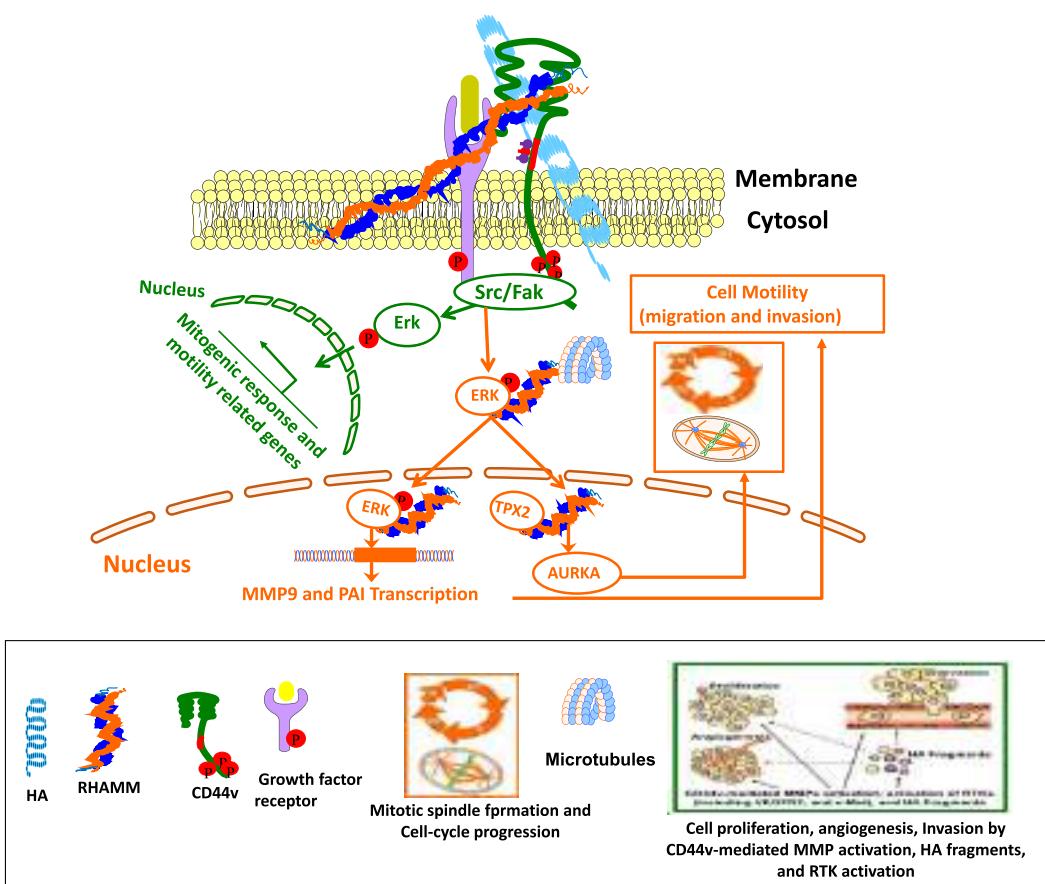


FIGURE 5 | Cross-talk between CD44 and RHAMM interaction with HA affects physiological and cellular functions. Green Track is for extracellular RHAMM signaling involving CD44-HA-mediated pathways. Red track is for intracellular RHAMM signaling. Studies suggest a model for functions of CD44 and RHAMM. The interaction of HA-CD44-RHAMM affects physiological and cellular functions. Cell surface RHAMM interacts with CD44, HA, and growth factor receptors (GFR) to activate protein tyrosine kinase signaling cascades that activate the ERK1/2 MAP kinase cascade in a c-Src/Raf-1/MEK-1/ERK1/2 dependent manner (depicted in

green track). In the absence of intracellular RHAMM, this signaling can stimulate the transcription of motogenic effectors to regulate a mitogenic response (cell proliferation/random motility). In the presence of intracellular RHAMM (red track), MEK-1/p-Erk1/2 also binds to a number of protein partners that allows activated RHAMM to enter the nucleus to regulate functions of microtubule dynamics via centrosome structure/function, and cell cycle progression via AURKA, a targeting protein for Xktp2 (TPX2). Activated RHAMM also controls expression of genes involved in cell motility such as PAI-1 and MMP-9.

(149). Similarly, overexpression of RHAMM controls severity of carcinogenesis (e.g., breast cancer, CRC, multiple myeloma) (239, 255, 279), and loss of RHAMM regulates peripheral nerve sheath tumor progression (280), suggesting duality of RHAMM performance to maintain cell behavior. Studies also indicate that the oncogenic role of RHAMM can be limited through the activation of p53 (281). Further, RHAMM and CD44v isoforms co-existed in 65% of the cancer cases with another 23% having either RHAMM or CD44v expression. Recent studies demonstrated that RHAMM- and CD44-mediated cell adhesion and motility appear reciprocal rather than overlapping (282), whereas concurrent expression of CD44 and RHAMM genes might confer tumorigenicity of gastric cancer cells (283).

This section has reviewed evidence for secretion of intracellular regulators of mitosis and differentiation in the determination of cellular migration, tumorigenesis, and inflammation by overlapping and independent interaction of CD44 and RHAMM with HA (Figures 3–5). The presence and functions of these

multipurpose HA-receptors have been examined both in diseased human tissues as well as in animal models of diseases. The modulation of these receptors may dramatically alter disease functions. The next section discusses the emerging cell-based strategies to target these molecules in inflammatory diseases, and particularly in cancer.

Therapeutic Approaches to Interrupt HA Interaction with Receptors

The inexorable course of progressive inflammation is similar in both the inflammatory diseases and in cancers. The semi-mutable properties of these inflammatory and cancer cells involve cell adhesion, proliferation, migration, and invasion, and the HA-CD44 interactions can regulate these processes. Due to the unavailability of specific inhibitors, the physiological functions of HA have been mostly deduced from the biological changes caused by HA-receptor antagonists that can block the HA-CD44,

TABLE 2 | HA-CD44-RHAMM function in inflammation and cancer.

Disease model	Function of HA in inflammation	Experimental approach by targeting HA-binding motifs	Reference
Bleomycin-induced lung Inflammation in α SMA-HAS2 ⁺ /CD44-null mice	Unremitting inflammation and death, and accumulation of both HMW and LMW HA	CD44 antibody (Ab)	(295)
Excisional wound skin injury model	Acute and chronic skin inflammation	12-mer Pep-1 peptide P15-1 peptide	(132, 297, 306, 307)
Staphylococcal aureus colonization of incisional skin wounds	Increased accumulation of CXCL1, CXCL2 and neutrophils after skin injury	Pep-35	(304, 305)
Bleomycin-induced lung injury mice model	Inflammation and fibrosis in lung injury	RHAMM (HABP) and RHAMM-like Peptide A	(295)
Disease model	Function of HA in cancer	Experimental approach by using HA-drug carriers	Reference
Bladder cancer	G2-M arrest and apoptosis Mitotic/oncogenic responses	HYAL-1-v1 HA (~10-12kDa)-PTX conjugated drugs	(312, 316-318)
EMT-6 tumor spheroids	Increasing the accessibility to the chemotherapeutic drugs	Bovine testicular or bacterial hyaluronidase	(314)
Pancreatic ductal adenocarcinoma	Reduction of metastasis	Soluble form of PH20	(60, 61)
Colon, breast, esophageal, ovarian prostate cancer	Mitotic/oncogenic characteristics	HA (~200 kDa)-Irinotecan conjugated drugs	(320-322)
Bone disease in cancer	Mitotic/oncogenic characteristics	HA (~750 kDa)-5-FU, doxorubicin	(323-325)
Colon cancer	Mitotic/oncogenic characteristics	HMW HA-bisphosphonate	(326)
CD44 positive cancer	Mitotic/oncogenic characteristics	Nanoparticle delivery of chitosan-HMW HA	(328-330)
CD44 positive cancer	Mitotic/oncogenic characteristics	Lipid-HMWHA-mitomycin C (HA-LIP)	(332-336)
CD44 positive HNSCC	Mitotic/oncogenic characteristics	Micelles to deliver paclitaxol, doxorubicin, Salinomycin	(338-340)
B16F10 murine melanoma and lung carcinoma cells	Mitotic/oncogenic characteristics	HA-nanocarrier to deliver doxorubicin, epirubicin	(335, 341)
Melanoma and breast cancers	Mitotic/oncogenic characteristics	Paclitaxel, mitomycin C, and various nanoparticles	(316, 341, 343-350)

and HA-RHAMM interactions. Here, we describe the possibility for therapeutic approaches to interfere with HA-CD44-RHAMM interactions (**Table 2**).

Role of HA, and HA-Receptors in Drug Conjugates

The success of HA as a carrier depends on the number of receptors available on the target cells and on the affinity between the homing ligand and the receptor. HA preparations have been approved by the Federal Drug Administration as a medical device.

HA-Preparations Used for Non-Inflammatory/Non-Tumorigenic Therapies

- (i) A transdermal drug delivery of insulin using micro needles (MNs) fabricated with 15% HA containing insulin is used in diabetes patients (284) (**Table 2**);
- (ii) Transcutaneous immunization (TCI) of tetanus toxoid (TT) and diphtheria toxoid (DT) uses mixtures of sodium HA separately with TT and DT to form the MNs (285-288);
- (iii) Antibodies against TNF- α or IL-1 β conjugated to HMW HA diffuse slowly thus providing a sustained delivery of the antibodies in the wound (84);
- (iv) Intra-articular injections have been used for the treatment of osteoarthritis of the knee, although further studies are required to establish its efficacy (289, 290); and

(v) HA has been approved as a surgical aid in eye surgery (291) and to improve skin elasticity (292).

Targeting HA and HA-Receptors in Anti-Inflammatory Therapies Using HA-Binding Peptide

Recent investigations have taken advantage of the peptide-based probes to develop imaging agents to target HA-binding regions of HA-receptors (**Table 2**). HA, CD44, and RHAMM are known to regulate immunity during tissue repair, including innate immune cells such as macrophages, and fibrogenesis (26, 28, 65, 112, 116, 132, 192, 210, 215, 259, 293-300). Thus, it is likely that HA-binding peptides can alter the HA-binding capacity of HA-receptors and modify disease processes such as tissue fibrosis, wound infection, contact hypersensitivity, and melanoma metastases in experimental models (293, 301-304).

CD44 and HA have been targeted for anti-inflammatory therapies using HA-binding peptides derived from a M13 phage-display library. One of these, Pep-1, which is a specific 12-mer HA-binding peptide, prevents leukocyte adhesion to HA (304) and inhibits leukocyte recruitment during contact hypersensitivity (304, 305). Pep-1 inhibits the binding of cells expressing HA-receptors (e.g., CD44) to immobilized HA substrate as well as the binding of soluble HA to cells expressing such receptors, implying that Pep-1 and HA-receptors compete for binding to the same ligand in acute skin inflammation (58). The peptide can also inhibit interleukin-2 induced vascular leak syndrome in mice

by reducing damage to the endothelium, although lymphocyte migration was not affected (305). Pep-1 also inhibited secretion of the proinflammatory chemokine MIP-2 from HA stimulated macrophages (62). Studies on therapeutic aspects are currently under investigation that include the inhibition of enzymes that cleave CD44 (306) and CD44 vaccination (307), which provides partial resistance to experimental autoimmune encephalomyelitis (307). To date, no therapies have yet been developed that focus on augmenting the function of CD44 in the resolution of inflammation.

Using BLAST and ClustalX2, database searches between RHAMM and microtubule binding domains in microtubule binding proteins revealed only a moderate sequence homology of 17–24% to the HA-binding domain of RHAMM (295). Among the peptides, P15-1 preferentially binds oHAs (<10 kDa) with a moderate affinity, and this peptide specifically mimics and blocks HA-RHAMM-induced FAK signaling, resulting in the healing of excisional wounds (132, 295). This peptide had no visible effect on incisional skin injury repair, which is consistent with genetic deletion of RHAMM, which affects excisional but not incisional skin injury (259, 295, 308). Pep-35, which encodes two RHAMM HA-binding sequences, also reduced *Staphylococcus aureus* colonization of incisional wounds (302) by increasing the number of neutrophils and their expression of CXCL1 and CXCL2 after injury (303). Short peptides of RHAMM [hydrazide group to bisphosphonate (HABP)] and RHAMM-like (peptide A) also reduced inflammation and fibrosis in lung injury models by reducing macrophage migration and accumulation, and by reducing hydroxyproline (collagen) content in the lungs of the bleomycin injury mouse model (293). Similarly, RHAMM HA-binding peptides inhibited arthritis formation in collagen-induced arthritis in mice (193). These studies suggest that blocking HA-RHAMM, or HA-CD44 interactions could have therapeutic benefits in inflammation and wound repair disease processes in light of the dual roles of CD44 in the inflammatory response as discussed above (202, 216, 309) in specific diseases.

HA-Drug Conjugates in Cancer

Enzymatic degradation based therapeutics of HA

Hyaluronidases are a class of enzymes that predominantly degrade HA (Table 2). Recently, Lokeshwar et al. have shown that the expression of HYAL-1-v1 in bladder cancer cells that express wild type HYAL-1 induces G2-M arrest and apoptosis (310). It has been shown that adhesion of monocytes to human coronary artery smooth muscle cells was also inhibited by bacterial HYAL (311). Similarly, commercial bovine testicular or bacterial HYAL act as an anti-adhesive compound on EMT-6 tumor spheroids (312), and HYAL disaggregated EMT-6 spheroids increased chemosensitivity to cyclophosphamide (312), and also improved the therapeutic effectiveness of these agents, i.e., by increasing the accessibility of solid tumors to the chemotherapeutic drugs. Unlike EMT-6 cells, HYALs have limitations as an anti-adhesive agent for other human tumors (313) and can have side effects that impact normal tissue functions.

Development of a recombinant soluble form of PH20 (60) has paved the way for drug delivery in otherwise non-penetrable pancreatic ductal adenocarcinomas where HA forms a formidable

barrier in the tumor stroma. Intravenous administration of PEGPH20 restored normal interstitial fluid pressure in the tumor by increasing vessel diameter. A prospective, randomized, placebo control trial in KPC mice with combined enzymatic and gemcitabine treatment has shown 83% increase in median survival rate. 80% placebo control mice died vs. 29% of Gem+PEGPH20 treated mice, and significant reduction of metastatic tumor burden was observed with combined therapy (61).

HA backbone-based conjugated drugs in cancer

Hyaluronan conjugated drugs are more soluble in water than the drugs alone (Table 2). For instance, the antimitotic chemotherapeutic agent paclitaxel (PTX) has low water solubility. Upon conjugation to HA, water solubility of the prodrug HA-PTX, and of HYTAD1-p20 (a HA-PTX conjugate renamed as ONCOFID-P by the pharmaceutical company Fidia) significantly increased CD44 dependent cellular uptake *in vitro* and *in vivo* in cancer cells, including bladder carcinoma cells (314, 315). Luo and Prestwich coupled PTX-N-hydroxysuccinimide ester (PTX-NHS) with HA of molecular weight ~11 kDa (316). PTX release from the hydrogel film was evaluated *in vitro* using selected anti-bacterial and anti-inflammatory drugs (317). The pharmaceutical company Fidia prepared ONCOFID™-S, another HA prodrug conjugate with SN-38, the active CPT11 (irinotecan) metabolite. The HA used had a molecular weight of ~200 kDa. *In vitro* and *in vivo* phase I and phase II clinical studies were initiated using ONCOFID-S in several CD44-overexpressing cancer cells, including colon, gastric, breast, esophageal, ovarian, and human lung cancer cells. In all these studies, these drugs reduced tumor cell growth and metastasis (318–320).

HA-encapsulated drugs

Another strategy for HA-based CD44 targeting utilizes the concept that the large volume domain of HA (molecular weight > 750 kDa) can non-covalently entrap small therapeutic molecules within its domain (Table 2). HA was then used as a macromolecular carrier for the irinotecan drug along with its targeting properties (321). Clinical trials of three HA formulations [termed hyaluronic acid chemo transport technology (HyACT)] have been undertaken in Australia. Phase I clinical evaluation of two formulations based on HA (HyACT) with 5-fluorouracil (5-FU) (known as HyFIVETM), and on HA (HyACT) with doxorubicin (DOX) (known as HyDOXTM) demonstrated reasonable cytotoxic efficacy without compromising safety of these formulations (322, 323).

HA-tailed drug carriers

These include the following HA conjugates with cytotoxic activity (Table 2).

- (i) Bisphosphonates (BPs) – where HMW HA is linked via a HABP (324).
- (ii) Carbonates (HA-pCB) – where *n*-propyl carbonate is linked to HA via an ester linkage (325, 326).
- (iii) Chitosan – where chitosan-HA nanoparticles (HA-CTNPs) containing 5-FU/oxaliplatin were prepared by the ionotropic gelation method. 5-FU/oxaliplatin loaded HA-CTNP formulation significantly enhanced cytotoxicity compared with

- either chitosan nanoparticles (CNTPs) alone or free 5-FU, or oxaliplatin in HT29 CRC cell lines, which overexpress CD44 (327, 328).
- (iv) Gagomers (GAG-mers) – where GAG-mers (GAG cluster of particles) are composed of lipid molecules that self assemble into particulate clusters in hydrophilic solutions, which are then covalently coated with HMW (1.2–5 MDa) HA. When tested in primary head and neck cancers and normal cells taken from the same patient (329), GAG-mers selectively bound only to the tumor cells to induce cytotoxic activity.
 - (v) Liposomes/lipoplexes (HA-LIP) (330–334) – where HMW HA was decorated on nano-sized encapsulated mitomycin C (MMC). The cytotoxic activity of the drug loaded into HA-LIP was found to be ~100-fold that of free drug in *in vitro* and *in vivo* tumor cells overexpressing the HA-receptors, but not in cells with low receptor expression levels. The HA-LIP conjugate can be used to deliver plasmid DNA and small interfering RNA (siRNA) to CD44 positive cancer cells (332, 335). The presence of HMW HA in the lipoplexes enhanced nucleic acid protection from degradation by DNase I or RNase VI. In case of LMW HA, the HA was linked to PE to form a conjugate in which only one PE molecule is linked to a HA molecule (333, 334). This procedure enables a controlled amount of HA to be introduced into the liposomes. oHAs were attached to PE and incorporated into the liposomes, which increased their recognition, cytotoxicity, and transfection efficiency by tumor cells expressing high levels of CD44 in a temperature-dependent manner.
 - (vi) Micelles – where the hydrophilic backbone of HA was conjugated via its carboxyl groups to amino functions of poly-L-histidine (PHis) or polyethylene glycol (PEG). These HA constructs form nanocomplexes by self-organizing into micelles, and they can carry anticancer drugs, including PTX. In addition, PTX when entrapped into the hydrophobic cores of the folic acid (FA)-conjugated HA-C18 micelles exhibited higher cytotoxic activity compared to Taxol in MCF-7 cells that overexpress both the folate receptor and CD44. The micelles of HA-PTX (336), HA-DOX (337), and HA-salinomycin (338) exhibited more pronounced cytotoxic effects on HA-receptor overexpressing cancer cells than on receptor deficient cells.
 - (vii) Nanocarrier – where HA was conjugated to a nanocarrier. These nanoparticles were able to deliver anticancer drugs, including epirubicin (339), DOX (333), PTX (314), and MMC (339), as well as siRNA, to CD44 overexpressing cells (340). In addition to the well-developed strategies described above, several multifunctional nanocompounds have recently been developed that combine therapeutic and diagnostic properties. These nanoparticles include quantum dots (341), carbon nanotubes (342) and nanodots (343), graphene (344), gold nanoparticles (345), iron oxide nanoparticles (346), and silica nanoparticles (347), and they have been found to acquire novel characteristics after their conjugation with HA (341–348) (**Table 2**).

Targeting CD44 in Cancer

HMW HA can interact with a number of CD44 receptors and be endocytosed. It is rapidly cleared from circulation by the liver hepatocytes (349), and any excess of the targeting compound can lead to adverse effects (350). This rapid clearance was circumvented by choosing oHAs long enough to bind to CD44 but too short to bind to the HARE receptor, which may permit targeting to cells that overexpress CD44. The minimum HA length required to interact with individual CD44 molecules is 6–10 monosaccharides (220) with moderate affinity.

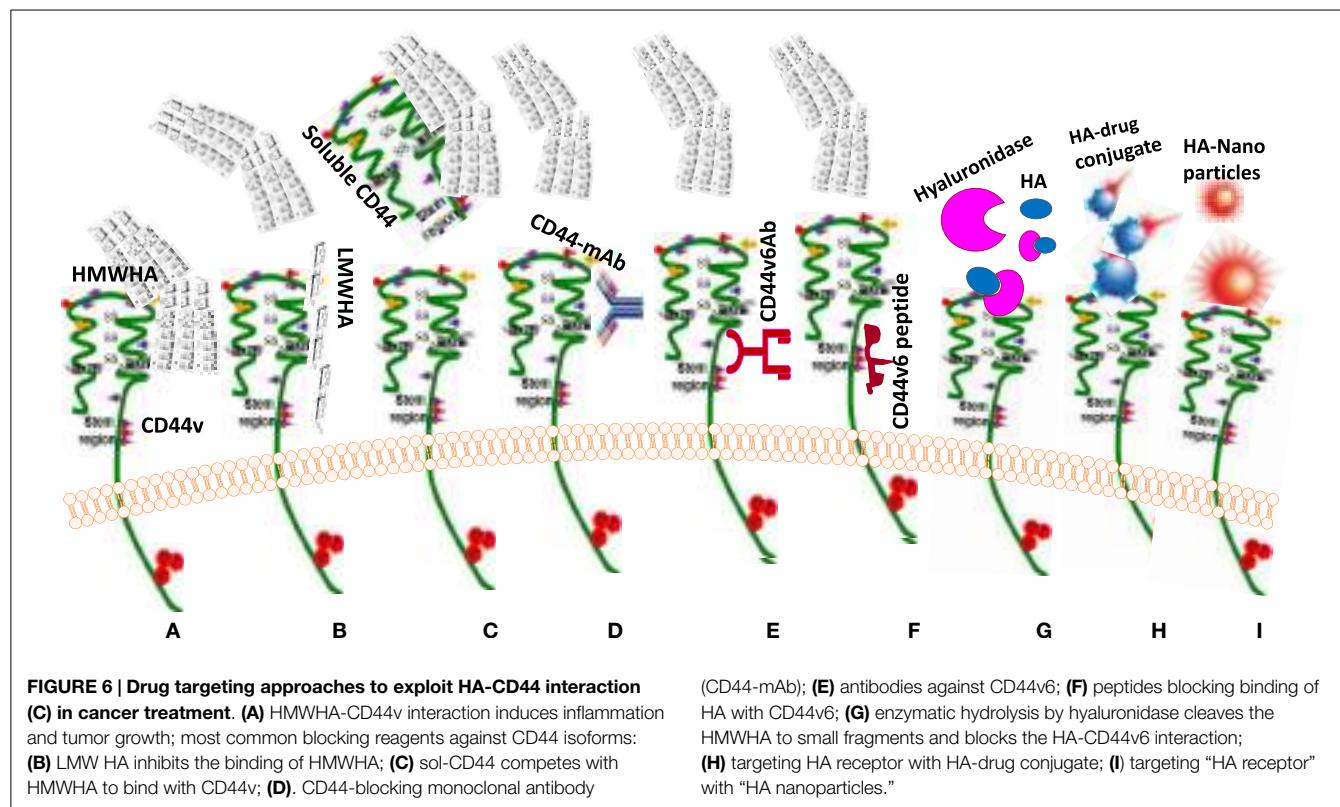
Interrupting HA-CD44 Interaction

As mentioned in the previous sections, the main property attributed to CD44 is its ability to bind HA, and this binding contributes to apoptosis resistance of cancer-initiating cells (6, 37, 351–353) (**Table 3; Figure 6**). There are also examples in which CD44 cross-linking initiates apoptosis. However, apoptosis induction by HA-CD44 cross-linking is largely restricted to non-transformed cells with immature leukocytes being most easily affected. Thus, together with the oncogenic transformation and the pronounced association of CD44 with oncogenes, HA-CD44 cross-linking initiates signals that promote cell survival in tumor cells. The cross-talk of CD44 with multidrug resistance genes accounts for an alternative CD44-mediated apoptosis resistance mechanism. This approach involves substituting multivalent interaction of HMW HA with CD44 with monovalent interaction of small oHAs [6–18 saccharide units (oHAs)] (217, 354). The oHAs inhibit HA-CD44 downstream cell survival and proliferation pathways, and they stimulate apoptosis and expression of phosphatase and tensin homolog (PTEN) (86, 355). The oHAs also sensitize cultured cancer cells to some chemotherapeutic drugs by inhibiting expression of MDR1 and other ABC transporters (96, 228, 241). While oHAs inhibit the growth of several tumors implanted as xenografts (86), they did not give consistent significant growth inhibition in adenoma growth in Apc Min/+ mice (Misra et al., unpublished results). There are several studies contradicting the cellular response of HMW and LMW HA. For example, in schwannomas cells, HMW HA inhibited tumor growth (356). In contrast, LMW HA can induce angiogenesis (80) and inflammatory responses in various cell types (76, 112, 357). These inconsistencies could be resolved if the HA preparations and size determinations could be done properly and if the receptors, or the signaling pathways were identified properly. Only a few studies define the receptors (93, 232). Thus, we developed siRNA and, even more advantageous, short hairpin RNA (shRNA), to target CD44v6 in colon cancer, and showed that they can successfully interrupt HA-CD44v6 interaction and signaling (~90–95%). We then developed a novel shRNA delivery approach to target HA-CD44v6 specifically in tumor cells (6, 94, 95, 248), which is discussed in the following sections.

Overexpression of sol-CD44 displaces exogenous HA from its binding to CD44 and thereby retards tumor cell growth both *in vitro* and *in vivo*, and sensitizes tumor cells to chemotherapeutic drugs (228, 229, 353, 358, 359). However, soluble forms of CD44 can differ in their capacity to bind HA, indicating that sol-CD44 can act as a competitive inhibitor of exogenous

TABLE 3 | CD44 function in cancer.

Disease model	Function of CD44 in cancer	Approaches to interfere the HA-CD44 interaction	Reference
Colon, breast and prostate cancer cells	Apoptotic resistance	HA oligosaccharides [6–18 saccharide units (oHAs)]	(86, 96, 219, 230, 243, 356, 357)
Schwannoma cells	Inhibition of cell survival	HMW HA	(358)
Xenografts of mammary and colon tumor cells	Cell survival	Soluble CD44	(166, 230, 231, 355, 360, 361)
HNSCC	<i>In vitro</i> and <i>in vivo</i> malignant properties	Anti-CD44-Mo-Ab conjugated with mertansine, or radionucleotide, activated anti-CD44 antibody (H90)	(152, 369–373)
AML cells	Tumor cell survival activities	CD44v6 peptides containing the v6 exon region	(373)
Colon cancer cells	Tumor cell survival activities	Penetratin-conjugated peptide, and peptide specific for CD44	(103, 374, 375)
Melanoma and prostate tumor cells	<i>In vitro</i> cell migration, invasion	Pep-1 (specific 12-mer HA-binding peptide)	(379)
Melanoma tumor cells	Melanoma tumor cells growing under both anchorage-dependent and -independent conditions melanoma tumor growth <i>in vivo</i> nude mice xenograft models	BHP (42 amino acid peptide containing three BX7B HA-binding motifs) peptide	(362)
Disease model	Function of CD44 in cancer	Systemic targeting by viral and non-viral vectors	Reference
Ornithine transcarbamylase deficient sparse fur mice	Tumor cell growth <i>in vitro</i> and <i>in vivo</i>	Viral vectors	(389)
K562 cells	Tumor cell growth <i>in vitro</i> and <i>in vivo</i>	Non-viral vectors	(390, 391)
Intestinal tumors in Apc Min/+ mice	Tumor cell growth <i>in vitro</i> and <i>in vivo</i>	Tissue-specific delivery of non-viral CD44v6 shRNA-nanoaparticle	(94)



HA-protein interactions. In these studies, sol-CD44 blocks HA-activated CD44 clustering and inhibits CD44-mediated recruitment of activated MMP-9 and invasion potential of cancer cells

(165). Similar to HA-binding sol-CD44, a peptide (42 amino acids in length) enriched with three HA-binding motifs (BX7B) possesses an antitumor activity in melanoma cancer (360).

Targeting with Anti-CD44 Antibodies

Anti-CD44 antibodies against highly expressed CD44v variants can effectively target drugs to cells expressing a selective CD44v, which can then inhibit and disrupt CD44 matrix interactions, alter CD44 signaling, and cause apoptosis (361) (**Table 3; Figure 6**). Antibodies against highly expressed variants can also be designed to selectively deliver a cytotoxic drug to cancer cells in two different ways. They can bind and neutralize the receptor by competitive inhibition of its ligand and hence stop the receptor-signaling cascade. They can also be attached to radioisotopes, toxins, or chemotherapeutic agents and target them to the required vicinity. The first concept has been effectively utilized for other receptors like anti-EGFR antibodies cetuximab, panitumumab, etc. Anti-CD44v6 conjugated with a cytotoxic drug mertansine has been extensively studied in early phase clinical trials for its value in translational medicine (362–366). Head and neck squamous cell cancer (HNSCC) patients were treated with three doses of mertansine conjugated bivatuzumab (humanized anti-CD44v6 monoclonal antibody [HAMA] labeled with technetium-99m) was first tested (151, 367, 368). However, the phase I clinical trials were rather disappointing in terms of accumulation and toxicity. Furthermore, dose limiting skin toxicity was observed, most probably due to CD44v6 expression in non-tumor skin areas, indicating limitations in the use of this antibody therapy. However, given the promising results of a phase I clinical study with the radionuclide-antibody conjugates (362–366), new similar conjugates of bivatuzumab with radioisotopes were found to be safe and effective in phase I trials (369, 370), and a conjugate of immunotoxin with bivatuzumab (369) was also found to be safe in the next clinical trial on 30 HNSCC patients. In this context, a previous study showed that activated anti-CD44 antibody (H90), when used in human acute myeloid leukemia (AML) cells, reduced the leukemic repopulation by altering the fate of AML leukocyte stem cells (AML LSC), and by abrogating AML LSC homing, leading to their death (371). This study suggests that activated anti-CD44 antibodies without conjugation with toxin are much safer and efficient for antitumor activity. Thus, CD44, particularly CD44 variant forms, remains a crucial target for tumor therapy (**Figures 4 and 5**). To address this issue, we have developed a novel tissue-specific (CD44v6) shRNA delivery strategy by a Cre-lox system. This technology is discussed in a following section.

Peptide-Based Strategies

It is becoming increasingly evident that future prospects for the treatment of inflammation/cancer should include the targeting of specific signaling pathways in tumor cells. (**Table 3; Figure 6**) CD44v6 is a co-receptor for VEGF/VEGFR-2 and HGF/c-Met, and mutational analysis of CD44v6 revealed that three amino acids in the v6 region are required for its co-receptor function for Met and VEGFR-2. These studies helped to identify CD44v6 peptides with the minimal length of five amino acids that contain the critical v6 exon region that can inhibit both VEGFR-2 and c-Met activation. This inhibits the co-receptor function of CD44v6 and the vascularization and tumor cell growth, migration and invasion (103, 372).

Serine phosphorylation of the human CD44 cytoplasmic tail at Ser323 and Ser325 enhances cell migration potency. Thus, a penetratin-conjugated peptide containing phosphoserine at residue 325 reduced *in vitro* migration of melanoma cells (373). Insertion of phosphorylated Ser325 (pSer325) or pSer323 and pSer325 in the peptides disrupted the activation of CD44/MMP-9 signaling complex in prostate cancer cells (374). Another peptide comprising eight amino acids that bind specifically to CD44 (375) and derived from human urokinase plasminogen activator (A6) inhibits migration, invasion, and metastasis of cancer cells by interfering with an uPA-independent signaling pathway (376).

The Pep-1 (specific 12-mer HA-binding peptide) peptide can reduce lung metastasis and prolong survival of mice injected with cell line-derived melanoma cells (377). Besides this Pep-1, BH-P, a 42 amino acid peptide containing three BX7B HA-binding motifs present in CD44 can exert antitumor effects by inhibiting the proliferation of melanoma tumor cells growing under both anchorage-dependent and -independent conditions and by inhibiting melanoma tumor growth in nude mice xenograft models (360).

Tissue-Specific Deletion of CD44variant Signaling

CD44 splicing can regulate interaction with HA (378, 379) (**Table 3; Figure 6**). HA is not only the essential component of the tumor matrix assembly (380), but also has a crucial role in cancer stem cell (CSC) niches, which are particularly rich in HA (381). Interference in matrix components alters signaling events of tumor-initiating cells such that tumor cells that express HA can induce expression of HA in other cells that make up the CSC niche (382, 383). In addition, CD44v6 isoforms are engaged in matrix assembly (384) and have been identified as markers of CSCs in colon cancer, and they account for the metastatic susceptibility of the tumors (385). In the intestinal mucosa, CD44 is a major direct target of β-catenin mediated transcription (177), and we have shown that CD44v6 also regulates β-catenin in colon cancer cells (93). Furthermore, CD44v4-v10, but not CD44s, is a crucial component of the intestinal stem cells in the crypts of ApcMin/+ mice, and controls tumor initiation and relapse by controlling the balance between cell survival and apoptosis (178). These studies indicate that CD44v6 targeting in colon cancer is a promising therapeutic approach. The inhibition of CD44 mRNA expression by inducing the expression of siRNA/shRNA in tumor cells is an alternative approach to the use of CD44-blocking antibodies to interfere with the function of CD44 proteins. This section discusses the fundamental aspects of a therapeutic approach targeting CD44v6 by means of colon cancer cell-specific delivery of shRNA. This approach addresses: (a) what to deliver (engineered therapeutic CD44v6 shRNA), (b) how to deliver (delivery strategies using non-viral transferrin (Tf)-coated PEG-polyetheleneimine (PEI) (Tf-PEG-PEI) nanoparticles for *in situ* cell-specific therapy), and (c) where to deliver (tumor cell targets, in particular, colon tumor cells for *in situ* cell-specific therapy). The technique of using shRNA in an expression vector is an alternative strategy to stably suppress selected gene expression, which suggests that the use of shRNA expression vectors holds potential promise for therapeutic approaches for silencing disease causing genes (386). There are two ways to deliver shRNA in

cancer cells, either using a viral vector or a non-viral vector. Viral vectors have been used to achieve proof of principle in animal models and, in selected cases, in human clinical trials (387). Systemic targeting by viral vectors toward the desired tissue is difficult because the host immune responses activate viral clearance. Systemic administration of a large amount of adenovirus (e.g., into the liver) can also be a serious health hazard and even caused the death of one patient (387). Nevertheless, there has been considerable interest in developing non-viral vectors for gene therapy.

Figures 6–8 illustrate the model for the uptake of non-viral vectors through Tf-PEG-PEI-nanoparticles carrying multiple functional domains. Non-viral vectors mediate unspecific interactions with non-target cells and blood components, which results in the rapid clearance from circulation. PEI has positive charges and binds to negatively charged plasmid DNA to form condensed particles. The PEG shields the condensed PEI-Plasmid particles from unwanted interactions and prevents clearance from circulation thus giving longer half-life (388). To increase the transfection efficiency of the shielded particles (plasmid DNA/PEG-PEI), different targeting ligands, such as peptides, growth factors and proteins, or antibodies, have been incorporated into the vectors (389). One such targeting ligand is Tf, an iron-transporting protein that is recognized by Tf receptors (Tf-Rs). Association

of Tf to polyplexes enhances transfection efficiency (389). This concept was tested by preparing non-viral vector Tf-PEG-PEI-nanoparticles with plasmids packed inside an outer PEG-PEI layer coated with Tf that binds with Tf-R with high affinity in the tumor cells (94, 389, 390) (depicted in the model in **Figure 7**). We found that the Tf-R is present at much higher levels on the tumor cells than on phenotypically normal epithelial cells (94). Tf-PEG-PEI-nanoparticles significantly enhance transfection efficiency of CD44v6 shRNA generator plasmids by promoting the internalization of the nanoparticles in proliferating and non-proliferating colon cells through receptor-mediated endocytosis (94, 389). Therefore, the uptake of Tf-PEG-PEI-nanoparticles carrying multiple functional domains (surface shielding particles Tf-PEG-PEI, CD44v6 shRNA generator plasmids, tissue-specific promoter driven Cre recombinase plasmids, and conditionally silenced plasmids) can overcome the intracellular barriers for successful delivery of the CD44v6 shRNA (94).

This CD44v6 shRNA plasmid delivery approach was tested for transfection of pSV-β-gal/Tf-PEG-PEI-nanoparticles in cellular models (6, 94, 248) (**Figure 7**). Following this experiment, we successfully demonstrated that the CD44v6 shRNA is localized into the colon tumor cells by an end point assay of CD44v6 expression and by perturbation of HA-CD44v6 interaction as reflected in the reduction in the number of tumors (94).

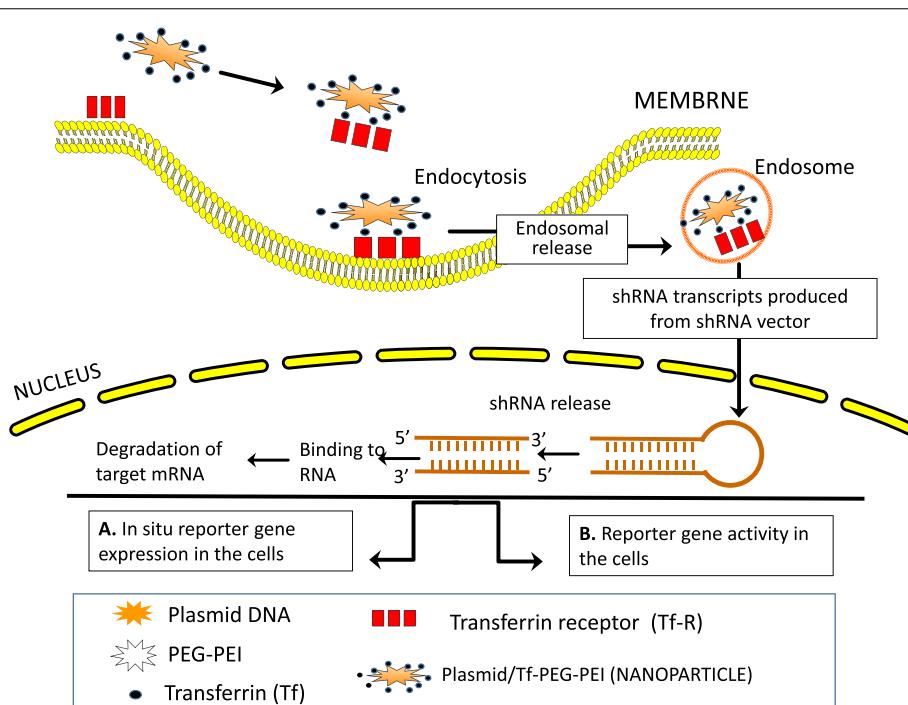
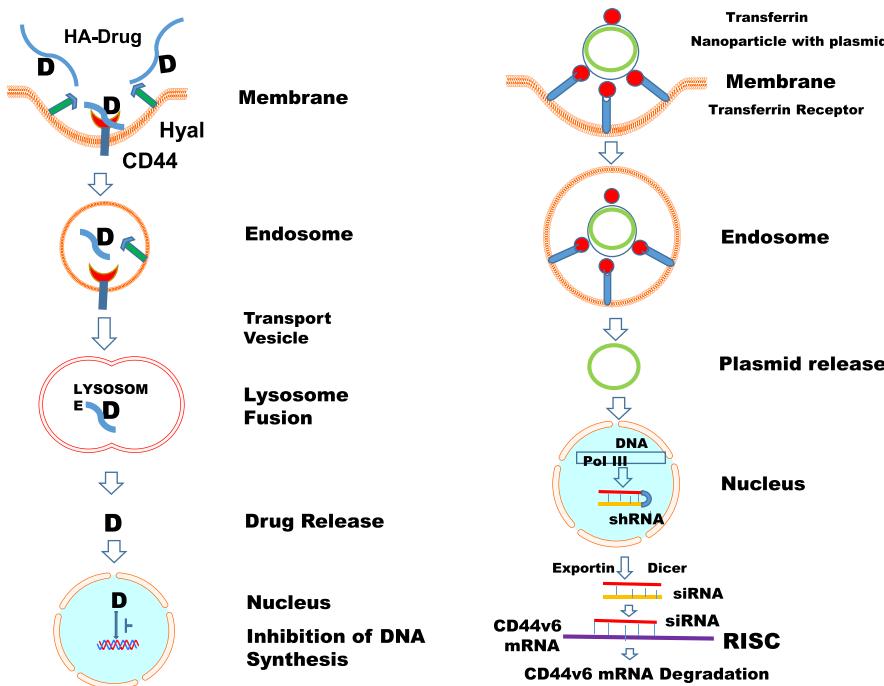


FIGURE 7 | Schematic illustration of cellular uptake of plasmid DNA/Tf-PEG-PEI (nanoparticles) polyplexes, their shielding from non-specific interaction, and the mechanism of action of shRNA.

Internalization of PEG-shielded and [transferrin receptor (Tf-R)]-targeted polyplexes into target cells occurs by receptor-mediated endocytosis after association of polyplex ligand Tf to Tf-R present on the target cell plasma membrane. Internalized particles are trafficked to endosomes followed by endosomal release of the particles and/or the nucleic acid into cytoplasm. Released siRNA will form a RNA-induced silencing complex and will be guided

for cleavage of complementary target mRNA in the cytoplasm. SiRNA (antisense) guide strand will direct the targeted RNAs to be cleaved by RNA endonuclease. Finally, plasmid/Tf-PEG-PEI-nanoparticles delivery in the target cell shows reporter gene expression and activity. The normal tissue cells are not affected because they do not make the targeted CD44 variant. Tf-PEG-PEI nanoparticle coated plasmids (pSico-CD44v6 shRNA/pFabpl-Cre) circulating in blood accumulate at tumor regions enhanced by the EPR effect. Endocytosis mediated by ligand-receptor interactions occurs because the nanoparticles are coated with the Tf-ligand for the Tf-R receptor on the tumor cell surface.

**FIGURE 8 |** Exploitation of HA-CD44 interaction for anticancer

therapy. Left panel shows a schematic diagram for cancer cell internalization of HA conjugated to a drug inhibitor of DNA synthesis. CD44 on the membrane traps the HA-drug conjugate and efficiently internalizes it by endocytosis and forms an endosomal vesicle. This is then transported to the lysosome and fused, and the internalized HA is degraded by hyaluronidase 1 (Hyal-1) into small HA oligosaccharides and then to monosaccharides by lysosomal glycosidases, which ultimately releases the conjugated drug. The drug then goes to the nucleus and inhibits the DNA synthesis. Right panel shows targeting the CD44v6mRNA in cancer cells by

CD44v6 shRNA. Plasmids that produce CD44v6 shRNA are coated with transferrin present on the outer surface of the nanoparticles. The transferrin molecules then target the transferrin receptors present in high amounts on the cancer cells. Upon internalization, an endosome forms and the plasmids are released to the nucleus where CD44v6 shRNA is produced by DNA pol III. The shRNA is then exported out of the nucleus by exportin, and the dicer enzyme converts shRNA into CD44v6 siRNA. One of the strands of siRNA will bind to CD44v6 mRNA and form a RISC (RNA-induced silencing complex) that is ultimately degraded. Adapted from our article in the International Journal of Cell Biology.

The tissue-specific shRNA delivery was made possible by the use of Cre-recombinase produced in response to a colon tissue-specific promoter, which deletes the interruption between the U6 promoter and the CD44v6 shRNA oligonucleotide. The newly developed cell-specific shRNA delivery approach by Misra et al. (94) confirmed that targeting the signaling pathways induced by HA-CD44v6 interaction inhibited distant colon tumor growth in Apc Min/+ mice. Our recent unpublished *in vivo* studies with the C57Bl/6 mice have now shown that systemic delivery of a mixture of two plasmids, prostate-specific Probasin-Cre/Tf-PEG-PEI-nanoparticles and floxed pSico-CD44v9shRNA/Tf-PEG-PEI-nanoparticles, can target both localized and metastatic prostate cancer cells. This novel approach opens up new ways to combat cancer and to understand tumorigenesis *in vivo* for the following reasons (Figure 7).

- Cell-specific shRNA to CD44variant (CD44vshRNA) is released by applying a tissue-specific promoter driven Cre-lox mechanism.
- This shRNA silences the expression of the selected CD44 variant in the target tissue cancer cells.
- This shRNA does not affect the normal target tissue cells, which rely on the standard CD44s and do not express the

targeted CD44variant, and therefore are not affected by the plasmids.

- The target CD44vshRNA will not be expressed in other types of cells because the tissue-specific promoter only unlocks the Cre-recombinase in the targeted tissue cells, which reduces potential side effects (94).
- The Tf-PEG-PEI-nanoparticles that carry plasmids are biodegradable and cleared from the system.
- This method inhibits the pathophysiological role of HA-CD44v interactions in cancer.
- It can establish diagnostic markers for the targeted cancer, including CD44variants, soluble CD44, and HA.
- It can identify HA-CD44v interactions as innovative novel therapeutic targets against cancer progression.

Thus, the conditional suppression of gene expression by the use of a CD44vshRNA expressing plasmid holds potential promise for therapeutic approaches for silencing HA-CD44variant signaling and downstream signaling pathways that promote disease causing genes (386).

Targeting RHAMM in Cancer

Under homeostatic condition, RHAMM expression is very low. Its expression is increased during pathogenesis (inflammation and

TABLE 4 | RHAMM function in cancer.

Disease model	Function of RHAMM in cancer	Approach using HA-binding peptide	Reference
Esophageal squamous cell carcinoma	Invasiveness	Pep-1	(393)
Multiple myeloma and myelodysplasia syndrome	Anti-apoptosis	RHAMM-R3 peptides	(394, 395)
Malignant peripheral nerve sheath tumors or multiple myeloma	Apoptotic resistance	RHAMM silencing	(396–398)
Neuroblastoma tumors	Apoptotic resistance	RHAMM-based immunotherapies targeting AURKA and BARD1	(399, 400)
Acute myeloid leukemia	Antitumor activity	RHAMM R3	(395, 401)

cancer) (132). Such a restricted expression of RHAMM makes it a suitable target for cancer and wound repair therapy with low toxicity (132).

RHAMM-Based Immunotherapies

Peptides that mimic either RHAMM itself or the HA sizes that bind to RHAMM have been shown to affect neoplastic processes as well as inflammation and wound repair (Table 4). HA targeting by Pep-1 blocked esophageal squamous cell carcinoma invasiveness by inhibiting HA-RHAMM induced effects (391). There is evidence that RHAMM-R3 peptides that are currently being tested in phase II clinical trials for multiple myeloma and myelodysplastic syndrome show efficacy and low toxicity in patients (392, 393). RHAMM silencing abrogated the self-renewing property of glioblastoma stem cells, and loss of RHAMM in malignant peripheral nerve sheath tumors or multiple myeloma sensitizes tumor cells to inhibitors (394–396). Neuroblastoma tumors are also sensitive to RHAMM-based immunotherapies due to the established roles of AURKA (397) and BARD1 [breast cancer gene 1 (BRCA1)-associated ring domain protein-1] (398), two components of the AURKA-BRCA1/BARD1-RHAMM-TPX2 centrosome module, within this refractory disease (267).

Cell-Based Therapies

In addition, cell-based strategies using peptide vaccination with a RHAMM-derived, highly immunogenic peptide, termed RHAMM R3, has proven safe and effective at generating CD8+ RHAMM-specific T cell cytotoxic cellular responses and antitumor activity in patients with AML, myelodysplastic syndrome, multiple myeloma, and, more recently, chronic lymphocytic leukemia in phase I/II trials (393, 399) (Table 4). These studies provide evidence that blocking RHAMM is a promising immunotherapy approach in patients with hematological malignancies. In another study, antitumor activity has also been reported for vaccination with dendritic cells expressing exogenous RHAMM mRNA in a mouse model of glioma (400).

A handful of RHAMM-based therapeutic studies in cancer suggest that more intense studies should be undertaken to determine how RHAMM signaling contributes to cancer progression. Although HA-receptor-mediated signaling is believed to be a promising anticancer/anti-inflammatory drug target, given the dual functions of HA, CD44 and RHAMM in the inflammatory/tumorigenic responses, caution must be taken in considering therapeutic targeting of these molecules, which should be designed for specific organs and specific diseases with consideration of the use of proper sizes of endotoxin free HA, which might provide a way to prevent side effects.

Conclusion

In the past few years, basic and clinical research on CD44 have identified the genomic DNA structure and alternative splicing pattern of CD44, which has led to a conclusion that CD44v is not one but a family of proteins, and that discrete isoforms are expressed and regulated at various stages of oncogenic transformation. Most, but not all, cancers overexpress discrete species of CD44v, which can be correlated with tumor aggressiveness. A challenging area of research would be to define what cellular functions are associated with the various CD44 isoforms that are overexpressed in various cancers before a CD44-based therapy can be undertaken. Considering the biological functions of HA particularly the connection of such function with molecular size of HA, the HA-binding proteins, its spatial and temporal distribution in tissues, and the cellular background and tissue stages, care must be taken to ensure the long-term safety of HA-based bioconjugates. Thus, this review defines the origins of evidence for a linkage between HA, CD44v, and RHAMM expression with inflammatory diseases, including malignancy, and emphasizes the most advanced and developed therapeutic strategies, those that have either been used for clinical trial or are nearly ready to get there. In addition, approaches used in various preclinical models are also briefly reviewed. Studies reviewed here identified strong prospects for anti-CD44 therapies. The HA-CD44 interaction system is illustrated in Figure 8 where we specify cancer therapeutical aspects (discussed in this review) that specifically perturb HA-CD44 signaling pathways. Interference with the function of HA-CD44 can inhibit the inflammation/malignant processes at multiple stages. This can be accomplished by perturbing HA-CD44 signaling pathways, by disruption of the HA matrix with HYALs to facilitate passive carrier uptake and providing a sustained source of drug at the tumor site, by targeting CD44 with a CD44-blocking antibody, or by tissue-specific targeting of specific variants of CD44v that are overexpressed in tumors (Figure 8).

Although overexpression of CD44 correlates with bad prognosis in patients with most human cancers (151, 178–184), it was also found that CD44 is extremely sensitive to changes in the microenvironment. For example, CD44 in breast cancer cells, neuroblastomas and prostate cancer may act as a metastatic suppressor gene (170, 185, 186), suggesting that the growth promoting pathways in these tumors are independent of CD44. These differential regulations should be considered carefully while designing CD44 as a target for therapeutic strategy.

For last two decades, several studies were dedicated to the use of CD44v, in particular CD44v6, as a therapeutic target. As discussed in Section “Targeting with Anti-CD44 Antibodies,” the

anti-CD44v6 antibody conjugated with mertansine showed dose limiting skin toxicity due to CD44v6 expression in non-tumor skin tissue. However, the scientific progress in the last few years provides strong support for using CD44v as a target for therapeutic strategies. This is due to the fact that: (1) CD44v6 is a marker for colon CSCs (180, 385); (2) CD44v6 can act as co-receptor for at least three RTKs (c-Met, VEGFR-2, and EGFR) (103, 236, 401), and many of the oncogenic functions of CD44v6 can be attributed to downstream signaling induced by these RTKs; and (3) CD44v6 is highly expressed in many cancers. Nevertheless, there is an urgent need to define which CD44v variants are present on the CSCs for the particular type of cancer, and then target them in signaling complex tissue specifically by non-viral vectors. Collectively, these studies suggest that future development of drug targeting approaches can use tissue-specific expression of CD44v-specific antagonists as well as inhibitors (agents that are currently used in the clinic) targeting CD44 isoforms and their co-receptors/ligands that

alter intracellular signaling in the inflammatory/tumor tissue microenvironment, as an effective and novel approach to regulate these diseases. Finally, since CD44 and RHAMM bind to HA, targeting against RHAMM may be an additional treatment option.

Author Contributions

SG and SM wrote the review. Dr. VH has reviewed the draft and final version, and the revised draft of the manuscript. Dr. RM has edited the final and revised version of the manuscript.

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Modulation of CD44 activity by A6-peptide

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Hyaluronan (HA) is a non-sulfated glycosaminoglycan distributed throughout the extracellular matrix that plays a major role in cell adhesion, migration, and proliferation. CD44, a multifunctional cell surface glycoprotein, is a receptor for HA. In addition, CD44 is known to interact with other receptors and ligands, and to mediate a number of cellular functions as well as disease progression. Studies have shown that binding of HA to CD44 in cancer cells activates survival pathways resulting in cancer cell survival. This effect can be blocked by anti-CD44 monoclonal antibodies. A6 is a capped, eight L-amino acid peptide (Ac-KPSSPPEE-NH₂) derived from the biologically active connecting peptide domain of the serine protease, human urokinase plasminogen activator (uPA). A6 neither binds to the uPA receptor (uPAR) nor interferes with uPA/uPAR binding. A6 binds to CD44 resulting in the inhibition of migration, invasion, and metastasis of tumor cells, and the modulation of CD44-mediated cell signaling. A6 has been shown to have no dose-limiting toxicity in animal studies. A6 has demonstrated efficacy and an excellent safety profile in Phase 1a, 1b, and 2 clinical trials. In animal models, A6 has also exhibited promising results for the treatment of diabetic retinopathy and wet age-related macular degeneration through the reduction of retinal vascular permeability and inhibition of choroidal neovascularization, respectively. Recently, A6 has been shown to be directly cytotoxic for B-lymphocytes obtained from patients with chronic lymphocytic leukemia expressing the kinase, ZAP-70. This review will discuss the activity of A6, A6 modulation of HA and CD44, and a novel strategy for therapeutic intervention in disease.

Keywords: A6, CD44, HA, CLL, metastasis, recurrence, resistant, ocular

INTRODUCTION

Mortality due to cancer is generally the result of metastasis of the primary tumor. Recurrence at distant sites following first-line therapy continues to be a major challenge. As a result, drugs that inhibit the metastatic process are of great interest. Metastasis and recurrence have been linked to a subpopulation of highly invasive tumorigenic cells that are characterized by the expression of CD44.

A6 has been shown to bind to CD44 and to exhibit anti-metastatic properties. Thus, A6 may serve as a therapeutic alternative for the treatment and prevention of metastatic disease.

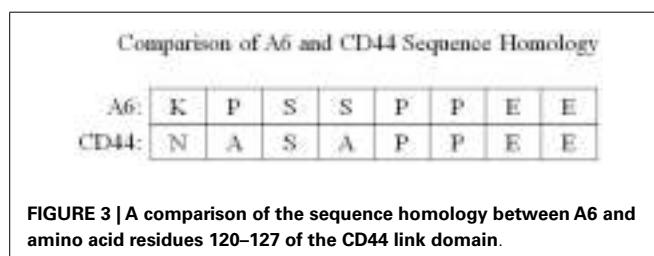
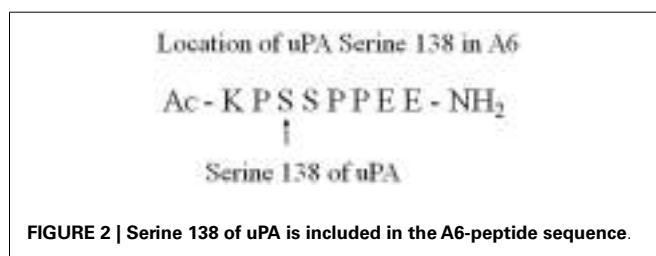
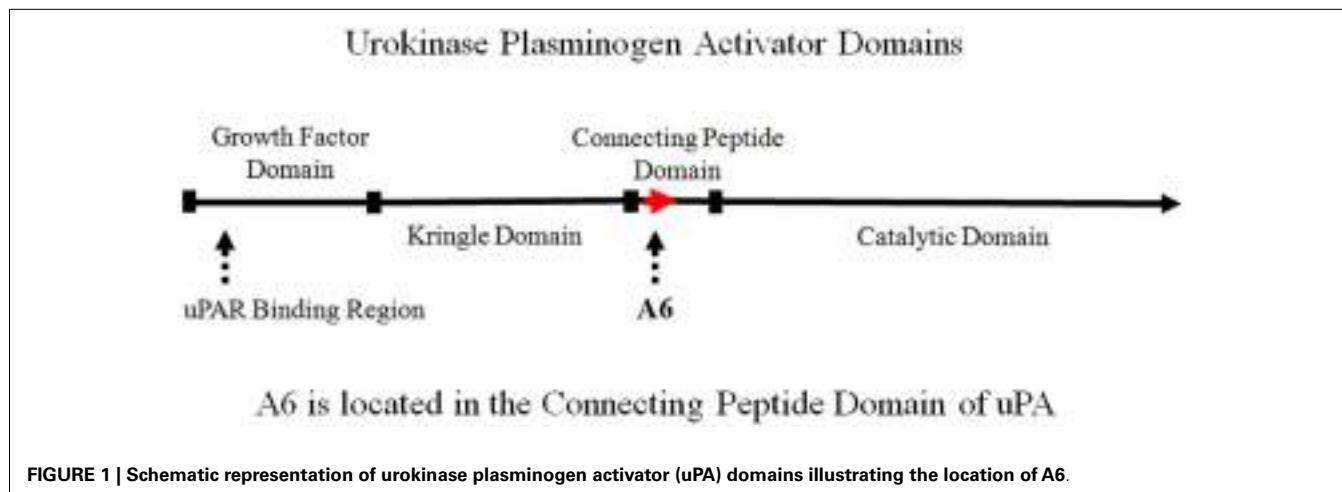
A6 BACKGROUND

A6 is a capped eight L-amino acid peptide (Ac-KPSSPPEE-NH₂) derived from amino acid residues 136–143 of the connecting peptide domain of human urokinase plasminogen activator (uPA).

The connecting peptide domain is located between the N-terminal growth factor domain and the C-terminal catalytic domain of uPA (**Figure 1**). The N-terminal growth factor domain of uPA binds to the uPA receptor (uPAR) to initiate the uPA/uPAR cascade, which is catalyzed by the C-terminal serine protease that activates plasminogen to plasmin. Numerous studies have shown that the binding of uPA to uPAR, initiates a cascade of events leading to proteolysis, degradation of the extracellular matrix (ECM), cell migration, cell invasion, metastasis, and angiogenesis (1–4). Further, the uPA system has been shown to play an important role in the growth and spread of solid tumors. Levels of uPA and

uPAR correlate with clinical outcome in a variety of malignancies. Specifically, the upregulation of the uPA system is associated with poor prognosis, and inhibition of the uPA system has been shown to block critical processes (e.g., cell migration, invasion, and angiogenesis) required for a broad range of proliferative diseases. This provides the rationale for development of inhibitors of this pathway (5–9).

The binding of uPA to uPAR has been localized to the N-terminal growth factor domain. Although the growth factor domain is known to initiate biological activity, the connecting peptide domain of uPA has also been shown to have biological activity independent of the growth factor domain (10, 11). uPA has been shown to bind to HEK 293 cells (transformed with uPAR) to stimulate migration (10). The binding of uPA peptide fragments composed of the growth factor domain and connecting peptide domain were evaluated to characterize domain-specific activity on the cell surface (10). Both the growth factor and connecting peptide domains were capable of stimulating cell migration independently. Furthermore, the binding of these peptide fragments to HEK 293 cells was inhibited by increasing concentrations of the uPA molecule. However, the connecting peptide domain itself did not inhibit growth factor domain binding, and growth factor domain did not inhibit connecting peptide domain binding. This indicated the presence of distinct binding sites for both the growth factor domain and connecting peptide domain, as well as a uPAR-independent signaling pathway (10). It was postulated that



the connecting peptide domain of uPA was functioning through interaction with a cell surface integrin receptor (10, 11).

The biological importance of the connecting peptide domain (amino acid residues 132–158) has been further demonstrated by the phosphorylation or substitution of serine 138, which results in the inhibition of uPA-induced cell migration without changing uPA binding to uPAR (12, 13). A6 comprises uPA amino acids 136–143. The serine 138 residue of uPA is included in the A6 sequence (Figure 2).

Phosphorylation or substitution of uPA serine 138 does not change the growth factor domain-mediated binding of uPA to uPAR, yet inhibits migration, which suggests that the connecting peptide domain region is important for regulatory function. This indicates that simultaneous concurrent interaction of uPA domains, with distinct surface receptors, is required for uPAR-dependent cell migration. This provided additional evidence to support the investigation of the A6-peptide.

The biological activity localized within the uPA connecting peptide domain prompted additional study. It was postulated that proteolysis by plasmin and matrix metalloproteinases (MMPs) such as MMP3 and MMP7 could excise a fragment from amino acids 136–143, corresponding to A6. Although there is no evidence that this proteolytic processing occurs, several peptides comprising various fragments of the connecting peptide domain sequence were synthesized. The A6 sequence was found to have activity, which led to the preclinical and clinical investigation of A6.

Interestingly, A6 shares sequence homology with a portion of the link domain of CD44 (CD44 amino acid residues 120–NASAPPEE-127) (14) (Figure 3). The CD44 gene is encoded by

20 exons in the mouse and 19 exons in humans: 5 constant exons are expressed at the 5' end, and 10 variant exons (mouse) or 9 variant exons (human) may be alternatively spliced within CD44 at an insertion site after the fifth constitutive exon, followed by the remaining constant exons at the 3' end (15, 16). The standard isoform of CD44 (CD44s) is the smallest isoform containing no variant exons, and the largest is CD44v1–10, which contains all of the variant exons. Although a CD44 specificity has not been determined for A6, it is important to note that the region of A6 sequence homology in CD44, through which A6 may act, is conserved in all CD44 isoforms as it is located within the first five non-variable exons of the molecule (15). This homology is important because the sequence straddles the CD44 splice junction of exons 3 and 4, includes a potential glycosylation site, and is involved with hyaluronan (HA) binding (14, 15, 17–21). The evaluation of A6 activity using alanine scanning mutagenesis demonstrated that there is a degree of substitution that can be accommodated without a substantial loss of A6 activity, and analysis of the CD44 homologous peptide sequence revealed activity similar to that of A6 (22).

A6 AND METASTATIC DISEASE

PRECLINICAL STUDIES

Preclinical studies have shown that A6 has anti-migratory, anti-invasive, and anti-metastatic properties. A6 has been shown to inhibit migration and invasion of breast, lung, glioma, ovarian, and prostate cancer cell lines *in vitro* in a dose-dependent manner (23–27), and to inhibit the growth and metastasis of breast, melanoma, glioma, lung, and prostate cancer cells in xenograft

models *in vivo* (14, 24–27). Interestingly, the combination of A6 with tamoxifen resulted in an inhibition of breast tumor cell growth greater than with either A6 or tamoxifen alone (24). A similar result was observed in glioma xenograft studies where the combination of A6 with cisplatin also inhibited tumor cell growth greater than with either A6 or cisplatin alone (26). These results are important because of the relationship between CD44 and chemoresistance.

IN VITRO STUDIES

Boyden chamber analyses demonstrated that A6 inhibited chemotaxis in a variety of human breast and ovarian cancer cell lines in a concentration-dependent manner (14). The IC₅₀ for the inhibition of chemotaxis of responsive cell lines was 10–100 nmol/L suggesting physiological relevance (14). Furthermore, A6 inhibition of chemotaxis was shown to correlate with the expression of CD44. This was demonstrated by flow cytometric analysis with four different anti-CD44 antibodies and five different human ovarian cancer cell lines. A6 produced more than 85% inhibition of migration in CD44-positive SKOV3 cells when compared to untreated control (14). Notably, A6 had no effect on the migration of CD44-negative A2780 cells. A6 was also shown to interfere with the binding of only one (DF1485) of the four anti-CD44 antibodies tested (14). A6 did not interfere with the binding of the anti-CD44 antibody, IM7, which blocks HA binding to CD44. These findings suggest that A6 does not produce a global non-specific change in CD44, but instead produces a subtle change to a specific epitope.

Because A6 inhibited migration of SKOV3 cells, this study also examined the direct interaction of A6 with CD44 (14). Human ovarian SKOV3 cells were bound and cross-linked to A6. Immunoprecipitation and immunoblotting of lysate preparations of cross-linked cells revealed that A6 was binding to CD44. To determine if this binding influenced CD44-mediated activity, and to determine if a functional relationship existed between A6 and CD44, intracellular signaling studies were conducted. A6 was shown to modulate FAK phosphorylation in CD44-positive SKOV3 cells, but not in CD44-negative A2780 cells. The study further demonstrated that the A6 modulation of FAK phosphorylation in SKOV3 cells was blocked by HA. These results show that a functional relationship exists between A6 and CD44 binding and CD44-mediated intracellular signaling (14).

IN VIVO STUDIES

Mammary

The effects of A6 in mammary tumor and metastasis models have been investigated. Studies with BALB/c (nu/nu) mice implanted with MDA-MB-231 human mammary carcinoma xenografts demonstrated that A6 inhibited tumor growth by 90% compared to control (23). An inhibition of metastasis was also noted. Additionally, the effect of A6 in Fisher rats inoculated with Mat B-III syngeneic mammary carcinoma cells was evaluated. A6 treatment inhibited tumor growth by 55% and markedly suppressed lymph node metastasis (23). Furthermore, the combination of A6 with tamoxifen in Fisher rats with Mat B-III syngeneic mammary carcinoma resulted in a 75% inhibition of tumor growth (24).

Prostate

A model of prostate cancer was used to evaluate the anti-metastatic effect of A6 in mice. Metastases to lymph nodes were measured following the orthotopic injection of human PC-3M-LN4 prostate cancer cells into the prostates of BALB/c (nu/nu) mice. The percentage of mice with lymph node metastases was reduced from more than 70% in the control group to as low as 22% in A6-treated animals (27). Additionally, A6 treatment significantly reduced lymph node volume by as much as 70%.

Glioblastoma

In animal models of glioblastoma, U87MG human glioma cells were implanted subcutaneously or intracranially in BALB/c (nu/nu) mice and the animals were divided into different treatment groups. A6 treatment suppressed subcutaneous U87MG tumor growth by 48% and prolonged the time to progression (TTP) following discontinuation of A6 treatment (26). In this study, the effects of cisplatin were also examined. Cisplatin treatment reduced tumor growth by 53%. Interestingly, the combination of A6 and cisplatin resulted in a 92% inhibition of subcutaneous tumor growth. This result was consistent with a U87MG intracranial xenograft study in which mice receiving a combination of A6 and cisplatin exhibited a significantly greater inhibition of tumor growth (98%) when compared to either A6 (44%) or cisplatin (82%). In this study, the combination therapy also significantly increased survival time over that for either drug alone. This was consistent with subcutaneous xenograft results.

Melanoma

The well-characterized B16-F10 lung metastatic model was employed to determine the ability of A6 to inhibit the colonization of secondary tissues by circulating cancer cells (14). B16-F10 melanoma cells were evaluated by flow cytometric analysis and were shown to express CD44. The IC₅₀ for A6 inhibition of chemotaxis in B16-F10 cells was 29 nmol/L, indicative of a responsive cell line. Melanoma cells were injected into the tail veins of C57BL/6 mice to simulate a burden of metastasizing cells and the lungs were then evaluated for lesions at day 11. Treatment with A6 reduced the number of lung metastases to 50% of control. Taken with previous results, this is important because it demonstrates that A6 not only inhibits the initial steps of the metastatic process (e.g., migration and invasion) but also inhibits the formation of secondary lesions after tumor cells enter the circulation.

Leukemia

A6 has also been evaluated for activity in hematological malignancies. Chronic lymphocytic leukemia (CLL) is characterized by the accumulation of mature monoclonal B cells in the blood and secondary tissues. CD44 is highly expressed in CLL cells and mediates the interaction between CLL cells and the microenvironment. CLL cells receive survival signals from the microenvironment, and one of these pathways is mediated by CD44. Binding of HA to CD44 has been shown to activate PI3K/AKT and MAPK/ERK-mediated survival pathways, and to induce expression of the anti-apoptotic protein Mcl-1, which promotes CLL cell survival (28). It has been shown that this effect can be blocked by an inhibitor of Mcl-1, or by anti-CD44 monoclonal antibodies, leading to apoptosis *in vitro* (29, 30).

Recent studies (31, 32) with human CLL B-cell lymphocytes have shown that A6 down modulates the expression of CD44 and ZAP-70 (a marker for an aggressive form of CLL), and inhibits B-cell receptor (BCR) signaling, resulting in a direct, dose-dependent, cytotoxicity *in vitro*. To evaluate the effects of A6 *in vivo*, an established CLL xenograft model was employed. ZAP-70^{pos} B-cell lymphocytes isolated from individual patients were injected into immune-deficient mice treated with A6 or vehicle control. A6 treatment resulted in up to 90% reduction in CLL burden (31, 32). Previously, A6 had not demonstrated cytotoxicity in solid tumor models of glioma, breast, and ovarian cancer (14, 22, 23, 25). However, in these CLL studies, A6 was shown to be directly cytotoxic for CLL B-cell lymphocytes. A6 is currently being evaluated for the treatment of CLL.

CLINICAL STUDIES

Several clinical studies have been conducted to evaluate the safety and efficacy of A6. These include safety studies in healthy volunteers as well as studies in patients with varying stages of metastatic disease.

Normal volunteers

A6 was administered to normal volunteers in a Phase 1a, double-blind, placebo-controlled, parallel-group clinical trial (33). Results showed that there were no systemic drug-related adverse events. No significant alterations in physical examinations, vital signs, electrocardiograms, or clinical laboratory testing, including coagulation parameters such as PT, PTT, fibrinogen, and thrombin time, were noted. Pharmacokinetic data in normal volunteers at the 150 and 300 mg/day single dose levels showed a $t_{1/2}$ of 1.8–2.0 h at both dose levels. Furthermore, no cumulative increase in concentration over time was detected. Following A6 subcutaneous administration twice daily for 6 days, no anti-A6 antibody production was detected at day 14 (33).

Advanced gynecologic cancer

A Phase 1b trial was conducted in women with advanced gynecologic cancer (34). Greater than 40% of patients dosed continuously with A6 experienced disease stabilization. The study used a sequential dose-escalation design, with the lowest-dose group (four patients) receiving A6 for cycles of 14 days “on” followed by 14 days “off,” a regimen not expected to produce any therapeutic effect. Twelve patients with advanced gynecologic malignancies that had failed standard therapy were treated with daily, uninterrupted A6. In this population, in which disease progression is expected, five patients (four of whom had ovarian or primary peritoneal carcinoma) achieved stable tumor measurements for at least 4 months, and one for greater than 12 months. Patients continued treatment until disease progression or unacceptable toxicity. Response was evaluated as defined by RECIST and the Gynecologic Cancer Intergroup (GCIG) CA-125 response criteria. A Kaplan-Meier retrospective analysis demonstrated that patients treated with daily A6 showed a delayed time to tumor progression relative to an effective control group (whose treatment was intermittent and, therefore, not expected to have beneficial effect) providing evidence of antineoplastic activity. Continuous treatment with A6 resulted in an increased TTP with a median TTP

of 78 days (95% CI 57, 365) compared to 44 days (95% CI 4, 62) in patients who received the intermittent therapy (log-rank p -value = 0.02). The safety outcome in this Phase 1b gynecologic cancer trial was excellent and showed no specific toxicity profile.

Asymptomatic progression of ovarian cancer

A randomized, double-blind, placebo-controlled Phase 2 clinical trial evaluating A6 in women with asymptomatic CA-125 progression of ovarian cancer (“marker-only relapse” or MOR) was conducted (35). Patients were in clinical remission after first-line chemotherapy with no evidence of disease following physical examination or imaging analysis, but had two consecutive, above-normal, increases of CA-125 (a biomarker for recurrence/poor prognosis). Because patients were clinically asymptomatic at the time of entry, the study was able to be placebo-controlled. The primary endpoints were time to clinical progression of disease and safety of A6. The secondary endpoints included changes in serum CA-125. This study enrolled 24 patients: 12 were randomized to daily self-administration of A6 at two doses, and 12 to matching placebo injections. Both groups were followed for up to 9 months. Although there were no complete responses, 36% of patients achieved stable disease. A6 treatment was not associated with CA-125 response. Results from a Kaplan-Meier analysis of progression-free survival (PFS) showed that treatment with A6 significantly prolonged TTP. Despite the small patient sample size, A6 therapy was associated with a statistically significant increase in PFS (log-rank p -value = 0.01) with a median PFS of 100 days (95% CI 64, 168) compared to 49 days (95% CI 29, 67) in patients who received the placebo. Furthermore, the safety profile of A6 was comparable to that of control (placebo) treatment.

Persistent or recurrent ovarian cancer

A Phase 2 trial was conducted in patients with persistent or recurrent epithelial ovarian, fallopian tube, or primary peritoneal carcinoma (36) to evaluate A6 in a patient population with a disease burden greater than that presented in the previously described MOR trial. Patients had received one prior platinum-based chemotherapeutic regimen and were allowed to have received one additional cytotoxic regimen for the management of recurrent or persistent disease. Patients received a 150 mg twice daily subcutaneous dose of A6 and continued on treatment until disease progression or unacceptable toxicity. Response criteria were as defined by RECIST. Primary measures of clinical efficacy were objective tumor response and PFS at 6 months compared to a historical Gynecologic Oncology Group (GOG) dataset based on a similar population of patients. Of the 31 eligible patients evaluated, no responses were observed; 6.5% were progression free for at least 6 months; and 36% of evaluable patients achieved stable disease. A6 was well tolerated but had minimal activity in patients with persistent or recurrent epithelial ovarian, fallopian tube, or primary peritoneal carcinoma under the conditions of this trial. Considering the relationship of A6 to CD44 and the relationship of CD44 to resistant and recurrent disease, it would be of interest to follow this study with a combination trial comparing standard-of-care to standard-of-care plus A6 in this difficult population.

A6 AND OCULAR DISEASE

A6 has been evaluated for the treatment of ocular disease (37). The focus of this application has been wet age-related macular degeneration (AMD) and diabetic retinopathy, which are characterized by neovascularization and vascular permeability. Since angiogenesis is known to involve HA and to be mediated by CD44 (38–40), A6 has been investigated for use as a therapeutic for these conditions. Angiogenesis is a multistage process involving cell migration and ECM remodeling, including the loss of cellular structure and function followed by invasion. Similar cellular changes are also observed early in the metastatic process. These cellular changes can be more accurately described in terms of an epithelial–mesenchymal transition (EMT). EMT is a process by which epithelial cells acquire mesenchymal-like properties, with reduced intercellular adhesion and increased motility, critical to many developmental, homeostatic, and pathological processes. The EMT process is a continuum leading to enhanced cell migration and invasion. Preceding migration, there is a loss of cadherin and epithelial adhesion, followed by disruption of the basement membrane and degradation of the ECM by MMPs (41–45). A6 has been shown to inhibit this process.

WET AGE-RELATED MACULAR DEGENERATION

Several *in vivo* studies have been conducted to evaluate the efficacy of A6 for the treatment of wet AMD. In the mouse model of laser-induced choroidal neovascularization (CNV), treatment with A6 resulted in a 95% inhibition of new vessel formation compared to the non-treated control group (46). Results employing a rat model of laser-induced CNV showed that subcutaneous injections of A6 produced a 70% reduction in CNV compared to non-treated controls (47). Finally, results from a primate model of laser-induced CNV demonstrated that intravitreal administration of A6 resulted in a 71% reduction in CNV relative to control (48). These studies demonstrate that A6 may be a promising candidate for the treatment of wet AMD.

DIABETIC RETINOPATHY

Research involving the use of A6 for treatment of diabetic retinopathy demonstrated that A6 treatment prevents the loss of vascular endothelial (VE)-cadherin and inhibits the increase in microvascular permeability in the retina of diabetic Brown Norway rats induced with streptozotocin (49). In the same study, similar results were observed using bovine retinal microvascular endothelial cells and showed that VE-cadherin degradation was associated with increased vascular permeability and the secretion and activation of MMP-2 and MMP-9. Treatment with A6 was shown to inhibit MMP-dependent VE-cadherin degradation and the loss of permeability. In addition, A6 prevented the secretion and activation of MMP-2 and MMP-9 (49). HA has also been shown to increase MMP-2 and MMP-9 expression in cell culture (50) and to promote CD44-EGFR interaction leading to MMP-2 secretion and enhanced cell motility (51). The ability of A6 to inhibit MMP activation may have important implications for the metastatic process.

The role of hepatocyte growth factor (HGF) in angiogenesis (52, 53) as well as the elevated intravitreous concentrations of HGF in diabetic patients has been described (54, 55). The

effect of A6 on HGF and its receptor, c-Met, in retinal angiogenesis has been examined (56). This study demonstrated that HGF was upregulated in the retinas of mice following hypoxia-induced retinal neovascularization. Furthermore, HGF was shown to stimulate retinal microvascular endothelial cell invasion *in vitro*, which is consistent with the angiogenic process. HGF-induced retinal endothelial cell invasion was reduced to control levels following treatment with A6 (56). Since CD44 functions as a co-receptor with c-Met, these results suggest a possible mechanistic pathway for A6.

DISCUSSION

The metastatic process involves migration and invasion of tumor cells from the local microenvironment, intravasation into the blood or lymph circulation, extravasation from circulation back into tissue, followed by metastatic colonization and growth or dormancy (57). Metastasis and recurrence have been linked to a sub-population of highly invasive tumorigenic cells, which have been shown to be resistant to chemotherapeutics. These tumorigenic cells are characterized by the expression of CD44, a multifunctional receptor involved in cell signaling, adhesion, migration, and proliferation. CD44 functions as a receptor, as a co-receptor (e.g., c-Met and EGFR), and as a platform for MMPs to enable many biological processes (58, 59). In addition, CD44 is known to mediate invasion and metastasis (60).

Chemotherapeutic resistance has been linked to a number of CD44 pathways including MDR1-dependent efflux of chemotherapeutics (61–65). This resistance results in expansion of invasive cells following first-line chemotherapy, which leads to recurrence. Studies have shown that targeting CD44 or related signaling pathways, using RNAi strategies (61, 62, 66, 67) or with anti-CD44 antibodies (68), will suppress tumor growth and relapse, and increase sensitivity of these cells to chemotherapeutics. In animal xenograft models, A6 has been found to enhance the activities of both tamoxifen to inhibit the growth of breast tumor cell growth (24) and cisplatin to inhibit the growth of glioma cells (26). This supports the concept that targeting CD44 may render tumor cells more sensitive to therapeutic agents.

A6 has been shown to bind to CD44 and to modulate CD44-mediated activity. A6 demonstrates anti-metastatic properties by inhibiting migration and invasion, which are early steps in the metastatic process. The mechanism by which A6 acts may involve inhibition of EMT, as observed in studies of ocular disease where A6 inhibited MMP activation and cadherin degradation (49). Rationale for this consideration is supported by evidence that HA is implicated in MMP activation (50, 51). A6 also acts later in the metastatic process to inhibit the formation of lesions resulting from the direct injection of cancer cells into the blood stream (14). This would indicate that A6 inhibits steps involving extravasation and/or metastatic colonization. This is important when considering recurrence following adjuvant therapy and the possibility of proliferation of dormant micrometastases long term.

Dormancy and micrometastases present a therapeutic challenge (69, 70). That subclinical micrometastases may be present long-term was demonstrated in a study involving 36 breast cancer patients found to be disease free from 8 to 22 years post-resection (71). This study demonstrated that in one-third of these

patients, with no evidence of disease, viable circulating tumor cells (CTCs) could be isolated. The CTCs were determined to be non-proliferative with a short half-life, but were found when repeated samples were taken up to 2 years after the patients entered the study. This suggested that CTCs were being continuously released from subclinical micrometastases. Long-term or maintenance therapy targeting recurrence is not practical when considering many cytotoxic agents. However, due to its superior safety profile (no immunogenicity, no dose-limiting toxicities, no serious side-effects), long-term or maintenance therapy with A6 may be an option. The use of A6 in this manner could introduce a new paradigm to cancer treatment.

As previously noted, A6 shares sequence homology with the link module of CD44. The link module of CD44 has been shown to be critical to HA binding and cell migration. When the CD44 link module was substituted with a homologous region of higher HA affinity (TSG-6), cells expressing this chimera bound HA, but failed to migrate and were described as tethered (20). A6 was shown to increase the binding of CD44-expressing SKOV3 cells to HA-coated plates (14). This effect was blocked with the anti-CD44 antibody, IM7. However, neither A6 nor IM7 had any effect on the binding of CD44-non-expressing A2780 cells to HA-coated plates. These results suggest that increasing adhesion may play a role in the anti-metastatic activity of the A6-peptide, and again illustrate correlation of A6 activity with CD44 expression. The study further demonstrated that A6 perturbed the binding of the anti-CD44 antibody, DF1485, to CD44-expressing SKOV3 cells. This was reported to be a partial inhibition, which did not result from a competition involving either A6 or CD44. Furthermore, the DF1485 antibody did not recognize A6 or inhibit the binding of an anti-A6 antibody to A6. It was postulated that A6 induces conformational changes in CD44, resulting in either a lowered affinity of the epitope for DF1485, or preventing DF1485 from binding (14). Regardless of the mechanism, the binding of A6 to CD44 results in a modulation of CD44-mediated intracellular signaling (14). This establishes a functional relationship between A6 and CD44 in CD44-expressing cells. This functional relationship was demonstrated by monitoring FAK phosphorylation in the presence and absence of A6 and HA in CD44-expressing and -non-expressing cell lines. Although HA is reported to have a rheostatic effect on CD44, with high molecular weight HA inhibiting tumor progression, and low molecular weight HA stimulating tumor progression (72–74), the extent to which A6 may differentiate these activities has yet to be studied.

The precise mechanism of A6 has not been defined, but compelling evidence from studies on metastatic and ocular disease supports action through a CD44-mediated pathway. Whether this is by direct action on CD44 or by modulating CD44 co-receptor activity remains unclear. A6 binding to CD44 and the effects of A6 on chemotaxis and intracellular signaling have been demonstrated in the absence of HA. This indicates that A6 has a primary effect, through CD44, that is independent of HA. However, because A6 increases adhesion of CD44-expressing cells to HA, it also suggests that A6 may interact with HA secondarily to A6 binding to CD44. The CD44 ligand-binding region that shares homology with A6 is likely to be critical to the mechanism by which A6 modulates the

activity of CD44. A6 may simulate the CD44 sequence and trigger a homotypic interaction resulting in modification of CD44 activity by inducing a conformational change in CD44, or CD44 dimerization, or both. As mentioned, the perturbation of DF1485 binding by A6 suggests that A6 may induce conformational changes in the receptor. Alternatively, A6 homology may simulate the CD44 sequence permitting it to influence a CD44-binding partner/co-receptor resulting in modulation of CD44-mediated activity. This is supported by A6 inhibitory activity on HGF and MMPs observed in ocular diseases. Finally, although A6 binds to CD44, the possibility cannot be excluded that A6 interacts with a protein independent of CD44 that initiates secondary modulation of CD44 activity.

CD44 is a complex multifunctional receptor modulating a variety of cellular processes. Although the mechanistic process is not completely defined, the studies described have demonstrated that A6 inhibits the metastatic process in a CD44-dependent manner. Because CD44 is associated with a chemoresistant phenotype, which is countered by inhibition of CD44 signaling, A6 is a candidate for inhibition of CD44-mediated resistance. In this case, A6 would be used in combination with a cytotoxic chemotherapeutic agent to inhibit metastases and to render resistant cells sensitive to chemotherapy. Certainly, the results from preclinical A6 combination studies in animals support this approach. Furthermore, due to the positive safety profile documented for A6, there would be a reduced likelihood of compounding toxicity. As such, A6 may potentially be combined with almost any chemotherapeutic. This safety profile also invites the use of A6 for longer-term maintenance therapy to prevent recurrence stemming from micrometastases surviving first-line standard-of-care treatment. A6 has demonstrated activity against CD44-expressing tumor cells and CLL cells, and is a candidate for the treatment of malignant disease and hematological malignancy. A6 has demonstrated clinical safety and efficacy, and by targeting CD44-resistant cells to prevent metastases and recurrence, has the possibility of creating a new paradigm for cancer treatment.

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The role of CD44 in disease pathophysiology and targeted treatment

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The cell-surface glycoprotein CD44 is involved in a multitude of important physiological functions including cell proliferation, adhesion, migration, hematopoiesis, and lymphocyte activation. The diverse physiological activity of CD44 is manifested in the pathology of a number of diseases including cancer, arthritis, bacterial and viral infections, interstitial lung disease, vascular disease, and wound healing. This diversity in biological activity is conferred by both a variety of distinct CD44 isoforms generated through complex alternative splicing, posttranslational modifications (e.g., N- and O-glycosylation), interactions with a number of different ligands, and the abundance and spatial distribution of CD44 on the cell surface. The extracellular matrix glycosaminoglycan hyaluronic acid (HA) is the principle ligand of CD44. This review focuses both CD44-HA dependent and independent CD44 signaling and the role of CD44–HA interaction in various pathophysiologies. The review also discusses recent advances in novel treatment strategies that exploit the CD44–HA interaction either for direct targeting or for drug delivery.

Keywords: CD44, hyaluronic acid, hyaluronidase, CD44-signaling

Introduction

CD44 is a glycoprotein that is widely expressed on the surface of many mammalian cells, which includes endothelial cells, epithelial cells, fibroblasts, keratinocytes, and leukocytes (1). Extensive alternative splicing of nine variable exons and different combinatorial insertions results in many distinct CD44 splice variants. CD44 standard is the shortest and most abundantly expressed isoform; the other variants are expressed in a cell-specific manner and in the pathophysiology of many diseases. CD44 has several important physiological functions in cell-cell and cell-matrix interactions including proliferation, adhesion, migration, hematopoiesis, and lymphocyte activation, homing, and extravasation (2). This diversity in cellular activity is a result of variable expression of CD44 variants, post-translation modifications such as N- and O-glycosylation, and binding by a variety of ligands (2). The diversity of isoform expression, posttranslational modifications, the abundance and spatial distribution of CD44 on the cell surface are likely to be important

Abbreviations: CSC, cancer stem cell; EMT, epithelial–mesenchymal transition; ERM, ezrin–radixin–moesin; GAS, group A *Streptococcus*; HA, hyaluronic acid; HCV, hepatitis C virus; HGF, hepatocyte growth factor; HMM, high molecular mass; ILD, interstitial lung disease; IPF, idiopathic pulmonary fibrosis; LMM, low molecular mass; MMP, matrix metalloproteinase; milk-HA, HA derived from breast milk; RHAMM, receptor for hyaluronic acid-mediated motility; TLR, toll-like receptor.

for the regulation of signaling; in particular, because high molecular weight hyaluronic acid (HA) can bind multivalently to CD44 (3–12).

The non-sulfated glycosaminoglycan HA is the principle ligand of CD44. HA is a polymer of repeating disaccharide units D-glucuronic acid and N-acetyl-D-glucosamine and can range in size from millions of Daltons to small oligosaccharides. The size of the HA is important for its physiological functions. HA of a high molecular mass (HMM) can naturally bind multivalently to more surface receptors across a larger area of the cell than low molecular mass (LMM) HA, and this difference in the number of bound HA receptors allows different sizes of HA to have different signaling effects. HMM HA is typically >500 kDa, and LMM HA is typically between 10 and 500 kDa. Because of the lack of a standard size for HMM and LMM HA, this review will list the molecular mass of the HA fragments and will refer to the size as HMM or LMM based on the classification used by study authors. HA is a major component of tissue matrices and fluids and is involved in a variety of physiological functions such as maintaining tissue hydration and osmotic balance, cell proliferation, adhesion, and migration (13). Much of its regulation of cellular function is mediated through CD44 and RHAMM signaling, although it has been shown to affect toll-like receptor signaling as well. Since HA also plays a role in the assembly and remodeling of extracellular matrix, these activities of HA are also likely to affect cellular function. HA is synthesized by HA-synthases HAS1, HAS2, and HAS3 in humans. HA synthesis occurs on the cytoplasmic side of the plasma membrane and it is then expelled into the extracellular space. HA is synthesized by many cells but mesenchymal cells are the major source of HA. HA is degraded by hyaluronidase family of enzymes, and the well-studied hyaluronidases are HYAL1, HYAL2, and PH-20/SPAM1 (13). Hyaluronidases degrade HA by hydrolyzing the linkages between the D-glucuronic acid and N-acetyl-D-glucosamine disaccharides.

CD44 has been implicated in a number of diseases such as cancer, arthritis, interstitial lung disease (ILD), vascular disease, wound healing, and infections by pathogens. CD44-HA signaling has been found to be involved in the pathophysiology of both malignant and non-malignant diseases. While several excellent reviews have been focused on CD44-HA signaling and its implications in cancer (3–12), this review focuses on both HA-independent (mainly) and HA-dependent functions of CD44 in the pathophysiology of various diseases. In addition, the review discusses various strategies used for targeting of CD44, and CD44/HA-based therapeutic interventions and devices for targeted imaging and treatment options.

CD44-HA Signaling and Human Disease

Infections

The importance of CD44-HA signaling has been implicated in a number of studies. CD44-HA signaling plays important roles in host defense against invading pathogens, specifically in activation and migration of lymphocytes (14). HA signaling through CD44 has also been linked to generation of antimicrobial peptides (15). Several other studies have shown that CD44-HA signaling can be

utilized by pathogens for progression of infections and resulting complications.

CD44-HA signaling has long been known to play a role in host defense through activation, homing, rolling, and extravasation of lymphocytes into inflammatory sites (16, 17). A recent study suggests that CD44-HA signaling also contributes to host defense during the early stages of infancy. CD44-HA signaling induces human β-defensin 2 (HβD2), which, in turn, enhances antimicrobial defense in the intestinal epithelium (15). The study found that HA derived from breast milk (milk-HA), at 2-week intervals up to 6-month postpartum when HA concentrations were highest, induced production of HβD2 in HT-29 cells, and mice fed with milk-HA also increased expression of MuβD3, the mouse ortholog of HβD2. The authors also fed CD44^{-/-}, TLR4^{-/-}, or wild type mice with milk-HA and found that MuβD3 expression was greatly reduced in CD44^{-/-} or TLR4^{-/-} mice when compared to wild type mice; HT-29 cells pretreated with anti-CD44 antibodies also showed inhibition of HβD2 expression upon treatment with milk-HA. *Salmonella* infection was shown to be greatly decreased in cells treated with milk-HA when compared to milk-HA pretreated with hyaluronidase. These results suggest that CD44-HA signaling is important in the establishment of intestinal epithelium resistance to invading pathogens during early infancy. In a previous study by the above-mentioned group, addition of LMM HA averaging 35 kDa was shown to upregulate HβD2 in HT-29 cells and in mice in a size-specific manner; similar expression was observed upon treatment in combination with HMM HA (2 MDa), but not with HA-2M alone (18). Interestingly, HA-35 up-regulation of HβD2 was shown to be toll-like receptor 4 (TLR4) dependent but not CD44 dependent. In the 2013 study, HβD2 up-regulation by milk-HA was both TLR4 and CD44 dependent. The 2013 study did not address whether breast milk HA of different sizes had any effect on HβD2 levels, and in the 2011 study CD44 dependency was not ascertained with HA-35 and HA-2M combination treatment. Other studies have also reported up-regulation of HβD2 and other antimicrobial peptides upon treatment with LMM HA (<200 kDa) in a CD44-independent manner (19, 20). TLR4 has been shown to complex with CD44 upon treatment with HA (21). Perhaps these studies together point to independent yet complementary mechanisms by which LMM HA (2.5 kDa) signals through TLR4, which may complex with CD44 in the presence of HMM HA (10 MDa), which has been shown to increase CD44 clustering (22).

Bacterial Infection

Group A *Streptococcus* (GAS) utilizes an intriguing method to escape host defenses and adhere to mammalian cells. The capsular polysaccharide of GAS comprises HMM HA that is similar in size to the HA synthesized by mammalian cells and tissues (23). It has been shown that GAS adheres to human keratinocytes through the binding of capsular HA polysaccharides to CD44 (24). An *in vivo* study by the same laboratory evaluated the importance of CD44 expression for GAS infection of the pharynx using C57BL/6 mice and K5-CD44 transgenic mice that expressed a CD44-antisense transgene (25). In this study, transgenic mice with reduced CD44 expression showed significantly lower GAS infection than mice with wild type CD44 expression. GAS infection was also reduced

by treatment with anti-CD44 antibodies and addition of exogenous HA. This study further reinforced the idea that CD44–HA binding is important for GAS infection.

A more recent study evaluated the importance of the molecular mass of HA for macrophage-mediated phagocytosis of GAS in both *in vitro* and *in vivo* murine models (26). In this study, ingestion of GAS by macrophages was inhibited by addition of HMM HA (i.e., 800–1200 kDa), while the addition of LMM HA (i.e., 25–75 kDa) increased GAS internalization. Similarly, GAS survival was increased in murine blood in the presence of HMM HA. Interestingly, the study showed that treatment with hyaluronidase, an enzyme that degrades HA in small fragments increased internalization of GAS by macrophages. The internalization of GAS by macrophages was not present in transgenic mice expressing a CD44-antisense transgene even in the presence of LMM HA, demonstrating that CD44 expression on macrophages is required for GAS internalization. The HMM HA in the capsular polysaccharide of GAS mimics tissue homeostasis and allows GAS to escape detection by the host immune system. Contrarily, LMM HA (>200 kDa) may function as an endogenous danger signal, activating the innate immune system (27). CD44 has also been shown to function as a primary phagocytic receptor via HA signaling (28). Taken together, LMM HA may mediate a signaling cascade that leads to macrophage recruitment and facilitate phagocytosis of GAS by binding to CD44 expressed on macrophages. These studies suggest that CD44–HA signaling is important for GAS infection. Whether CD44–HA signaling aids in host defense of GAS infection depends on the molecular mass of HA, further exemplifying the intricacy of CD44–HA interaction and signaling in disease pathophysiology.

Helicobacter pylori infection is the major risk factor for gastric cancer, which is the second leading cause of cancer-related death in the world (29). Chronic infection by *H. pylori* has been shown to result in atrophy of acid-secreting parietal cells, which leads to increased proliferation of stem/progenitor cells in the isthmus (30). The increased proliferation of isthmus stem cells is believed to be one of the contributing factors that lead to neoplasia. A recent study showed that proliferation of isthmus stem cells after atrophy of parietal cells is a result of a signaling cascade that involves CD44–HA-mediated activation of ERK and STAT3 (31). The study showed that in CD44 knockout mice, or mice treated with PEP-1, an inhibitor of CD44–HA interaction, there are significantly less proliferating isthmus stem cells than in wild type mice after infection with *H. pylori*. CD44–HA signaling was also shown to be important in the progression toward gastric cancer after a complication of *H. pylori* infection. It would be interesting to see the impact of HA size in isthmus stem/progenitor cell proliferation after parietal cell atrophy. Parietal cell atrophy possibly leads to breakdown of HA to small fragments, which then induce to proliferative signaling cascades; in that case, addition of HMM HA may serve to restore homeostasis and inhibit proliferation.

Pneumonia is caused by infection of lung parenchyma by numerous bacteria and is a leading cause of morbidity and mortality. A number of studies have implicated CD44 in the progression of bacterial infection and in the amelioration of lung inflammation in pneumonia models. The role of CD44 in the acute phase (6 h after infection) of pneumonia caused by *Escherichia coli*

and *Streptococcus pneumoniae* differed significantly as reported in a previous study (32). The study showed that when compared to wild type mice, *E. coli*-induced pneumonia in CD44-deficient mice causes increased lung inflammation, as evidenced by increased neutrophil accumulation, migration, and increased mRNA levels of inflammatory genes. However, such was not the case in *S. pneumoniae*-induced pneumonia. The differences in *E. coli* versus *S. pneumoniae*-induced pneumonia may be because the latter expresses hyaluronidase, which breaks down HA. This would decrease CD44–HA-mediated signaling and the downstream induction of inflammatory pathways. The study found that HA levels were decreased in the lungs after Streptococcal infection. A lower dose of infection with *S. pneumoniae* may have given a clearer picture of CD44's role in the inflammatory response to infection by this bacterium. A later study explored this scenario and found transient increases in inflammation in CD44 knockout mice at lower doses of *S. pneumoniae*-induced pneumonia (33). This study also looked at the role of CD44 in prolonged lung infection (10 days) by *Streptococcus*. The study found that CD44 knockout mice had less bacterial outgrowth and dissemination, and higher survival rates compared to wild type mice at lethal doses. Another study by the same laboratory found similar results for *Klebsiella pneumoniae*-induced pneumonia (34). Together these studies show that CD44 signaling is important for reducing inflammation in the lungs, and may increase bacterial dissemination and outgrowth. It is possible that CD44 anti-inflammatory signaling plausibly decreases the clearing of bacteria, and therefore, allows for their growth and dissemination to other sites. To this effect, CD44 has been previously shown to play an important role of resolving lung inflammation (35).

Viral Infection

CD44–HA signaling has also been shown to play an important role in the course of HIV and hepatitis C (HCV) infections. HIV virions have been shown to acquire functional CD44 from host cells (36–38). In a recent study, CD44–HA signaling was shown to affect the infectivity of HIV in unstimulated primary peripheral blood mononuclear cells, unstimulated CD4⁺ T cells, and M7-Lue cells (39). In this study, HIV with virions expressing CD44 showed decreased infectivity of unstimulated peripheral blood mononuclear cells, unstimulated CD4⁺ T cells, and M7-Lue cells when treated with exogenous HA. Interestingly, this decrease of infectivity was not seen in Jurkat-E6.1 cells, which do not express CD44 nor was this decrease in infectivity observed using HIV with virions that did not express CD44. The study also showed that HIV infectivity was increased upon addition of hyaluronidase, suggesting that endogenous HA plays a protective role against HIV infection. Cells treated with hyaluronidase had decreased HA at the cell surface and were five times more susceptible to HIV infection than cells not treated with hyaluronidase. The study also showed that exogenous HA reduces HIV infectivity of CD4⁺ T cells by reducing the activation of protein kinase C- α via CD44. These results suggest that CD44–HA interactions are important for HIV infection and that addition of exogenous HA may interfere with this interaction. Furthermore, this inhibition of infectivity by exogenous HA may be mediated by the inhibition of the protein kinase C- α pathway and is dependent on the thickness of

HA at the cell surface. It would be interesting to see if these results can be replicated *in vivo*, and whether the reduction of infection is dependent on the average molecular weight of exogenous HA and/or HA at the cell surface.

The expression of gamma interferon-inducible protein 10 (IP-10) has been shown to be elevated in chronic HCV patients and is a predictor of treatment outcome (40). In a study aimed at elucidating what role TLRs play in the production of IP-10 in cells infected by and actively replicating HCV, CD44–HA signaling was shown to be involved in IP-10 production (41). In this study, CD44 expression was found to be increased in cells harboring viral replicons. Blocking viral replication led to a reduction in CD44 expression. Furthermore, HA stimulation of cells with actively replicating HCV virus increased IP-10 production; contrarily, knockdown of CD44, TLR2, or MyD88 greatly reduced IP-10 production. These results suggest that in infected cells, HCV induces IP-10 production via CD44–TLR2–MyD88 interactions. This study also links HCV replication to increased expression of CD44. An interesting follow-up study may elucidate whether HCV replicon mediated up-regulation of CD44–HA signaling and increased production of IP-10 leads to leukocyte recruitment and the establishment of chronic inflammation in the liver, leading to HCV-associated fibrosis, cirrhosis, and hepatocellular carcinoma.

These studies further illustrate the complex roles that CD44–HA signaling plays in human infections by pathogens. In the case of HIV infection, it appears that exogenous and endogenous HA protect host cells from infection in a CD44-dependent manner. The opposite is seen in the case of HCV infections; overexpression of CD44 by HCV replicating cells may contribute to overproduction of IP-10, exacerbation of liver damage, and to a poor treatment outcome in chronic HCV patients.

Interstitial Lung Disease

CD44–HA signaling has been implicated in the progression of ILD, which includes idiopathic pulmonary fibrosis (IPF) and systemic sclerosis associated ILD. CD44–HA signaling was shown to play a role in the development of progressive lung fibrosis by activation of myofibroblasts with an acquired invasive phenotype (42). In this study, HAS2-overexpressing transgenic mice showed increased deposition of HA and accumulation and up-regulation of CD44 mRNA and protein in lung myofibroblasts after bleomycin-induced lung injury, resulting in increased lung fibrosis and fatality. However, HAS2 knockout mice did not have increased HA deposits, up-regulation of CD44 or myofibroblast accumulation, and did not show signs of pulmonary fibrosis. Interestingly, the study also found that fibroblasts isolated from HAS2-overexpressing transgenic mice showed increased invasive capacity in Matrigel™ when compared to controls and HAS2-null mice. This increase coincided with increased matrix metalloproteinase (MMP) levels and decreased tissue inhibitors of metalloproteinases levels in HAS2-overexpressing transgenic mice and also in the fibroblasts from IPF patients. The results from this study suggests that HAS2 overexpression, post-lung injury, leads to dysregulated CD44–HA signaling, resulting in a signaling environment conducive to fibroblast activation and invasion leading to the development of IPF. It would be important to determine

if this HA-mediated signaling environment is dependent on the molecular weight of HA. For example, addition of exogenous HA of specific mass to isolated IPF fibroblasts may further elucidate the nature of CD44–HA signaling in progression of lung fibrosis. Another important study would be to evaluate the respective roles of CD44 and receptor for hyaluronic acid-mediated motility (RHAMM), which are expressed on fibroblasts (43), in the development of IPF.

A recent study evaluated the relationship and function of CD44v6, TGF- β 1, and hepatocyte growth factor (HGF) in ILD (44). The study found that lung fibroblasts from bleomycin-treated mice and from ILD patients had elevated levels of CD44v6 and c-Met, both of which were expressed upon TGF- β 1 induction. CD44v6 was shown to increase collagen-1, and therefore, enhance lung fibrosis. TGF- β 1 induction and lung fibrosis were shown to be abolished by HGF. One caveat of this study is that HGF levels are increased in fibroblasts from ILD patients, which is contradictory to the finding that HGF negatively regulates TGF- β 1 induction, and therefore inhibits lung fibrosis. However, HGF concentrations in ILD fibroblasts were much lower than the concentrations used in the study.

In summary, these studies reveal a causative role of CD44 and its isoform CD44v6 in ILD. Furthermore, HA is important in the progression of fibroblast invasion and lung fibrosis. Interestingly, a recent study found that disruption of the HA matrix or inhibition of HA synthesis actually increased deposition of fibronectin and collagen-1 by myofibroblasts in which TGF- β 1 was induced, and HAS2 expression was increased in these myofibroblasts (45). These results together suggest that disruption of endogenous HA or tissue matrix homeostasis may cause dysregulated deposition of fibronectin and up-regulation of HA by fibroblasts that have constitutively active TGF- β 1 signaling. Up-regulation of HAS2 expression in these fibroblasts leads to aberrant CD44–HA signaling, which may lead to activation of more fibroblasts with invasive phenotypes.

Wound Healing

Hyaluronic acid concentrations in tissues undergoing wound repair are elevated for several weeks after injury (46). The primary source of this HA is keratinocytes, but other tissue specific cells can produce the glycosaminoglycan as well. One of the functions of HA in wound repair is to provide a scaffold for cell migration toward injury sites such as hepatic stellate cells migrating toward a liver epithelial injury utilizing CD44v6 (47).

A recent study in a rat bladder regeneration model found that HA deposition in a regenerating bladder after partial cystectomy was required for proper wound healing (48). CD44 expression also increased as the wound healed, mimicking the deposition pattern of HA. The function of the HA–CD44 interaction could simply be to provide a scaffold for tissue repair, but other research shows that LMM and HMM HA found in sites of injury may have direct effects on regeneration. In a mouse acute myocardial infarction model, it was found that IL-6 production in the heart induced HA synthesis, and that HA was required for the differentiation of fibroblasts into myofibroblasts (49). HA binding to CD44 also leads to the production of MCP-1 and CCL5, which recruit neutrophils to the heart and establish a cardio-protective

environment. It has previously been shown that CD44 and ERK1/2 complexes exist in fibroblasts and are required for scratch repair (43).

It is not just the presence of HA and CD44 that shape the wound healing process, but also the size and longevity of the HA fragments. HMM HA ($>10^6$ Da) is typically present in prenatal wound healing, resulting in healing with minimal inflammation and scar formation (50–53). HA oligosaccharides (six to eight oligomers) are typically present in adult wound healing, and results in inflammation and scar formation (54–57). If HA oligomers are not removed from tissue during wound repair then tissue destruction occurs from unending fibrosis and inflammation (21). The negative impact of prolonged accumulation of LMM HA on wound repair is primarily caused by its ability to bind to and activate TLR4. In a mouse sterile injury model, it was found that MD-2, a TLR4 accessory protein that increases TLR4's ability to bind lipopolysaccharide, and CD44 worked together to bind LMM HA on monocytes (21). This prolonged TLR4 signaling-induced production of TGF- β 2 and MMP-13, resulting in tissue damage much the same as if lipopolysaccharide was present in the wound. This finding expands upon earlier research showing that CD44 can associate with MyD88 signaling complexes and TLRs in lipid rafts (58).

A recent study using normal human dermal fibroblasts also shows that LMM HA (4.3 kDa) induces the production of IL-6, IL-8, CXCL1, CXCL2, CXCL6, and CCL8 whereas, HMM HA (1.1×10^6 Da) can inhibit the production of IL-6 and IL-8, indicating potential TLR and MyD88 involvement (59). Closer examination of the effect of specific sizes of HA has revealed that six oligomer fragments of HA are capable of promoting wound closure, accumulation of M2 macrophages, and production of TGF- β 1 without inducing myofibroblast differentiation in a scratch wound repair model (60). Conversely, HA fragments of 40 kDa were found to actively inhibit wound closure and cause the accumulation and differentiation of myofibroblasts.

In conclusion, HA binding to CD44 is required for proper wound healing, but the size and longevity of the HA has a large effect on the outcome. Future research on the size and location of exogenous HA that can be used to aid in wound healing will be of great medical benefit. Surgical scarring could be a thing of the past if small oligomers of HA could be introduced into a wound after incision, but the fragments would need to have a short biological half-life in order to prevent fibrosis and inflammation from occurring.

CD44–HA Signaling and Cancer

CD44 signaling has been shown to be important in cancer metastasis and tumor growth, but can be broken down into two primary areas. HA-independent signaling relies on interactions between CD44's intracellular domain and cytoskeletal proteins or membrane associated kinases. HA-dependent signaling relies on two CD44 molecules being cross-linked, allowing other CD44-associated signaling proteins to interact with each other. Further, CD44 is used as a marker for cancer stem cell (CSC) detection in a variety of cancers without much research into the function of CD44 in stem cells beyond adhesion.

HA-Independent Signaling

CD44 signaling that is HA independent is largely reliant on CD44's position in lipid rafts. Association between CD44 and other signaling proteins are often regulated through CD44's interaction with ezrin–radixin–moesin (ERM) protein family members that initiate association with cytoskeletal elements.

A recent study examines the role of CD44 in Wnt pathway signaling using *Xenopus* embryos (61). In this study, CD44 was found to be a positive regulator of Wnt pathway activation by associating with LRP6. In this model, a comparison between various CD44 splice variants showed that all isoforms of CD44 are capable of association with LRP6, but some isoforms such as CD44v6 may be associated with higher levels of Wnt activity. The Schmitt study also shows that while the extracellular domain of CD44 interacts with LRP6, the intracellular domain interacts with and coordinates a protein complex with the rest of the Wnt pathway downstream targets through cytoskeletal arrangement. HA-independence was verified in this study by utilizing hyaluronidase treatment and an anti-CD44 antibody that blocks the HA binding domain. This recent finding is significant because CD44 variant isoform expression is correlated with tumor progression in colorectal cancer and high Wnt activity, which is the signaling pathway known to control colorectal cancer progression (62–64).

A similar signaling pathway is found in hepatoma, colon adenocarcinoma, and cervical carcinoma cells, which results in the ability to scatter and spread (65). The Orian-Rousseau study illustrates the fact that CD44v6 is required for c-Met activation upon HGF stimulation. The extracellular domain of CD44v6 is required for autophosphorylation of c-Met, but the intracellular domain is required for further downstream signaling. The interactions between CD44's intracellular domain and ERM protein family members facilitate the association of GRB2 and SOS. In this way, CD44v6 serves as a linkage between the cell surface c-Met protein and the MAPK/ERK pathway members that are associated with the cytoskeleton. Recently, it was discovered that CD44v6 is also required for internalization of activated c-Met so that c-Met can be recycled or degraded (66). Hasenauer et al. showed that the Met receptor was linked to the actin cytoskeleton via CD44 and Ezrin, which allowed for endocytosis of activated Met in order to sustain regulated cell migration and branching. ERM protein family interaction with CD44 and the cytoskeleton can be disrupted by decreasing the amount of available phosphatidylinositol 4,5-bisphosphate (67). This finding can be exploited depending on the context through activation or inhibition of phospholipase C proteins, which decrease the amount of phosphatidylinositol 4,5-bisphosphate available upon activation.

Another recent novel finding is the association of CD44 with PP2A, a phosphatase known to dephosphorylate Raf, MEK, and Akt (68). In this study, EL4 T cell lymphoma cells were found to show decreased ERK phosphorylation and increased apoptosis upon ligation of CD44 with an anti-CD44 antibody that also blocks the HA binding domain. The CD44 ligation induced the binding of PP2A to the intracellular domain of CD44 as well as PP2A's activation. The activated PP2A then dephosphorylates ERK1/2 without causing its degradation, resulting in activation of the mitochondrial death pathway. It is not known if the

interaction of PP2A and CD44 requires the ERM protein family or cytoskeleton.

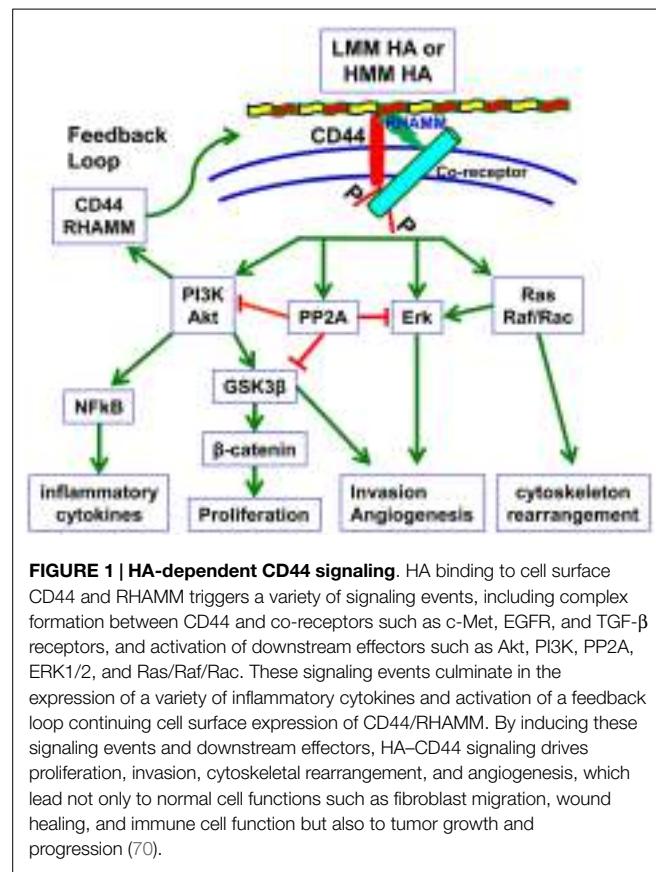
In summary, CD44 standard and variant isoforms are closely associated with many signaling pathway proteins that drive tumor development and progression in many types of cancer. These associations exist and activate signaling cascades without direct binding of CD44 to its ligand, highlighting the importance of CD44 as a co-receptor and emphasizing its importance and potential in targeted cancer therapy. The findings discussed above may not tell the complete story of HA-independent CD44 signaling. Experiments often rely on antibodies against the HA binding domain of CD44 and hyaluronidase treatment to study HA-independent CD44 signaling, but it is not possible to completely eliminate endogenous HA. Hyaluronidase treatment alone may simply cleave HA within the extracellular matrix and induce oligomer binding to CD44. Blocking antibodies compete with endogenous HA for receptor binding, and may not completely prevent HA binding; these antibodies may also cross-link CD44 on the cell surface in a similar manner to HA binding, resulting in a phenomenon that could be observed with exogenous HA. Truncated CD44 expression or use of CD44 knockout mice are two potential ways to verify HA-independence, but this proves a complicated feat to perform in cell lines that already express high levels of CD44 and human samples.

HA-Dependent Signaling

CD44 does not exist without ligand binding capabilities, and the interaction between CD44 and its major ligand HA induces signal transduction as well (Figure 1). HMM HA (>950 kDa) has been shown to promote proliferation of decidual cells during the early stages of pregnancy by activating the PI3K/Akt and ERK1/2 signaling pathways (69). Several recent studies have also focused on the function of CD44 isoforms in various types of cancer upon the addition of HA.

A recent study has shown that when fibroblasts are allowed to adhere onto a surface coated in HA; CD44, CD36, PP2A, and CDK9 are upregulated (71). PP2A expression increases in these fibroblasts, mimicking what was seen in T cell lymphoma in the Rajasagi study. The Yang study shows that natural conditions resulting from HA polysaccharides binding and cross-linking CD44 on fibroblast surfaces increase the expression of PP2A. The Rajasagi paper demonstrates PP2A association with CD44 through therapeutic intervention with anti-CD44 antibodies and no exogenous HA. One of the more thoroughly investigated aspects of HA-dependent CD44 signaling is in its role in facilitating epidermal growth factor receptor signaling.

The above two studies illustrate the fact that the signaling pathways seen in HA-dependent CD44 signaling are almost identical to those seen in HA-independent signaling. CD44 isoforms are associated with downstream pathway molecules such as PI3-kinase, SRC, SOS, and GRB2 through the ERM family (65, 72–74). This association allows for CD44 in lipid rafts to essentially bring all of the necessary signaling components of multiple pathways with them. When HA binding to CD44 occurs it may bring CD44 in close proximity to signaling receptors such as Erbb2, EGFR,



or TGF- β type 1 receptors, allowing for direct association and interaction between receptors and their signaling complexes in a tightly localized lipid raft (75–78).

Recent work has highlighted novel areas of CD44-HA interaction. A study in MDA-MB-468 breast cancer cells has shown that CD44 cross-linking and signaling upon HA binding activates c-Jun nuclear translocation (79). Activated c-Jun induces the transcription of microRNA-21, which increases the amount of Bcl-2 and upregulates inhibitors of the apoptosis family of proteins. The HA-dependent CD44 signaling also induces chemoresistance. This finding can help transition CD44 research from the more classical viewpoint that it serves as a marker without much functional role into a biomarker that has a known and exploitable function.

CD44 does not bind to HA alone. RHAMM also binds to HA in a complex with CD44 in order to facilitate signal pathway involvement. In prostate cancer cells, HA and HA fragments were shown to induce growth factor receptor signaling, including the PI3K/Akt pathway (80, 81). However, knockdown of both RHAMM and CD44 with siRNAs was required to achieve complete inhibition of HA-mediated signaling events. RHAMM, CD44, and ERK1/2 are in a signaling complex and can be co-immunoprecipitated from MDAMB231 and Ras-MCF10A breast cancer cells; this complex promotes sustained high basal motility (82). The Hamilton study also used anti-RHAMM antibodies to block MEK activation, illustrating the important of RHAMM as a co-receptor with CD44. RHAMM has also been shown to be

required for CD44-HA signaling in head and neck cancer and cementifying fibroma (77, 83).

CD44 as a Cancer Stem Cell Marker

CD44 has lately emerged in CSC or cancer initiating cell studies as a biomarker. A large number of CSC studies use CD44 standard or variant isoform expression as a typical marker (84–87). Current research is focusing on the role of CD44 signaling during epithelial–mesenchymal transition (EMT).

A recent study using mouse pancreatic tumor cells shows that CD44 is expressed on a specific population of pancreatic cancer cells (88). CD44 expression drives up-regulation of Snail1, resulting in increased MT1-MMP, which facilitates invasion. MT1-MMP is also known to directly associate with CD44 at the cell surface, where CD44 aids in the recycling of MT1-MMP so that cells can continue to degrade the extracellular matrix (89, 90).

An in-depth look at current literature for CD44 and EMT reveals that most studies use CD44 as a marker instead of a potential mechanism. A recent study shows that microRNA-106b is upregulated only in CD44⁺ gastric cancer cells undergoing EMT and that TGF- β signaling was also elevated (91). Coupled with the established link between CD44 expression and other microRNAs, as well as the relationship between CD44 and TGF- β , one could make the case for further study into the potential for CD44 expression to be the cause of this phenomenon rather than a simple marker for EMT. A recent study of a mouse mammary epithelial cell line (NMuMG) and a triple negative human breast cancer cell line MDAMB231 shows that CD44 couples with VCAM-1 in order to initiate EMT as well as chemoresistance (74). Physical association between VCAM-1 and CD44 resulted in increased expression of ABCG2, a drug efflux pump responsible for inducing resistance to chemotherapy (92).

A recent study of human primary prostate cancer tissues and lymph node metastases revealed that CD44v6 was highly upregulated in tumors and CSCs (93). Knockdown of CD44v6 with siRNA in DU145, PC3M, and LNCaP cells increased sensitivity to doxorubicin, methotrexate, docetaxel, and paclitaxel. CD44v6 knockdown also reduced tumor sphere formation and proliferation. The Ni study also showed that CD44v6 was responsible for activation of PI3K, Akt, mTOR, and Wnt signaling pathways, which were driving EMT in these cell lines.

A study by Kinugasa et al. also showed that CD44 was important for maintaining the stemness of cancer cells, but in an indirect manner (94). Cancer-associated fibroblasts from B16 melanoma tumors were found to express high levels of CD44, and co-culture of human colorectal cancer HT29 cells and human lung carcinoma LLC1 cells with these fibroblasts resulted in drug resistance, tumor sphere formation, and tumor growth. Cancer-associated fibroblasts generated from CD44 knockout mice did not result in a sustained CSC state. The pathways regulated by CD44 in these fibroblasts have yet to be determined, but the studies from Ni and Kinugasa illustrate the complex nature of CD44 signaling in cancer.

Several studies do not directly examine the role of CD44 in CSCs, but focus on its absence or presence in response to gene silencing and other treatments. When STAT3 is knocked

down in MCF7-HER2 breast cancer cells, the expression of CSC markers CD44, Oct-4, and Sox-2 was downregulated, resulting in decreased tumor sphere formation (95). Potential CD44-PI3K and CD44-ERK signaling would be eliminated in the case of CD44 downregulation, which could explain the absence of tumor sphere formation in these cells. Treatment of human T24-L bladder cancer cells with Silbilin results in a decrease in CD44 expression, spheroid colony formation, side population presence, and a reversal of EMT (96). Silbilin also inhibited β -catenin and ZEB1 signaling, possibly through inhibition of GSK3 β phosphorylation. As discussed above, CD44 is involved in the Wnt pathway and therefore β -catenin, but CD44 can also positively and negatively regulate GSK3 β (83, 97). Therefore, CD44 may be a central molecule in several signaling pathways, but many studies still examine it only as a marker of stemness.

In conclusion, changing the perspective of CD44 expression from that of a simple marker to a protein, which causes cancer growth and progression, will pave the way for future therapeutic intervention. The studies highlighted above that focused on the role of CD44 as a signaling molecule rather than a marker without a function, are a fragment of the potential research yet to be unlocked. Continued discovery of novel roles for CD44 in cancer development will help expand the mounting potential of CD44 as a prime therapeutic target.

Targeting HA Receptors

Since the discovery that HA receptor, CD44 is a stem cell marker, targeting of CD44 for anti-cancer therapy has been attempted using DNA vaccines, anti-CD44 monoclonal antibodies, and nanoparticle-mediated delivery of CD44siRNAs. In addition, HA-coated nanoparticles or anti-CD44 conjugates have been used to target CD44⁺ cells for therapy.

CD44 Vaccines

CD44 cDNA or targeting of CD44-expressing cells has been used to generate tumor immunity in experimental models. A recent study has examined the concept of “foreignizing” tumor cells by specifically delivering foreign antigens to target CD44^{hi} tumor cells using a polymeric ovalbumin (foreign antigen) and HA delivery system (98). In this study, the polymeric conjugate was accumulated on CD44^{hi} tumor cells. Furthermore, the surface class I MHC antigens on these tumor cells displayed an OVA_{257–264} peptide. When these tumor cells were injected in mice, which were immunized with a vaccinia virus expressing ovalbumin, tumor growth was reduced due to OVA_{257–264} peptide specific cytotoxic T-lymphocytes. CD44 cDNA vaccination also has been delivered by implanting virtual lymph nodes in immunocompetent animals to generate anti-CD44 antibodies. These virtual lymph nodes are generated by subcutaneous injection of a silicon tube filled with a segment of hydroxylated-polyvinyl acetate wound dressing sponge in which CD44-standard or CD44-variant cDNA is inserted. Using this model, CD44-standard form vaccination was shown to reduce autoimmune encephalomyelitis (99).

In a similar approach of using virtual lymph nodes, the effects of CD44 vaccination on tumor growth and lung metastasis were evaluated in a mouse mammary adenocarcinoma model (DA3 cells). Vaccination was achieved by injection of

virtual lymph nodes loaded with human CD44 variant (v3–10) or CD44-standard cDNAs. Immunized animals expressed antibodies against human CD44 variant and CD44-standard forms. The vaccination against CD44 variant (v3–10) was more effective than vaccination with the CD44-standard isoform in eliminating tumor growth in 75% of the vaccinated mice and slowed tumor growth in the remaining animals. Furthermore, metastasis was eliminated in all animals. Since CD44-standard form did not generate the same immunological response against tumors, it suggested that CD44 variant (v3–10) and not CD44s was functional in promoting tumor growth and metastasis in DA3 cells. It is noteworthy that in this study human CD44 (hCD44) was injected in order to break the tolerance to mouse CD44 (mCD44). Therefore, mouse CD44 (mCD44) should be injected to human patients to break CD44 tolerance, but this is not practical in clinical setting (100). The virtual lymph node and CD44 cDNA approach has also been used to induce resistance to insulin-dependent diabetes. In this study, both CD44 standard and CD44v3–v10 cDNAs induced resistance to diabetes to the same extent, and the resistance was antibody mediated (101). As in the study by Wallach-Dayan et al., this study also used human CD44 cDNA to break the self-tolerance to mouse CD44, and the use of mouse CD44 cDNA did not generate a humoral response. This may be a likely reason as to why the virtual lymph node approach, involving CD44 cDNA vaccination has not been translated into clinical trials. Another approach for CD44 vaccination involves dendritic cell vaccination. In the B16 melanoma lung metastasis model, dendritic cells were pulsed with anti-CD44 coated apoptotic B16 melanoma cells. Such opsonized B16 melanoma cells were readily endocytosed by dendritic cells. Following vaccination of mice with dendritic cells, animals were challenged with subcutaneous injection of B16 cells. In vaccinated animals, both lung metastasis (50% reduction) and tumor growth were inhibited. Moreover, 60% of the animals remained tumor-free for 8 months. In this model, vaccination-induced B16 cell-specific CD8 T cells (102).

In summary, although the high expression of CD44 in CSCs, and other cell types render CD44 as an attractive molecule for a targeted vaccine therapy, the use of CD44 vaccination has been confined to pre-clinical studies in very limited number of cancer and non-cancer models.

CD44 siRNA Delivery

In a few studies, CD44 has been targeted for therapy using specific siRNAs. A challenge with this approach is the alternatively spliced isoforms of CD44. Based on the sequence, the siRNAs may downregulate only certain CD44 variants. The most common isoforms targeted by siRNAs are CD44-standard and CD44v6. Most commonly these CD44 siRNAs have been delivered to tumor cells using nanoparticles. For example, biodegradable poly D,L-lactide-co-glycolide acid nanoparticles have been used to simultaneously deliver CD44 and FAK siRNAs to ovarian cancer xenografts. Knockdown of both genes reduced tumor growth by inhibiting angiogenesis and proliferation index in tumors (103). More recently, a nanoscale-based drug delivery system was tested to inhibit the growth of an ovarian cancer xenograft model. The nanoscale delivery system contained a modified

polypropyleniminedendrimer as a carrier, paclitaxel, a synthetic analog of luteinizing hormone-releasing hormone peptide for targeting tumor cells, and siRNA targeted to CD44 mRNA. This dendrimer was able to downregulate CD44 mRNA and protein expression and inhibit tumor growth without toxicity, suggesting that the targeted delivery of CD44 siRNA along with chemotherapeutic agents may be explored for cancer therapy (104). However, this study did not specify which CD44 isoforms were targeted by the siRNAs. Delivery of CD44 siRNAs by coating them on microneedles for self-delivery was shown to reduce the expression of CD44 in human skin xenografts in immunocompromised mice (105). Similarly, a CD44v6 targeting siRNA encapsulated into polyethylene glycol-poly-L-lysine micelles was shown to accumulate into tumor tissues and reduces tumor growth in a pancreatic xenograft model (106).

Targeting CD44 for Delivering Antitumor Therapies

Since CD44 is overexpressed in a variety of tumor cells, HA-coated self-assembling nanoparticles or liposomes have been tested for the delivery of siRNAs and/or chemotherapy drugs in pre-clinical xenograft models. The siRNAs reported so far include those specific for the multidrug resistance (MDR) protein or proteins in the apoptosis pathway (e.g., bcl-2, survivin). The advantage of CD44-targeting HA-coated nanoparticles for siRNA delivery is that these nanoparticles are biodegradable and reasonably specific to tumors. For the proper function, encapsulation and stabilization of HA-coated nanoparticles, HA has been combined with various materials including, poly-L-lysine-graft-imidazole-based polyplexes (107), lipids of varying carbon chain lengths/nitrogen content and polyamines (108), protamine sulfate interpolyelectrolyte complexes (109), near infrared dyes for imaging (110), chitosan(CS)-triphosphate (111), poly(dimethylaminoethyl methacrylate) for cross-linking of siRNAs (112) and polypropylenimine dendrimer (104). Some studies have confirmed that the uptake of the HA-coated nanoparticles or liposomes by tumor cells is mediated by HA-receptor mediated internalization. In these studies, incubation of tumor cells with soluble HA inhibited uptake of these materials and non-CD44-expressing cells did not show preference in up-taking the HA-coated liposomes when compared to the control liposomes (110, 113).

The efficacy of different HA-coated nanoparticles to deliver siRNAs has been studied in xenograft models, where the preferred model is a subcutaneous implantation of tumor cells and intravenous delivery of the nanoparticles. For example, HA-poly(ethyleneimine)/HA-poly(ethylene glycol) has been used to deliver MDR1 siRNA to OVCAR8TR (established paclitaxel resistant) tumors. Following the downregulation of P-glycoprotein, these xenografts become sensitive to paclitaxel treatment (114). HA nanoparticles have been loaded with a near infrared dye (amphiphilic carbocyanine dye that strongly absorbs and fluoresces in the near infrared region) to visualize the distribution of HA-coated nanoparticles in various tissues, by live animal imaging. In this study, intravenous delivery of the nanoparticles and imaging showed that HA-cisplatin nanoparticles had favorable safety profile for targeted delivery of cisplatin to tumors, while the HA-poly(ethyleneimine)/HA-poly(ethylene glycol) nanoparticles

were efficient in delivering the siRNAs (e.g., survivin) to cisplatin resistant but CD44-overexpressing tumors (110).

In the nanoparticles that deliver the siRNAs, polymeric compounds (e.g., polyethyleneimine or polyethyleneglycol) form ionic complexes with the siRNAs; however, these nanoparticles are not stable. To improve stability while retaining biodegradability, a HA-graft-poly(dimethylaminoethyl methacrylate) nanoparticle has been designed. In this nanoparticle, siRNAs are cross-linked via a disulfide linkage (112). These nanoparticles were shown to efficiently accumulate in the CD44-overexpressing murine melanoma tumor tissues and the cross-linked siRNAs had 50% more stability than the uncross-linked siRNAs. One study has designed nanoparticles involving HA and protamine sulfate interpolyelectrolyte complexes for delivering miR-34a to breast cancer cells *in vitro* and in the MDAMB231 subcutaneous xenografts. The nanoparticle delivery of miR-34a reduced tumor growth by 70% and showed TUNEL positive cells in tumor tissues (109).

For the delivery of anti-cancer drugs such as doxorubicin, a photochemically triggered cytosolic drug delivery system based on combining pH-responsive HA nanoparticles containing doxorubicin has been developed (98). The pH responsiveness of these nanoparticles leads to doxorubicin release, and results in significant antitumor efficacy both *in vitro* and *in vivo*. Similarly, paclitaxel loaded hyaluronate-cholanic acid nanoparticles with FlammaTM-774 fluorescent dye imaging have been used to track the targeting of these nanoparticles to tumors upon intravenous injection (115). The nanoparticles showed accumulation and retention up to day 6 in tumors (SCC7 squamous cell carcinoma model) upon intravenous injection, with little accumulation in other organs. Furthermore, the Paclitaxel loaded nanoparticles decreased tumor growth by over 60%, while free Paclitaxel at the same concentration (5 mg/kg) had little effect on tumor growth. Amphiphilic cholesteryl-succinyl hyaluronan (Chol-Suc-HA) conjugates self-assembled into docetaxel-loaded nanoparticles in the aqueous environment have been evaluated both *in vitro* and *in vivo*. While all docetaxel-loaded Chol-Suc-HA nanoparticles showed high drug loading, uniform particle size distribution and stability *in vitro*, nanoparticles with higher degree of substitution of the hydrophobic moiety had significantly more stability in plasma and antitumor efficacy in breast cancer xenografts (116). Similarly, mTOR inhibitor rapamycin chemically conjugated to HA nanoparticles via a novel sustained-release linker, 3-amino-4-methoxy-benzoic acid was found to slow down the clearance of rapamycin by 8.8-fold in immunocompetent mice bearing CD44-positive 4T1.2neu breast cancer cells. In this pre-clinical model, rapamycin conjugated HA nanoparticle inhibited tumor growth and increased survival (117).

Hyaluronic acid-coated magnetic nanoparticles have been tried for the delivery of chemotherapeutic drugs to tumors. An advantage of these nanoparticles is their tracking by magnetic resonance imaging. In an ovarian cancer model, the HA-coated superparamagnetic iron oxide nanoparticles laded with doxorubicin delayed, as well as, reduced tumor growth and increased survival (118).

In summary, HA-coated nanoparticles can be conjugated to a variety of materials for the delivery of siRNAs or chemotherapy drugs. Although it is clear that the nanoparticles are taken up by tumor cells or tissues via receptor-mediated endocytosis, it is less

clear whether the nanoparticles are taken up only through CD44-mediated endocytosis. This is because a variety of tumors also overexpress RHAMM. Since in most studies, the efficacy of these nanoparticles has been evaluated in immunocompromised mice, it is unclear what the distribution will be of these nanoparticles in an immunocompetent host. The latter is of importance since CD44 was originally known as a “lymphocyte homing receptor” and is highly expressed in both B- and T-lymphocytes. However, since the major (and probably the only) CD44 isoform expressed in lymphocytes is the standard form, siRNAs specifically directed to the variant isoforms should have the specificity for targeting tumors. Nevertheless, the long-term toxicity, stability, and tissue distribution of the various HA-coated nanoparticles will need to be evaluated in relevant hosts before these nanoparticles can be tested in clinical trials.

Targeting of CD44 Protein

The efficacy of anti-CD44 antibodies has been evaluated in murine models of autoimmune and inflammatory diseases including thrombocytopenia and arthritis. Immune thrombocytopenia is an autoimmune bleeding disorder characterized by a low platelet count and the production of anti-platelet antibodies (119, 120). While the standard treatment for immune thrombocytopenia is passive infusion of immunoglobulins, several anti-CD44 antibodies have been shown to ameliorate immune thrombocytopenia. However, some anti-CD44 antibodies (i.e., IM7, IRAWB14.4, 5035-41.1D, KM201, KM114, and KM81), which reduce serum-induced arthritis can themselves induce thrombocytopenia in murine models (120). It has been suggested that since CD44 is not expressed in human platelets, anti-CD44 antibodies should not induce thrombocytopenia in patients. A fully humanized rat anti-CD44 monoclonal antibody, PF-03475952 was found to cause a dose-dependent decrease in symptoms in a mouse model of collagen-induced arthritis. This monoclonal antibody was found to be safe in pharmacological assays. The antibody was suggested as a treatment for inflammatory diseases such as rheumatoid arthritis (121); however, no further studies were conducted on this antibody and the antibody does not appear to have entered in clinical trials. In a genome-wide association study, CD44 was found to be a functionally associated gene in type 2 diabetes patients (122). The same study found that intraperitoneal administration of an anti-CD44 antibody in a murine model of high fat diet induced type 2 diabetes, decreased blood glucose levels, and macrophage infiltration in adipose tissues (123). Furthermore, daily injection of the anti-CD44 antibody decreased blood glucose levels, weight gain, liver steatosis, and insulin resistance to the levels lower than anti-diabetes drugs metformin and pioglitazone (122).

Anti-CD44 antibodies have also been evaluated as an anti-cancer therapeutic. In chronic lymphocytic leukemia cells, which express high levels of CD44, a humanized monoclonal antibody specific for CD44 (RG7356) was found to be cytotoxic to leukemia B cells without affecting the viability of normal B cells. Systemic administration of this antibody caused complete clearance of leukemia xenografts. Interestingly, the effects of the antibody were not neutralized in the presence of HA, suggesting that CD44 may have functions other than binding to HA, which play a role in leukemia (124). The bio-distribution of the same antibody has

been evaluated in the CD44(+) and CD44(−) xenograft bearing mice and normal cynomolgus monkeys, following radiolabeling with ^{(89)Zr} (125). The study found that while the uptake of the ^{(89)Zr}-RG7356 antibody in CD44⁺ xenografts was ~9-fold higher than in CD44(−) xenograft, the uptake in CD44(+) xenograft was similar, regardless of whether the xenograft was responsive or non-responsive to the anti-CD44 antibody. Similarly, the antibody was detected in the spleen, salivary glands, and bone marrow, most likely because these organs express high levels of CD44 (125). Therefore, for targeting tumors a large dose of an anti-CD44 antibody might be needed and furthermore, simply the presence of the antibody in tumors may not be indicative of its efficacy. In pancreatic cancer, where CD44 expression correlates with poor prognosis, intravenous delivery of an anti-CD44 monoclonal antibody H4C4 has been shown to completely inhibit tumor growth and metastasis in two different pancreatic xenograft models. In the same study, H4C4 was also able to eliminate tumor initiating cells, as well as, tumor recurrence following radiation treatment. This suggests that targeting of CD44-overexpressing pancreatic CSCs may improve outcome in pancreatic cancer patients who undergo radiation therapy (126).

In addition to the therapeutic uses of various anti-CD44 antibodies, a study has used an anti-CD44 antibody for tumor imaging. For example, a chimeric monoclonal antibody U36 and its F(ab')2 and Fab' fragments that recognize the CD44v6 isoform have shown potential for radioimmuno-therapy and radioimmuno-targeting of experimental tumors (127, 128). A ^{99m}Tc-labeled U36, when used in conjugation with a single-photon emission computed tomography has been shown to detect all primary tumors of head and neck squamous cell carcinoma. In clinical trials, this antibody labeled with ¹⁸⁶Re-labeled could detect up to 66% of breast cancer lesions (129). However, these techniques do not visualize micro-tumors, tumor nodes with necrosis, or tumors containing keratin or fibrin. Another humanized anti-CD44v6 antibody VFF18 labeled with a near infrared dye IRDye800Cw has shown promise in tracking ductal carcinoma *in situ* in mouse xenografts (130).

As in the case of HA-coated nanoparticles or liposomes, anti-CD44 antibody-drug conjugates have also been used either for imaging of tumors or for delivering chemotherapeutic agents to experimental tumor models. For example, anti-CD44 antibody conjugates have been used to deliver radioisotopes or mertansine for the treatment of CD44-expressing tumors. In these studies, disease stabilization was observed in breast or head and neck tumor patients; however, dose-limiting toxicity was observed along with the distribution of the antibody in the skin, where high levels of CD44 are expressed (131). In phase I studies, maximum tolerated dose, safety, and efficacy of an immuno-conjugate BIWI 1 (bavituzumabmertansine), consisting of a highly potent anti-microtubule agent coupled to an anti-CD44v6 monoclonal antibody, was evaluated in head and neck cancer patients. In this study, while three patients showed a partial response, the binding of BIWI to CD44v6 on skin keratinocytes caused serious skin toxicity with a fatal outcome, leading to early termination of this trial (132). More recently, nanoparticles and liposomes containing an anti-CD44 antibody, as well as, imaging reagents (e.g., cDNAs

for monomeric red fluorescence protein or luciferase) have been used to target tumor detection and imaging (86). Anti-CD44v6 single chain variable fragment [scFv(CD44v6)] screened out from the human phage-display library has been used for the targeting of arsenite ion (As) encapsulated nanoparticles to CD44-positive cells. Upon intravenous delivery, these nanoparticles specifically accumulated in the PANC-1 tumor xenografts for up to 2 days and completely inhibited tumor growth (133). However, none of these reagents have been evaluated in clinical trials.

The HA binding domain is highly conserved in all CD44 isoforms and, therefore, attempts have been made to target this domain using specific mono-thiophosphate-modified aptamers. These aptamers bind specifically to CD44-expressing tumor cells with high efficacy. However, the *in vivo* bioavailability of these aptamers has not been examined in detail (134).

In summary, targeting of CD44 by siRNAs or antibodies for therapy and the use of HA-coated nanoparticles or of anti-CD44 antibody conjugates for the delivery of therapeutic agents have been examined as attractive strategies in the treatment of cancer and chronic diseases. In addition, anti-CD44 antibodies have also been used for imaging purposes. However, the majority of the studies are limited to pre-clinical models. In limited clinical studies, the use of anti-CD44 antibodies has resulted in significant toxicity. In addition, the HA-coated nanoparticles may also deliver the cargo to tissues, which express other HA receptors. Therefore, treatment and imaging strategies that target CD44 will have to be carefully evaluated for their effects on normal cells, and the immune system. Furthermore, the risk versus benefit must be carefully evaluated before CD44-targeting strategies are translated to the clinic.

Conclusion

CD44 is a transmembrane protein with a variety of functions depending on its ligand binding, co-receptor associations, and cytoskeletal interaction. The normal functions of LMM HA binding with CD44 coupled with CD44's inherent interactions with secondary signaling complexes allow for wound healing, modulation of the immune system, and developmental angiogenesis. Irregular or prolonged CD44 and CD44-HA interaction, however, can lead to fibrosis, scarring, immunopathology, and tumor growth. The high expression of CD44 and high production of HA in tumor environments make them both prime targets for therapeutic intervention. The presence of CD44 at high concentrations in the skin and other tissues and the necessity of HA production for proper wound healing complicates potential clinical intervention strategies. Further understanding of the complexities of CD44 HA-dependent and independent signaling are required for developing highly specific targeted therapeutics that avoid the previously seen serious adverse reactions.

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CD44, hyaluronan, the hematopoietic stem cell, and leukemia-initiating cells

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CD44 is an adhesion molecule that varies in size due to glycosylation and insertion of so-called variant exon products. The CD44 standard isoform (CD44s) is highly expressed in many cells and most abundantly in cells of the hematopoietic system, whereas expression of CD44 variant isoforms (CD44v) is more restricted. CD44s and CD44v are known as stem cell markers, first described for hematopoietic stem cells and later on confirmed for cancer- and leukemia-initiating cells. Importantly, both abundantly expressed CD44s as well as CD44v actively contribute to the maintenance of stem cell features, like generating and embedding in a niche, homing into the niche, maintenance of quiescence, and relative apoptosis resistance. This is surprising, as CD44 is not a master stem cell gene. I here will discuss that the functional contribution of CD44 relies on its particular communication skills with neighboring molecules, adjacent cells and, last not least, the surrounding matrix. In fact, it is the interaction of the hyaluronan receptor CD44 with its prime ligand, which strongly assists stem cells to fulfill their special and demanding tasks. Recent fundamental progress in support of this "old" hypothesis, which may soon pave the way for most promising new therapeutics, is presented for both hematopoietic stem cell and leukemia-initiating cell. The contribution of CD44 to the generation of a stem cell niche, to homing of stem cells in their niche, to stem cell quiescence and apoptosis resistance will be in focus.

Keywords: CD44, hematopoietic stem cells, leukemia-initiating cells, bone marrow niche, homing, adhesion, dormancy, apoptosis resistance

Introduction

CD44, first described as a lymphocyte homing receptor (1), is expressed by a wide range of hematopoietic and non-hematopoietic cells (2). Interest in CD44 increased considerably, when it was noted that the insertion of alternatively spliced exon products in the CD44 standard or CD44 hematopoietic isoform (CD44s) strikingly affects the molecules function, such that expression of CD44 variant isoforms (CD44v) induces a metastatic phenotype in locally growing tumor cells (2, 3).

Abbreviations: ASC, adult stem cells; bFGF, basic fibroblast growth factor; BM, bone marrow; BMP, bone morphogenetic protein; BM-Str, BM stroma cells; C, complement; CD44s, CD44 standard isoform; CD44v, CD44 variant isoforms; CIC, cancer initiating cells; ECM, extracellular matrix; ERM, ezrin, radixin, moesin; ESC, embryonic SC; FN, fibronectin; GAG, glucosaminoglycan; GEM, glycolipid enriched membrane microdomains; HA, hyaluronic acid; HAS, hyaluronan synthase; HGF, hepatocyte growth factor; HSC, hematopoietic SC; ICD, intracellular domain; kd, knockdown; ko, knockout; LIC, leukemia initiating cells; MSC, mesenchymal SC; M ϕ , macrophages; OPN, osteopontin; PCCD4, programmed cell death 4; PDGFR, platelet-derived growth factor receptor; RTK, receptor tyrosine kinase; TGF, transforming growth factor; TPO, thrombopoietin; VEGF, vascular endothelial growth factor; wt, wild type.

At the time, it was surprising that a leukocyte marker is engaged in solid tumor metastasis formation. As the hematopoietic system is the only organ that components repeatedly shift between sessile and mobile states, we argued that metastasizing tumor cells may transiently take over part of the program of hematopoietic cells and that this programmatic shift, which is independent of oncogene transformation, depends to a considerable degree on CD44 and its activities (4). This hypothesis received strong support by the recovery of cancer- and leukemia-initiating cells (CIC/LIC), which are defined by their capacity to take over part of the program of stem cells (SC). In fact, CD44s/CD44v are CIC/LIC markers (5, 6) and, most importantly, CD44 was the first marker defined as a CIC/LIC biomarker. This implies that CD44 is engaged in fulfilling special SC-related tasks in CIC/LIC (7). These particular tasks include, besides growth upon serial transplantation in xenogeneic models, self-renewal and recapitulation of the heterogeneous phenotype of the parental tumor, reflecting the differentiation capacity of CIC/LIC. It also includes, at least for a subset of CIC/LIC, the capacity of SC to transiently shift from a sessile toward a mobile state, which is required for metastasis formation (7–9). Furthermore, like SC, CIC/LIC are highly apoptosis resistant (6, 7) and may profit from the crosstalk with the surrounding (5, 6). Notably, too, CIC/LIC are heterogeneous (10) and genetically unstable (11). This is in line with their disputed, not mutually exclusive origin from adult stem cells (ASC), from oncogene-transformed committed progenitors or from cell fusion particularly with macrophages (Mφ) (12). Despite their heterogeneous origin, CIC/LIC share many features with hematopoietic SC (HSC), like relative quiescence, longevity, drug resistance, and support by the surrounding that for SC is called the niche. Finally, there is strong evidence that CD44/CD44v is engaged in many of the activities, which CIC/LIC share with ASC (13).

CD44 is a quite abundant expressed molecule. Thus, the question arises, what qualifies CD44 for this multitude of very special tasks. This review outlines that two features of CD44 mostly account for the molecule's contribution to SC maintenance: first and most important, CD44 crosstalks with the surrounding/the niche. Second, CD44 is located in membrane subdomains, which are particularly prone for collecting signal transduction molecules, proteases, and cytoskeletal components, and foster concerted activities. HSC and LIC were chosen as prominent examples. Based on the largely overlapping activities of CD44 in CIC and LIC, some references to CIC are included, as far as deeper insight was gained with the latter.

CD44 Structure and Ligands

CD44 are glycoproteins encoded by a single gene (14). CD44 molecules vary in size due to *N*- and *O*-glycosylation (15, 16) and insertion of alternatively spliced exon products in the extracellular domains of the molecule (17). The smallest, hematopoietic isoform (CD44s) is present on the membrane of most vertebrate cells (3). CD44 has seven extracellular domains, a transmembrane, and a cytoplasmic domain (18). The latter is encoded by exons 9 or 10 (19). Between domains 5 and 6 up to 10, variant exon products can be inserted by alternative splicing (15).

CD44 is a member of the family of cartilage link proteins (15, 16). The *N*-terminal region forms a globular structure. Conserved cysteins are important for the stability of the extracellular domain, and two cysteins in the flanking region account for correct link domain folding (19). This globular structure contains binding sites (AA 32–132) for collagen, laminin, fibronectin (FN), and cell surface receptors like E-selectin and L-selectin (20–22). Importantly, CD44 also is the major receptor for hyaluronan (HA) (23). HA binds to a basic motif (AA 150–158) within the globular structure, but outside of the link domain (23, 24). Though the HA binding motif is present in all CD44 isoforms, not all CD44⁺ cells bind HA. However, HA-binding can be induced by CD44 cross-linking, which indicates that HA-binding depends on conformational changes or a redistribution of CD44 in the cell membrane (25). CD44 also has two binding sites for other glycosaminoglycans (GAG) (26). The *N*-terminal globular domain is followed by a stretch of 46 AA, which comprises exon products 5–7. This stretch of 46 AA forms a stalk like structure (27). It is heavily glycosylated and contains putative proteolytic cleavage sites (28). Variable exon products are inserted in the stalk like region (29). The transmembrane region supports CD44 oligomerisation and contributes to incorporation in glycolipid-enriched membrane microdomains (GEM) (30). The cytoplasmic tail of CD44 contains binding sites for the cytoskeletal proteins ankyrin and ezrin, radixin, moesin (ERM). Ankyrin mediates contact with spectrin and is involved in HA-dependent adhesion and motility (31). ERM proteins are engaged in regulating migration, cell shape, and protein resorting in the plasma membrane (32). The *N*-terminus of activated ERM proteins binds to a motif between the transmembrane region and the ankyrin binding site, and their C-terminus binds to F-actin. Thereby, ERM proteins link CD44 to the actin cytoskeleton (33). Merlin, an additional ERM family member, which lacks the actin-binding domain, might be involved in stabilizing the junctional-cortical actin interface through its *N*-terminal domain (34). The binding of CD44 to cytoskeletal linker proteins influences signaling pathways downstream of CD44, which expands the range of CD44-mediated functions.

Finally, CD44 O-glycosylation, the transmembrane region, and the cytoplasmic tail affect the membrane subdomain localization. Depending on the activation state, CD44 is recruited into GEM (35), which has great bearing on the interaction of CD44 with extracellular ligands and the association with other transmembrane and cytoplasmic molecules (36, 37). These associations are most crucial for the accessory functions of CD44 in migration and signal transduction. This is a sequel of the inner membrane side organization of GEM, which favors harboring adapter and signal transducing molecules like src family members (38). Some of these cytoplasmic adapter and signaling molecules are constitutively associated with GEM-located CD44 (39). GEM are also prone for internalization (40).

Unlike CD44s, CD44v is only expressed on subpopulations of epithelial and hematopoietic cells, particularly during embryonic development, hematopoietic cell maturation and activation, and in some carcinoma and leukemia with a tendency toward over-expression in CIC/LIC (41, 42). Several of the CD44v exon products can contain specific post-translational modifications. These include a heparan-sulfate site in exon v3, which serves for the

binding of heparin-binding proteins like basic fibroblast growth factor (bFGF) (43); CD44v6 contains a binding site for hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), and osteopontin (OPN) (44–46). OPN also binds to CD44v10 (47). Via these cytokine/chemokine binding sites, CD44v takes over a central and coordinating role in receptor tyrosine kinase (RTK) activation (48).

Briefly, CD44 is the major HA receptor. It has a multitude of additional ligands and associates with transmembrane and cytoplasmic molecules. This is due to several glycosylation sites, variant exon product sequences, the insertion into GEM, and the cytoplasmic tail structure. Noteworthy, HA binding contributes to the GEM recruitment of CD44. Beyond forcing CD44 associations, this has bearing on CD44 internalization.

HSC/LIC CD44 and Stem Cell Genes

CD44 is a marker of ASC, including HSC and of a large range of CIC and LIC (7, 49). In addition, there is some evidence for pronounced CD44v expression in SC (50). Thus, the question arose, whether CD44 is engaged in stem cell gene expression and/or whether CD44/CD44v expression is regulated by stem cell genes.

Embryonic SC (ESC) are characterized by expression of a set of master SC transcription factors, Oct4, Sox2, and Nanog (51), as well as distinct chromatin organization and epigenetic signatures, which govern the intrinsic ability to self renew and to differentiate into multiple lineages (52). Polycomb genes, which have a role in transcriptional repression through histone modification, associate with the promoter and regulatory regions of target genes in ESC. Expression of master gene transcription factors and epigenetic regulation are maintained in HSC and LIC (53, 54), which share with ESC Oct4, Nanog, and Myc overexpression (55, 56), and Notch, Wnt, and Hedgehog signaling pathways, important in shaping tissue structure, cell fate, and identity (57). In fact, leukemia recurrence was prevented by deletion of the polycomb gene *Bmi1*, important for HSC self-renewal (58).

As discussed below in concern of HSC quiescence, there are links between CD44, Nanog, and Myc expression, and CD44 is a target of the Wnt and Notch pathways in HSC and LIC (59–61). However, there is no evidence that CD44 plays a central role in regulating master gene transcription factors in HSC/LIC (62–64).

Besides master SC transcription factors, miRNA were recognized as key regulators of self-renewal and SC fate (65). This includes hematopoiesis, HSC being lost upon abrogation of Dicer, which was ascribed to miR-125a (66). Additional miRNA overexpressed in HSC either promote HSC engraftment (miR-125b-5p, miR-126-3p, miR-155) or are disadvantageous (miR-196b, miR-181c, miR-542-5p, let7e) (67). Notably, miRNA profiles differ significantly between HSC and lineage committed progenitors, and miRNA profiles in hematological malignancies differ from those of HSC and progenitor cells. In addition, miRNA profiles are selective for distinct leukemia [review in Ref. (68–72)]. Nonetheless, there are some common trends: miR-15a, miR-29b, miR-34a, miR-151, and miR-204 frequently act as tumor suppressors, and miR-155, miR-96, miR-24, miR-21, miR-32, miR-106-25, and let-7 as oncomir (73). However, the engagement of HA/CD44 on

miRNA regulation in HSC and LIC remains to be elaborated in detail. So far, there are only sporadic hints toward a mutual impact.

HA-crosslinked CD44v3 binds Nanog, Oct4, and Sox2, which promotes miR-302 expression (74) a key player in controlling SC self-renewal and pluripotency (75). Also, binding of HER2 to CD44 leads to upregulation of MTA-1 (metastasis-associated-1), which induces silencing of the miR-139 promoter, accompanied by increased CXCR4 expression (76). HA-CD44v6 binding promotes PKC ϵ activation, and this increases Nanog phosphorylation and nuclear translocation, where Nanog associates with Drosha and an RNA helicase p68, which leads to oncogenic miRNA-21 transcription and a reduction in the expression of the tumor suppressor programmed cell death 4 (PCD4) (77). CD44v6-associated overexpression of miR-21 (78) induces pre-B-cell lymphoma (79), and is frequently observed in CML (80). Analyzing the impact of CD44v6 on the miRNA profile in metastasizing CIC (81) revealed CD44v6-dependent downregulation of the tumor suppressors let-7b, let-7d, let-7e, miR-101, and miR-34a. The latter, which suppresses tumor growth by CD44 downregulation (82), is abundantly expressed in CD44v6 knockdown (CD44v6^{kd}) cells, which argues for CD44v6 to be engaged in miR-34a silencing. On the other hand, metastasis-promoting miR-494 and miR-21 and apoptosis-regulating miR-24-1 (83–85) are abundant only in CD44v6-competent cells. miRNA transcription and/or posttranscriptional regulation also were affected by CD44v6-associated MET (86), which supports miR-103 transcription (87). MiR-103 expression was only high in CD44v6-competent cells.

We are also far away from a comprehensive view on the regulation of CD44 via miRNA. MiR-199a binding to the CD44 3'-UTR suppresses tumorigenicity, multidrug resistance, and migration (88, 89). The CD44 3'-UTR binds additional miRNA that target extracellular matrix (ECM) mRNA, like miR-328, miR-491, miR-671, and miR-512-3p. In fact, transfection-induced CD44 3'-UTR overexpression is accompanied by collagen I and FN upregulation (90). However, stressing the need for further studies, opposing findings have also been reported, such as downregulation of CD44 by pro-metastatic miR-373/520c (91).

Finally, aberrant and alternative splicing is frequently observed in CIC/LIC, and CD44 ranks first in the affected genes (92). However, no mutations were found in *cis* acting CD44 splice elements (93). Thus, a genetic basis for CD44 alternative splicing in malignancies remains questionable.

Taken together, though links between CD44 and master SC genes, dominating SC signaling pathways, and epigenetic regulation of SC genes were described, HSC do not essentially depend on CD44. This could have been expected, as HSC are not or not seriously affected in panCD44^{ko} (94), CD44v10^{ko} (95), CD44v7^{ko}, or CD44v6/v7^{ko} (96–98) mice.

On the other hand, it is already known since 1990 that CD44 is required for the development and maintenance of early hematopoietic progenitors. In long-term bone marrow (BM) cultures, tightly packed clusters of small cells, so called cobble stone areas, develop below a stroma layer. These cobble stones contain cells with the capacity for long-term reconstitution. When cultures contain anti-CD44, HSC clusters do not develop (99). Furthermore, CD44 is a reliable LIC marker in many malignancies (100),

and the first LIC biomarker that blockade severely affected LIC maintenance, e.g., anti-CD44 drives LIC into apoptosis (101, 102). Thus, the essential contribution of CD44 relies on the communication of SC/HSC and LIC with the surrounding. In the following sections, those features of HSC are discussed that depend on or are modulated by the surrounding. This includes the requirement for a niche to maintain quiescence and to receive signals that drive out of quiescence toward differentiation. The latter frequently is associated with changes in motility. Finally, HSC are relatively apoptosis resistant. It also will be discussed, where LIC, which resemble HSC in many respects, become less dependent on the surrounding or respond differently due to the oncogenic transformation.

The Endosteal Niche

The fate of a cell in the developing organism is determined by its position (103, 104). SC reside in specialized locations, the niches, which minutely regulate their activity (105). Niches are composed of epithelial and mesenchymal cells and extracellular substrates. They govern location, adhesiveness, retention, homing, mobilization, quiescence and activation, symmetric and asymmetric division, and differentiation (106). Accordingly, a niche might prevent tumorigenesis, which would argue against CIC/LIC profiting from a niche. However, there is ample evidence that a preformed niche supports CIC/LIC survival and homing (105) and regulates the balance between quiescence and growth (107). Beyond this, a niche can support reprogramming of non-CIC toward CIC by exposing them to an embryonic microenvironment (108). CD44 plays a central role in the crosstalk between SC/malignant SC and the niche, which includes an active contribution of CD44 in niche assembly.

The Composition of HSC and LIC Niches

A niche for HCS, where they receive instructions particularly in respect to their lifelong capacity for self-renewal, was first proposed by Schofield in 1978 (109). Only 25 years later, it was uncovered that osteoblasts lining the surface of the bone play a major role (110). Additional cellular components of the endosteal niche are mesenchymal stem cells (MSC), osteoclasts, Mφ, fibroblasts, and adipocytes (111, 112). Interestingly, MSC, too, are influenced by their surrounding. Thus, it was expected that MSC from different tissue fulfill equivalent biological activities. On the contrary, when implanting MSC from BM, white adipose tissue, umbilical cord or skin, only BM-derived MSC spontaneously formed a BM cavity, which was progressively replaced by hematopoietic tissue and bone and permitted homing and maintenance of long-term murine and human HSC (113). Matrix components of the endosteal niche are HA, FN, laminin, and collagen that are secreted by endosteal niche cells and support HSC adhesion, quiescence, and self-renewal. Prominent cytokines and chemokines secreted by BM stroma cells (BM-Str) and/or captured by the BM stroma are thrombopoietin (TPO), SDF1, OPN, and parathyroid hormone. TPO promotes HSC quiescence (114). SDF1 supports quiescence and affects apoptosis resistance (115). OPN is engaged in lodgment to the endosteum (116), and parathyroid hormone supports trabecular network formation of osteoblasts and HSC expansion (117).

Hematopoietic SC avail on a second niche, the vascular niche, which is located in proximity to endothelial cells (118). Though components and activities of the endosteal and the vascular niche are partly overlapping (119), distinct to the endosteal niche, the vascular niche plays a major role in HSC homing and hematopoietic progenitor egress. The vascular niche also supports hematopoietic progenitor expansion and maturation. In line with these special duties, reticular cells in the vascular niche express IL6, HGF, OPN, and SDF1 at high or higher levels than cells in the osteogenic niche (120).

Thus, possibly distinct to ASC in solid organs, HSC dispose of two niches. The requirement for two niches might be linked to the general feature of cells of the hematopoietic system that are not sessile and circulate through the body to fulfill upon request their tasks *in loco*, and thereafter patrol again through the organism. Noteworthy, HSC/LIC CD44 contributes to the establishment of both BM niches.

CD44 Contributes to the Generation of the BM Niches

The Contribution of CD44 to Matrix Assembly

Stem cells niches, including the osteogenic niche in the BM, are particularly rich in HA (121). HSC synthesize and express HA, and HA expression correlates with HSC adhesion to the endosteal niche (122). Similarly, CD44 contributes to building an HA coat on endothelial cells, which facilitates binding of mobilized HSC to endothelial cells as well as HSC homing (123, 124). Furthermore, perturbation in matrix components alters cell shape and intracellular tension, which results in shifts in signaling events that affect gene expression (125). This could be particularly important in the BM niche, where HA delivery by HSC/LIC can induce expression of HA in niche cells (126). Furthermore, CD44 is involved in matrix assembly (127) such that the HA-CD44 association modifies the matrix to support colonization (128).

In concern about CD44v, there is evidence for an engagement of CD44v6 in matrix assembly. A CD44v6 knockdown (^{kd}) in a highly metastatic tumor line revealed a striking reduction in metastatic capacity, which was, at least, partly due to an altered tumor matrix (81). CD44v6^{kd} cells secrete a matrix not supporting adhesion of CD44v^{wt} or CD44v6^{kd} cells, whereas both cells readily adhere to the CD44v^{wt}-matrix. In fact, HA synthase 3 (HAS3) expression is strongly reduced in CD44v6^{kd} cells (86), where high HAS3 expression frequently correlates with aggressiveness of carcinoma and leukemia (129). On the opposite, the CD44v6^{kd} cells abundantly secrete hyaluronidase such that the matrix contains a lower amount of HA and exclusively low molecular weight HA (130), which significantly affects adhesion and the catcher activity of the matrix (Figure 1A).

Briefly, HSC CD44 contributes to the generation of HSC niches mostly via HA provision, where the composition of HA varies depending on the expression of CD44v isoforms. CD44/CD44v strengthens HAS3 expression and, by not yet defined mechanisms, prohibits hyaluronidase activity.

CD44 Contributes to the Catcher Activity of the Niche Matrix

CD44 is a transmembrane proteoglycan, which allows for the local concentration of glycosaminoglycan-associating proteins (131).

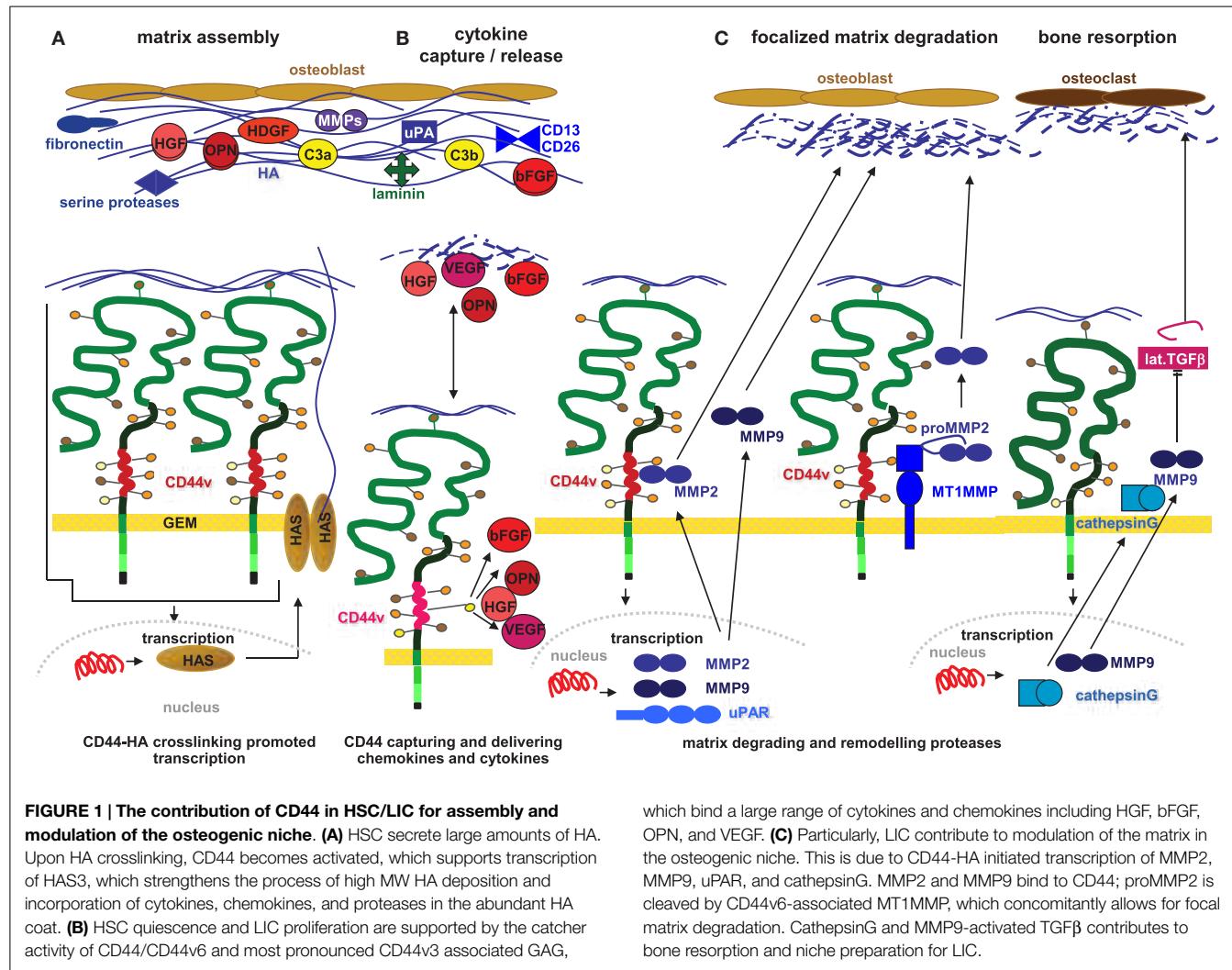


FIGURE 1 | The contribution of CD44 in HSC/LIC for assembly and modulation of the osteogenic niche. **(A)** HSC secrete large amounts of HA. Upon HA crosslinking, CD44 becomes activated, which supports transcription of HAS3, which strengthens the process of high MW HA deposition and incorporation of cytokines, chemokines, and proteases in the abundant HA coat. **(B)** HSC quiescence and LIC proliferation are supported by the catcher activity of CD44/CD44v6 and most pronounced CD44v3 associated GAG,

which bind a large range of cytokines and chemokines including HGF, bFGF, OPN, and VEGF. **(C)** Particularly, LIC contribute to modulation of the matrix in the osteogenic niche. This is due to CD44-HA initiated transcription of MMP2, MMP9, uPAR, and cathepsinG. MMP2 and MMP9 bind to CD44; proMMP2 is cleaved by CD44v6-associated MT1MMP, which concomitantly allows for focal matrix degradation. CathepsinG and MMP9-activated TGF β contributes to bone resorption and niche preparation for LIC.

Of special interest for HSC and LIC is the binding of OPN to CD44v3, CD44v6, and CD44v10 (47, 132, 133), where OPN secretion is further stimulated by HA (134). OPN is chemotactic and haptotactic, and as such important for the recruitment of HSC into the niche (135). On the other hand, the OPN-CD44 interaction exerts a feedback on the donor cell, which supports migration. Thus, $p53^{ko}$ CD44 ko mice have the same rate of primary tumor development as $p53^{ko}$ mice, but tumors do not metastasize (136). Similarly, a blockade of CD44v10 strongly reduced OPN delivery by leukemic cells, which was accompanied by pronounced retention of HSC in the niche (137). CD44v6 also binds VEGF and HGF (44, 45, 138, 139). In the hypoxic environment of the osteogenic niche, HIF1 α acts as a regulator to prevent HSC proliferation and exhaustion, where it is supported by VEGF, a target of HIF1 α (140). Instead, leukemic cells, which were supported by VEGF-activated endothelial cells in the vascular niche, gain in cytotoxic drug resistance (141). In concern about HGF, it is worthwhile noting that a subpopulation of HSC responds to HGF by migrating toward skeletal muscles (142). CD44v3 binds bFGF that stimulates proliferation of underlying mesenchymal cells in the developing limb and affects BM MSC (143, 144). This might

be due to bFGF inducing changes in HAS and hyaluronidase isoform expression (145). A direct contribution of CD44v3-bound bFGF to the activity of bone MSC remains to be explored (**Figure 1B**).

Finally, CD44v6 can directly contribute to the composition of the niche matrix (130). CD44v6 supports transcription of hepatoma-derived growth factor, which stimulates the growth of fibroblasts, endothelial cells, and vascular smooth muscle cells, and recruits MSC (146). CD44v6 also promotes clusterin secretion that influences chemokine secretion and initiates stromal changes affecting intercellular communications (147). In addition, the complement (C) components 3a and 3b are absent in a CD44v6 kd matrix, but are abundantly delivered by CD44v6 wt cells (86). These findings are well in line with the innate immune system, particularly C3, cooperating with CD44 in HSC to strengthen the HSC CD44 – niche interaction [review in Ref. (148)] (**Figure 1A**).

CD44 Modulates HSC Niches

CD44 concentrates MMPs at the cell surface, where the production of uPAR, MMP2, and MMP9 is concomitantly stimulated by

the interaction between HA and CD44 (86, 149). MMP9 transcription is actively supported by the CD44 intracellular domain (ICD), which binds to a MMP9 promoter response element (150). By a not yet defined mechanism, CD44v6 also is involved in uPAR transcription (86). CD44 aggregation via HA binding facilitates MMP binding (150). Furthermore, proMMP2 becomes activated through CD44v-associated MMP14, which is located in the leading lamella. As cell-bound MMPs are protected from their inhibitors, this allows for focal degradation of the ECM to form space for invading LIC (151). LIC also stimulate osteoclasts to secrete cathepsinG and MMP9 to resorb bone to create a niche. CathepsinG, primarily secreted by osteoclasts (152), is another transcriptional target of HA-CD44 signaling (153). Transforming growth factor β (TGF β) activation through CD44-associated MMP9 promotes angiogenesis, invasion (154), and enhances osteoclast activity and bone resorption (155) (**Figure 1C**).

Taken together, HSC require a niche and CD44 contributes to niche assembly. The most prominent CD44 contribution relies on the stimulation of HA provision via pronounced HAS activation. CD44 also supports retaining growth factors and chemokines that are supplied by the different niche elements. This facilitates message delivery from the niche toward the HSC/LIC. There is evidence for a contribution of CD44v particularly in cytokine/chemokine retention. It remains to be explored whether this provides a pronounced profit from the niche for CD44v expressing LIC. CD44 also contributes to modulating the niche by hyaluronidases and proteases that transcription is promoted by CD44 or that become activated via direct or indirect associations with CD44. There is no evidence that LIC contribute to establishing a niche. Rather, LIC are suggested to make use of the HSC niche. It is still disputed whether LIC displace HSC from their niche or actively remodel/destroy the niche (156), such that HSC die by neglect. High hyaluronidase secretion by CD44v6 $^{+}$ LIC could favor the latter.

CD44 Supports Adhesion, Homing, and Migration of HSC and LIC

CD44, HSC, and LIC Adhesion to the Bone Marrow Stroma

One of the prime functions of the osteogenic niche is the retention of HSC to instruct for longevity and quiescence, which requires firm HSC adhesion. This task is mainly taken over by HA (157). HA binding initiates or, at least, influences most activities of CD44 (158). The importance of CD44 as an adhesion molecule for HSC and LIC has been amply demonstrated (157, 159). The particular engagement of the CD44-HA interaction was confirmed by the finding that HSC adhesion can be blocked by anti-CD44, soluble HA, or hyaluronidase (160).

CD44 adhesion to its ligand(s) induces up-regulation of additional adhesion molecules, mostly integrins, which strengthen HSC adhesion (161). This was intensely explored for the association of CD44 with $\alpha 4\beta 1$ (162, 163). Upon activation by HA adhesion, the two molecules directly associate (164), which is accompanied by $\alpha 4\beta 1$ -promoted stronger adhesion to FN and laminin (165). HSC $\alpha 4\beta 1$ additionally supports the direct contact with stroma cells via ICAM1 binding (166). In line with the contribution of CD44 and $\alpha 4\beta 1$ to the adhesion of HSC to the

osteogenic niche, anti-CD44 and anti- $\alpha 4\beta 1$ dislodge HSC from the BM niche (167, 168) (**Figure 2A**).

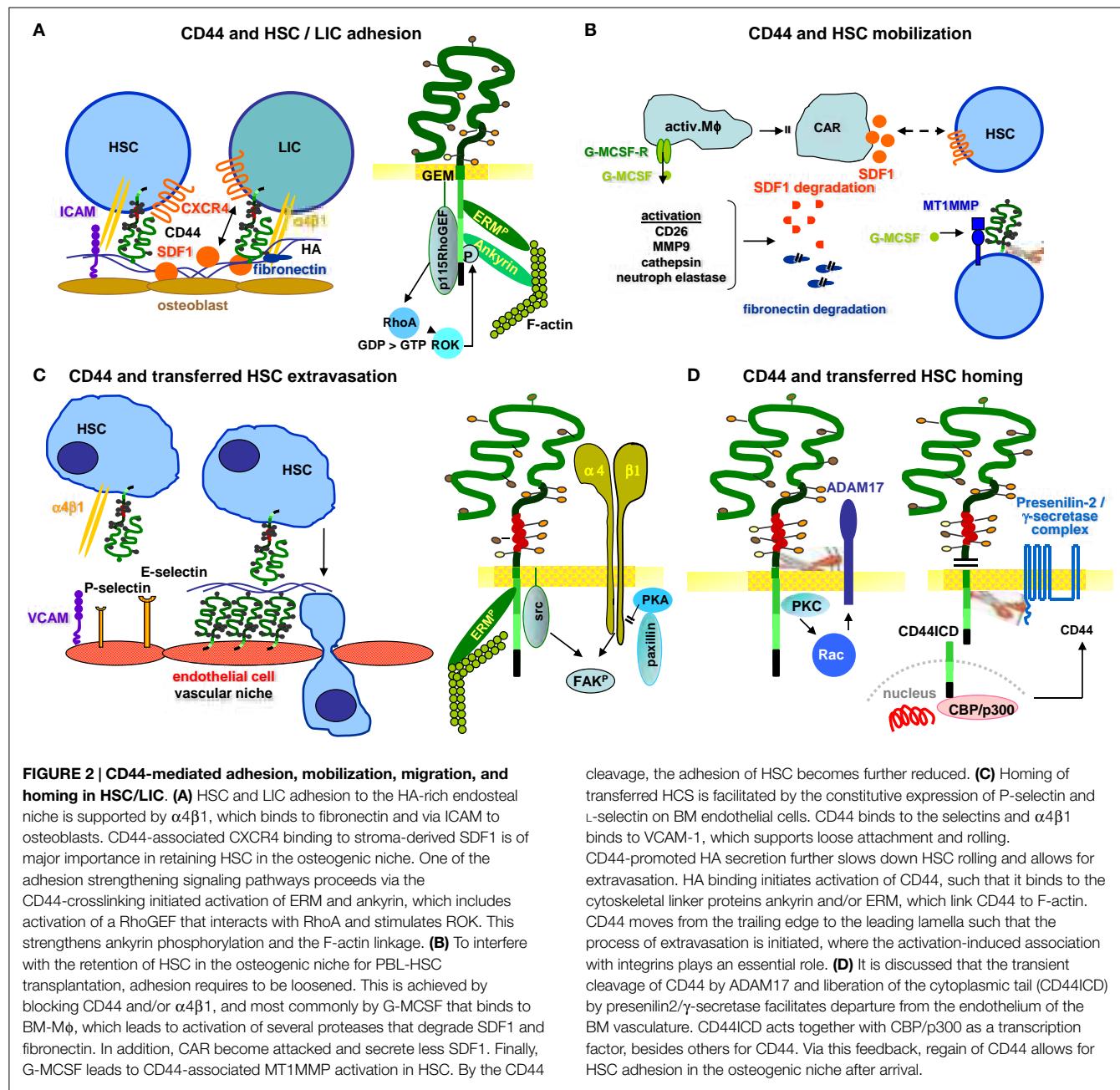
CD44 also participates in LIC embedding into the endosteal niche, such that targeting CD44 is considered a new strategy to eliminate persistent and drug-resistant LIC. In AML, CD44 is required for the transport of LIC to the HSC niche, and anti-CD44 antibodies alter the fate of the LIC by inducing differentiation (101). In a mouse model of CML, BCR-ABL1-transduced progenitors from CD44-mutant donors were defective in BM homing, which resulted in decreased engraftment and impaired CML-like disease induction (100). These studies provided additional evidence that LIC may be more dependent on CD44 for settlement in the osteogenic niche than HSC (101). Studies in a murine model confirmed leukemia cell homing and growth retardation by a CD44-specific antibody (169). However, during reconstitution, a panCD44-specific antibody more efficiently interfered with HSC than leukemia cell settlement in the BM niche (82).

The latter topic, how to avoid replacement of HSC when attacking LIC, was recently approached in an elegant study aiming to find selective ligands for LIC in CML and AML. Alteration of the niche by osteoblastic cell-specific activation of the parathyroid hormone receptor attenuates BCR-ABL1 oncogene-induced CML-like myeloproliferative neoplasia, but enhances MLL-AF9 oncogene-induced AML, possibly through opposing effects of increased TGF- $\beta 1$ on the respective LIC. These results, though providing a first and very important hint toward sparing niche embedded HSC, also demonstrate that niches differ for distinct LIC (170). The use of CD44v specific antibodies could be an alternative for blocking LIC, but sparing HSC embedding in the niche. Unfortunately, our data so far point toward a dominating role of CD44s in niche embedding. Thus, adhesion of CD44v6/v7 ko and of CD44v7 ko HSC to BM-Str is unimpaired (97, 171).

Facing competition for the niche, it should be remembered that LIC might remodel the niche such that it no longer serves the requirements of HSC (172). This possibility did not yet receive appropriate attention. Nonetheless, growing awareness and elaboration of the differentiation potential of distinct SC populations, particularly of BM derived MSC, might finally allow reconstituting/replacing a niche, which was distorted by LIC.

Distinct to the contribution of HSC/LIC CD44 on adhesion, little is known on the engagement of stroma cell provided CD44. Anti-CD44v6 strikingly hampered stroma formation in rat long-term BM cultures, but had no impact on HSC embedding in a preformed stroma (173). In addition, recovery of HSC from wt mice in the BM of CD44v7 ko mice is severely impaired (97). *In vitro* studies confirmed that HSC poorly adhere to long term CD44v6/v7 ko and CD44v7 ko BM-Str (171, 174). Thus, BM-Str CD44 should not be neglected as it contributes to matrix assembly. A comprehensive evaluation is missing. However, the availability of conditional ko mice will facilitate answering the question.

There is overwhelming evidence confirming the important finding of Miyake that HSC require CD44 for embedding in the BM niche, and that dislodgement by anti-CD44 severely affects hematopoiesis. In view of the essential role of CD44 to anchor HSC in the osteogenic niche, care should be taken on the therapeutic use of anti-CD44 to drive LIC out of the niche. Despite promising results, further refinement is required to guarantee



unimpaired hematopoiesis. First trials to replace anti-CD44 have been successful, but point toward no single strategy being effective in distinct LIC. Additional studies are also needed to elaborate options for correcting niches, which were distorted by LIC.

CD44 and HSC Mobilization

Transplantation of hematopoietic progenitor cells provides in many instances an ultimate chance of curative leukemia therapy. It has become obvious that the transfer of peripheral blood HSC appears advantageous, yet it requires HSC mobilization. In 1976, Richman et al. described an increase of HSC in the blood of patients, who had undergone chemotherapy (175). Later, a similar increase was observed after the administration of recombinant

growth factors (176). In fact, both G-CSF and chemotherapy mobilize HSC through the same mechanisms, with chemotherapy increasing the level of endogenous G-CSF (177). Though the precise mechanism remains to be elucidated, HSC mobilization obviously does not proceed directly, as HSC do not express the G-CSF receptor, which is expressed by BM macrophages (M ϕ) (178). Activation of M ϕ could result in a reduction of Nes $^+$ MSC and SDF1-abundant reticular cells (CAR), and their provision of SDF1 such that the SDF1-CXCR4 bond becomes loosened (179, 180). SDF1 is secreted by several BM-Str and its interaction with CXCR4 on HSC plays a key role in retention and trafficking. CXCR4 expression is enhanced through signaling cascades involving cAMP, PI3K, several GTPases, and PKC ζ (181). PKC ζ induces

motility, adhesion and survival, and MMP2 and MMP9 secretion (182). Disruption of the SDF1-CXCR4 axis is the major mechanism leading to HSC release from their niche (182, 183). Alternatively, not mutually exclusive, upregulated expression of proteases may be involved, which can affect SDF1 (184) via MMP9 (185) or CD26 (186), cathepsin G and K, and neutrophil elastase (183). The same proteases also may account for VCAM1, FN, and OPN degradation (187). In addition, CD44 cleavage via MMP14 can contribute to HSC mobilization, where G-CSF leads to increased MMP14 expression in HSC (188, 189). Activation of the C cascade and plasminogen also contributes to HSC mobilization (190, 191) (**Figure 2B**).

Although G-CSF efficiently mobilizes HSC in most instances, some patients do not or insufficiently respond. In addition, G-CSF treatment may be accompanied by maturation of the most primitive progenitors, and this impairs HSC homing and recovery of hematopoiesis. Therefore, additional approaches for HSC mobilization have been searched for, in particular, mobilization via a blockade of adhesion molecules expressed by CD34⁺ cells. As described above (167, 168), concomitant application of anti-CD44 and anti- $\alpha 4\beta 1$ most efficiently mobilizes HSC. Notably, most of the mechanisms suggested accounting for G-CSF-induced HSC mobilization might become initiated directly via the CD44 blockade. First, as CD44 is associated with CXCR4, SDF1-CXCR4 binding becomes loosened by a CD44 blockade (192). Second, antibody crosslinking of CD44 contributes to the activation of MMP9 and MT1MMP, which both are associated with CD44 (193).

In brief, mobilization, though mostly approached via G-CSF, can be achieved by directly loosening adhesion of HSC to the niche via anti-CD44 and/or anti- $\alpha 4\beta 1$.

CD44 and HSC Homing

With the therapeutic transfer of mobilized HSC, the question arose on their homing. Transferred HSC preferentially home into the BM, where they search for the osteogenic niche (194). Homing is facilitated by the unique feature of BM endothelium that constitutively expresses the endothelial P- and E-selectins and VCAM1 (195). Proinflammatory cytokines stimulate CD44 expression in endothelial cells and strengthen their binding to HA. This promotes the arrest of HSC, which bind via CD44 to HA captured by endothelial cells (196). HSC express P-selectin ligands and CD44 as well as VCAM1 receptors such as $\alpha 4\beta 1$ (197–199). Function blocking antibodies and targeted deletions confirmed the contribution of these adhesion molecules to slowdown HSC, which allows for firm adhesion and extravasation (200, 201) (**Figure 2C**).

CD44 also is involved in the extravasation of the endothelial cell-attached HSC (202). CD44-HA binding initiates the interaction of the CD44 cytoplasmic tail with the actin cytoskeleton through ankyrin and ERM proteins (196, 203), guiding CD44 to the leading edge of migrating cells (204). Thus, cells expressing CD44 with a truncated cytoplasmic tail retain HA-binding capacity, but loose the capacity to migrate on HA (204). One of the central events in CD44-mediated cytoskeletal reorganization appears to be Rac1 activation. Lamellipodia formation on HA-coated plates can be inhibited by a CD44 blocking antibody,

but also by transfection of a dominant-negative mutant form of Rac1 (205). Upstream regulators of Rac1 are Vav1 and Vav2, phosphotyrosine-dependent guanine exchange factors of Rho GTPases. Vav phosphorylation is mediated by src kinases (206). As GEM-located CD44 associates with src (207), cytoskeleton reorganization most likely is initiated via src activation. Another mediator of CD44 signaling is RhoA. The RhoA-specific p115RhoGEF interacts with CD44 and regulates HA-mediated CD44 signaling via the serine-threonine Rho-Kinase (ROK), a downstream target of RhoA. ROK phosphorylates CD44, which promotes enhanced ankyrin binding (208). Another pathway of CD44 promoted motility proceeds via the association with $\alpha 4\beta 1$. By associating with $\alpha 4\beta 1$, CD44 gains access to FAK (focal adhesion kinase) and $\alpha 4\beta 1$ gains access to src kinases and ERM proteins, such that the integrin–paxillin association becomes weakened and the GEM-integrated CD44-ezrin-integrin-FAK complex moves toward the leading edge (164, 209).

Cell motility is additionally supported by CD44 cleavage via a disintegrin and metalloproteinase domain (ADAM) protein and MMP-14 (210). CD44 cleavage is stimulated by Ca⁺⁺ influx, which triggers ADAM10 activation after proADAM10 dissociation from calmodulin. ADAM17, which colocalizes with CD44 at Rac-regulated membrane ruffling areas, becomes activated by PKC and Rac and contributes to CD44v cleavage (211, 212). Thus, the rapid activation of membrane-integrated proteases by CD44-HA binding contributes to a shift toward motility by CD44 cleavage. CD44 cleavage is tightly regulated, in part, by the missing activation of CD44-associated proteases after CD44 cleavage, and in part by cleavage-promoted CD44 transcription. After ectodomain cleavage, CD44 becomes accessible to the presenilin/ γ -secretase complex, which triggers intramembrane CD44 cleavage, setting free the CD44 ICD (CD44-ICD). CD44-ICD acts as a cotranscription factor that potentiates CD44 (28), MMP9, and MMP3 transcription (150) (**Figure 2D**).

As leukemia therapy frequently relies on autologous HSC transplantation, it became important to know whether LIC compete with HSC not only for the niche but also for homing. The described signaling pathways promoting HSC extravasation do not fundamentally differ for LIC. However, there are subtle differences. This was explored in an elegant study for BCR-ABL1-induced CML-like myeloproliferative neoplasia. Expression of $\alpha 4\beta 1$, $\alpha 5\beta 1$, LFA1, and CXCR4 did not differ between BCR-ABL1(+) progenitors and HSC, but expression of P-selectin glycoprotein ligand-1 and of L-selectin was lower than in HSC. Deficiency of E-selectin in the recipient BM endothelium significantly reduced engraftment by BCR-ABL1-expressing SC. Destruction of selectin ligands on leukemic progenitors by neuraminidase reduced engraftment. BCR-ABL1-expressing L-selectin-deficient progenitors were also defective in homing and engraftment, and an L-selectin-specific antibody decreased the engraftment of BCR-ABL1-transduced SC. These results establish that BCR-ABL1(+) LIC rely to a greater extent on selectins and their ligands for homing and engraftment than HSC. Thus, a selectin blockade may be beneficial in autologous HSC transplantation for CML and perhaps other leukemia (213).

After extravasation, transplanted HSC should reach the osteogenic niche. As mentioned, HSC synthesize HA (214), and HA expression supports HSC migration toward the endosteal niche (189). In the endosteal niche, SDF1 promotes adhesion through CD44-associated CXCR4 accompanied by rac1 and cdc42 activation (182, 192, 215). These findings confirm the key role of HA and CD44 in SDF1-dependent HSC anchorage within specific niches (189). Finally, space is created by activated ROK, which phosphorylates the Na-H-exchanger1. Hyaluronidase-2 and cathepsinB become activated in the acidic milieu and support ECM degradation (206, 207).

Unfortunately, AML share the homing mechanisms with HSC (216, 217). However, the problem can possibly be circumvented in leukemia highly expressing CD44v6, as CD44v6 expression is low in HSC and HSC homing is dominated by CD44s. Analyzing migration of HSC from CD44v6/v7^{ko} and CD44v7^{ko} mice toward HA and BM-Str revealed impaired migration toward FN, possibly due to CD44v6 directly binding to FN (218) or being promoted by the CD44v6- α 4 β 1 association (174). Furthermore, HSC migration toward IL6 is strikingly impaired by anti-CD44v6 (219). Migration of CD44v7^{ko} and CD44v6/v7^{ko} HSC toward SDF1 was reduced to background levels, which indicates major importance of these two splice variants in migration along a SDF1 gradient (220). Binding and migration toward OPN is also impaired in CD44v6/v7^{ko} HSC. The finding fits the selective CD44v6 binding of OPN, which triggers migration and invasion (221, 222). Finally, BM-Str CD44v7 supports HSC migration (97). Thus, CD44v6/v7 are engaged in HSC migration toward chemokines/cytokines and BM-Str. Another protein selectively trapped by CD44v6 is C3 (81, 130). As elegantly elaborated by the group of Ratajczak [review in Ref. (223)], C3 can drive CXCR4 into lipid rafts, where it associates with CD44v6. Thereby, the CXCR4-SDF1 axis becomes strengthened, which helps retaining HSC in the niche. In a similar attempt elaborating homing of multiple myeloma to the BM, the authors explored differences in a stroma-dependent and a stroma-independent line. Only the stroma-dependent line expressed IGF-1R and CD44v6, where IGF-1 promoted chemotaxis toward BM-Str and CD44v6 supported adhesion. By modulating the culture conditions, the authors demonstrated that BM-Str promotes up-regulation of IGF-1R and CD44v6 in multiple myeloma, which facilitate homing and support adhesion to BM-Str (224).

Thus, the particular BM endothelium supports the egress of transplanted HSC into the BM. The engagement of CD44 relies on the provision of HA and the binding of CD44 to L-selectin, binding being supported by the association with integrins. Once attached to the endothelium, CD44 promotes activation of rac and rho, which initiate the shift toward a migratory phenotype. Migration is strengthened by activation of CD44-associated proteases. The proteases create space and cleave adhesion molecules including CD44, which fosters migration toward the endosteal niche. Settlement in the niche follows the path that underlies the preferential retention of HSC in the osteogenic niche. In most instances, LIC and HSC use the same adhesion molecules and signaling pathways for migration. Nevertheless and notably, there are some discrete differences between HSC and LIC, which may help elaborating protocols for preferential homing of transplanted HSC.

CD44 and the Crosstalk Between HSC and the Niche

CD44 does not only contribute to niche assembly but, importantly, there is a feedback from the niche toward HSC and LIC, which also involves CD44. Two aspects of this crosstalk will be in focus, the engagement of CD44 in (i) HSC quiescence and (ii) stress resistance, which both are linked to the osteogenic niche. Different to the engagement of CD44 in HSC and LIC homing and migration, mostly HSC rely on CD44-promoted quiescence. Instead, both HSC and LIC profit from CD44 in apoptosis resistance. Though the CD44-mediated crosstalk with the niche and the GEM location of CD44 are important for HSC and LIC apoptosis resistance (225, 226), the dominating mechanisms differ.

In advance of discussing the impact of the niche on CD44-promoted quiescence and apoptosis resistance, exosomes need to be mentioned. Unfortunately, their impact on HSC and LIC has not yet been explored in detail. Many cells including HSC and LIC secrete small vesicles, called exosomes, which are supposed to be most efficient intercellular communicators (227–229), where miRNA transfer via exosomes can lead to target cell reprogramming (230). This was demonstrated for ESC exosomes reprogramming hematopoietic progenitors through miRNA delivery (231), and for the transfer of miRNA between different cells of the hematopoietic system as well as from CIC into BM-Str (232). Although the impact of CD44 on the exchange of exosomal miRNA between HSC/LIC and niche cells has not been elaborated, the impact of exosomal CD44v6 on miRNA transfer points toward the engagement of CD44 (78) and unquestionable demonstrated the strong impact of exosomal miRNA. Thus, a more comprehensive knowledge on the transfer of HSC exosomal miRNA should be approached, and can be expected to open new therapeutic options.

CD44 and HSC Quiescence

The quiescent state is critical for preserving self-renewal capacity and stress resistance of HSC. Besides intrinsic regulatory mechanisms, where p53 plays a dominant role, there are extrinsic microenvironmental regulatory mechanisms, which include angiopoietin-1, TGF β , bone morphogenetic proteins (BMP), TPO, N-cadherin, integrins, Wnt/ β -catenin, and OPN (233).

Angiopoietin is secreted by osteoblasts and binds to Tie2 on HSC, which supports maintenance of quiescence and prevents cell division. Furthermore, the Tie2-angiopoietin interaction promotes cobblestone formation in long-term BM cultures (234). Besides strengthening adhesion of HSC to BM-Str (234), possibly via CD44, I am not aware of a particular linkage between the angiopoietin-Tie2 axis and CD44 signaling.

TGF β are potent inhibitors of HSC proliferation. TGF β disruption increases circulating progenitor cells, and a bolus injection of TFG β 1 inhibited early progenitor proliferation. TGF β -mediated quiescence of HSC may be due to alteration in cytokine receptor expression and upregulation of cyclin-dependent kinase inhibitors (235). The engagement of CD44 relies on its interaction with the TGF β R1 (236). TGF β cooperates with HA-activated CD44 to induce expression of the NADPH oxidase (237), which could help regulating redox signals in HSC.

Bone morphogenetic proteins, secreted by osteoblasts (238), potently inhibit HSC proliferation. BMP bind to their serine threonine kinase receptors on HSC, which leads to transphosphorylation and kinase domain activation, initiating phosphorylation of Smad 1, 5, and 8 that concomitantly associate with Smad4 and translocate to the nucleus. In the nucleus, they act as cotranscription factor regulating expression of target genes such as Runx1 and GATA2, which operate during specification of hematopoiesis (239) and regulate HSC quiescence (110, 240, 241). The linkage to CD44 is based on the association of CD44 with Smad1 (242). Alternatively, and BMP-independent, Smad1 can become phosphorylated via galectin-9, where galectin-9 binding to CD44 promotes formation of a CD44/BMP receptor complex with concomitant BMP receptor activation (243).

Binding of TPO to its ligand (MPL) is critically involved in HSC steady-state maintenance with an over 150-fold reduction of HSC in TPO^{ko} mice. Posttransplantation HSC expansion was highly MPL-and TPO-dependent. Accelerated HSC cell-cycle kinetic in TPO^{ko} mice is accompanied by reduced cyclin-dependent kinase inhibitor *p57kip2* and *p19INK4D* expression (244). The activity of TPO becomes strengthened by glucosaminoglycans in the matrix. Though this was demonstrated for megakaryocytopoiesis (245), it may have bearing on TPO affecting HSC embedded in the osteogenic niche.

Wnt signaling has emerged as an important factor in HSC quiescence, self-renewal, and differentiation (246). Wnt, secreted glycoproteins, binds to their sevenpass transmembrane receptors (Frizzled) (247) and low-density lipoprotein receptors LRP5 and LPR6 (248), which become phosphorylated and form an activated Frizzled/LPR receptor complex. The Frizzled/LPR receptor complex promotes dephosphorylation of β -catenin, which in the absence of Wnt signaling is phosphorylated and associated with a so-called destruction complex (249). Dephosphorylated β -catenin translocates to the nucleus, and together with LEF/TCF initiates transcription of Wnt target genes. In the non-canonical pathway, mostly Wnt5a signals via Frizzled using as coreceptor ROR, which leads to RhoA/Rac and JNK activation. In the Wnt-Ca⁺⁺ pathway, G-protein signaling is activated with upregulation of IP3-mediated release of intracellular Ca⁺⁺ and activation of PKC, which triggers nuclear translocation of NFAT and NF κ B (246). Though Wnt is a potent morphogen (250), Wnt effects are highly context and dose-dependent (251), which makes it difficult to define precisely its role in HSC maintenance. To circumvent these difficulties, the group of Scadden used an osteoblast-specific promoter for expression of the Wnt paninhibitor Dickkopf1 (Dkk1). Binding to the coreceptor LRP5/6 leads to internalization of the complex (252). Inhibition of Wnt signaling in HSC resulted in reduced p21Cip1 expression, increased cell cycling, and a continuing decline in the reconstitution capacity of HSCs. Notably, though the effect on HSC was microenvironment-dependent, HSC did not recover, when transferred in a normal host (253). Furthermore, Wnt-inhibition affected activation of the Notch target, Hes-1. This finding suggests that Notch and Wnt coordinately regulate HSC quiescence. Indeed, elevated Hes-1 and p21 expression correlate with the maintenance of HSC quiescence (254). The importance of Notch signaling was confirmed by inhibition of Notch signaling diminishing the capacity of HSC to

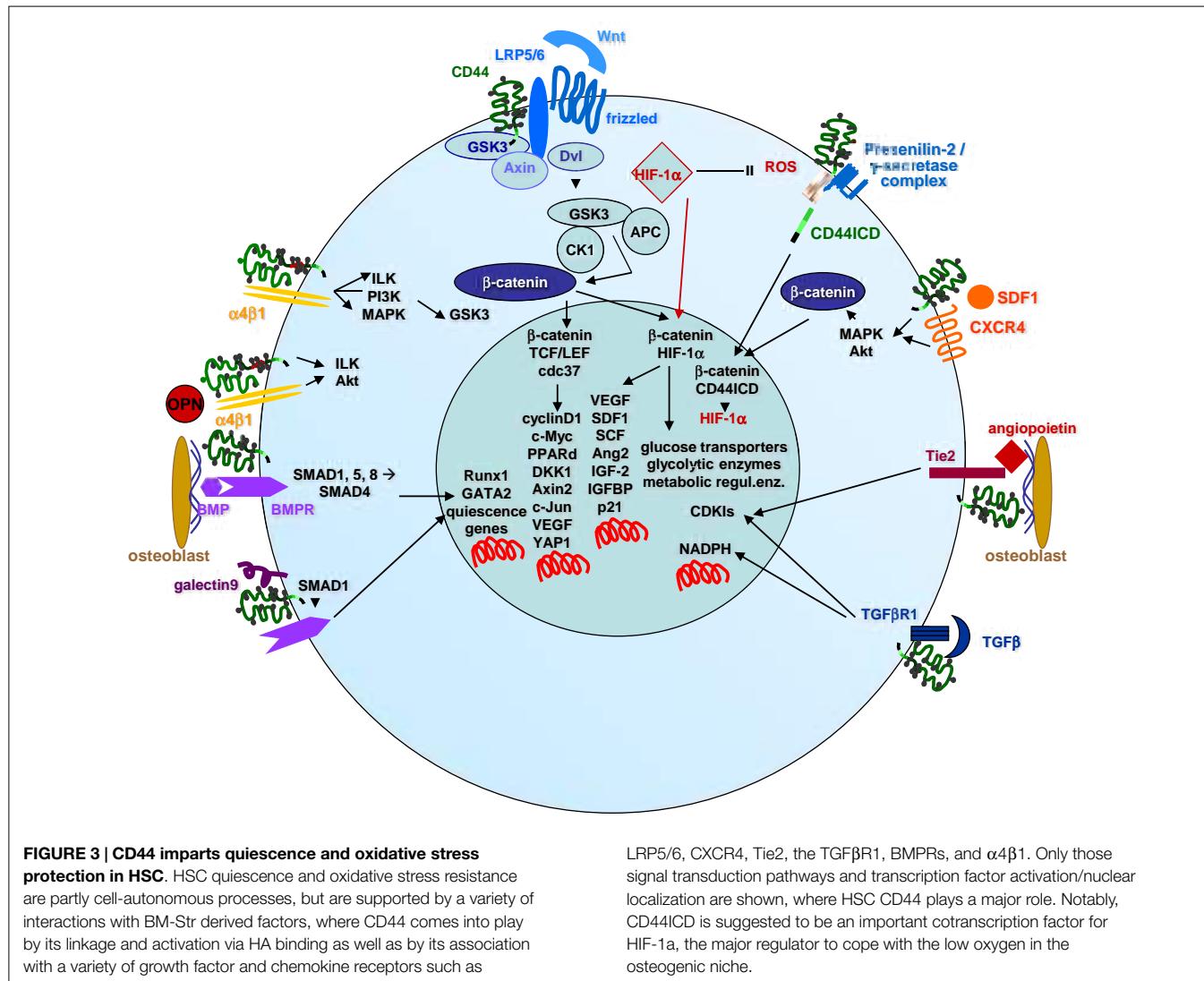
maintain an undifferentiated state. As proliferation and survival were not affected, the authors suggest that Notch "may act as a "gatekeeper" between self-renewal and commitment" (255).

Wnt signaling, in fact, is not only important for HSC [review in Ref. (62)], but the association of CD44 with Wnt signaling is also amply demonstrated for LIC [review in Ref. (61)]. However, as mentioned, Wnt effects are context dependent. Highlighting the differences to HSC, one example will be given. The cytoplasmic domain of GEM-located CD44 associates with the Wnt receptor LRP6, whereby LRP6 becomes recruited into the plasma membrane, which strengthens Wnt signaling and the accumulation of β -catenin in the nucleus, where down- and upregulation of CD44 directly affected Wnt signaling. Importantly, this activity of CD44 does not require CD44-HA crosslinking (256).

Osteopontin also negatively regulates the number of HSC in the BM niche. OPN^{ko} mice display a significantly increased number of HSC, but not of committed progenitors (257). OPN also can modify primitive hematopoietic cell number and function in a stem cell-non-autonomous manner. This conclusion derived from the observation that the BM microenvironment of OPN^{ko} mice was sufficient to increase the number of HSC, which was accompanied by an increase in stromal Jagged1 and angiopoietin-1 expression and a reduction of primitive hematopoietic cell apoptosis (116). The authors discuss that the ECM plays a dynamic role in governing HSC responsiveness to expansion signals. Whether the signals are transferred via CD44-associated integrins (258) or directly via OPN binding to CD44 (133) remain to be explored. Instead, OPN binding to CD44v6 in CIC promotes activation of the PI3K/Akt pathway and promotes tumor growth (259). This is mentioned to remember that in concern of signal transduction, the peculiarities of HSC frequently do not allow a direct comparison to oncogene transformed CIC or LIC.

Finally, the interaction of SDF1, expressed by developing stroma in fetal bones, with HSC CXCR4 is critically for retaining HSC in the quiescence-protecting niche (260). Originally, it was noted that SDF1^{ko} and CXCR4^{ko} embryos have greater impairment of myelopoiesis in the BM than in the fetal liver, which suggested that SDF1 and CXCR4 are primarily involved in colonization of the BM by HSC during embryogenesis (261). Later on, elegant work with conditional CXCR4^{ko} mice implicated stromal SDF1 and its receptor in maintaining the pool of quiescent HSC. Conditional CXCR4^{ko} mice have a significantly increased pool of HSC in G1 compared to wt mice. This may be due to an altered environment with upregulation of cytokines, which promote HSC cycling and differentiation (115). Interestingly, actively signaling CXCR4 is associated with GEM localization (262). This implies that CXCR4 signaling sensitivity can be modulated by colocalization with other signaling molecules, including Rac1 (263). Notably, HA-crosslinked, GEM-located CD44 directly interacts with CXCR4, such that SDF1-CXCR4 signaling is abrogated in CD44^{kd} cells (264). Less is known about the impact of the niche on the resting versus cycling state of leukemia. However, it can be expected that due to oncogene transformation LIC are less susceptible to quiescence promoting signals from the niche or may even distinctly respond.

Figure 3 summarizes those signaling pathways in HSC, where CD44 is actively involved in maintaining HSC quiescence and



HSC stress protection. Thus, CD44 becomes stimulated by several key molecules engaged in HSC quiescence. Alternatively, CD44, particularly when crosslinked by HA, associates with quiescence regulating molecules.

Last, not least, the importance of the CD44-HA interaction was also demonstrated with HAS3^{ko} mice. HSC homing into the osteogenic niche depends on the HA coat of endothelial cells, and is significantly retarded in HAS3^{ko} mice (265). Furthermore, HA is required for the generation of HSC during differentiation of ESC (266). Finally, HSC seeded on HA rarely proliferate and retain multipotency (267, 268). All these findings strengthen the upmost importance of the CD44-HA crosstalk in HSC maintenance.

CD44 and HSC Stress Resistance

The distinction between HSC and LIC also accounts for a second phenomenon, the response to low oxygen pressure according to the location of HSC in niches (110), characterized by low oxygen concentration (269). Though LIC compete with HSC for the niche, they are not dependent on low oxygen and instead

remodel the niche toward accumulation of inflammatory myelofibrotic cells, which drive LIC expansion, but compromise HSC maintenance (270). Notably, too, the metabolic status of HSC residing in a hypoxic BM environment also differs from that of their differentiated progeny (271).

Hematopoietic SC maintains redox homeostasis by low oxygen production due to the minimal metabolic rate (271, 272). Low metabolic rate maintenance further relies on asymmetric cell division, where the daughter cell, which remains in the SC state, inherits a very low level of energized mitochondria (273, 274). Furthermore, HSC generate energy mainly via anaerobic metabolism maintaining a high rate of glycolysis, which limits the production of reactive oxygen species. The hypoxia responsive regulatory pathways in HSC resembles that in other cells, HIF1 α being the master regulator driving the metabolic machinery toward anaerobic glycolysis (275). HIF1 α , stabilized under hypoxic conditions (276), reprograms glucose metabolism via transcriptional activation of genes encoding glucose transporters, glycolytic enzymes, and metabolic regulatory enzymes, thereby switching from oxidative to glycolytic metabolism (277). One

of the mechanisms proceeds via the HSP GRP78 and its ligand Cripto, HIF1 α binding to the Cripto promoter (278). The importance of HIF1 α orchestrating molecular responses, which maintain redox homeostasis in the face of changing O₂ levels, was demonstrated by the loss of reconstitution capacity of HSC in HIF1 α^{ko} mice (279). HSC dispose on additional regulatory molecules, including polycomb, DNA damage-related, and anti-oxidant proteins that participate in ROS regulation (280). Notably, maintenance of the hypoxic state of HSC is not restricted to the location in a poorly vascularized niche, but is dictated by cell-specific mechanisms derived from their glycolytic metabolic profile (281). Finally, the cotranscription factor CD44-ICD promotes expression of HIF2 α (282), as well as of additional hypoxia-related genes, like aldolase c, 6-phosphofructose-2-kinase, pyruvate dehydrogenase kinase-1, and pyruvate dehydrogenase, which are directly associated with aerobic glycolysis (283–285). The authors point out that the repeatedly observed impact of the CD44-ICD on HIF expression in SC suggests a more active role of CD44-ICD in SC maintenance and protection as previously anticipated (282).

In concern about the engagement of CD44 and its ligands in protection from oxidative stress, it also was described that neural SC reside undifferentiated in a HA rich matrix, but proliferate and differentiate upon hyaluronidase upregulation (286). Additional contributions of CD44 and HA on stress protection may be shared by HSC and LIC and are described below.

Taken together, HSC circumvent stress, which would drive them into proliferation and exhaustion, mostly by a minimal metabolic rate and the generation of energy via anaerobic metabolism. The main contribution of CD44 relies on the cotranscription factor activity of CD44-ICD.

CD44, LIC, and Apoptosis Resistance

Besides contributing to circumvent stress, CD44 also actively promotes apoptosis resistance. From this activity of CD44, which does not appear to be of major importance for HSC stress resistance, LIC make profit. As to my knowledge, CD44-mediated apoptosis resistance proceeds in LIC and CIC via overlapping pathways, some examples of CIC will be included, where corresponding experiments have not yet been performed with LIC. There are two major mechanisms of CD44-mediated apoptosis protection: (i) initiation of signal transduction by CD44 crosslinking via HA, which frequently involves CD44v and associated RTK (287), and (ii) the crosstalk of CD44 with multidrug resistance genes that also is HA-dependent (288).

Apoptosis resistance initiated by the cooperation of CD44- or CD44v with RTK mostly proceeds via activation of anti-apoptotic proteins. CD44 coimmunoprecipitates with all ERBB family members. The association of CD44 with ERBB2 and ERBB3 mediates heterodimerization and activation of the receptor in response to neuregulin, which strongly promotes CIC apoptosis resistance (289, 290). The impact of CD44 on ERBB2 activation is strikingly HA-dependent. CD44 crosslinking via HA initiates association of CD44 with ERBB2, which becomes phosphorylated. The complex, located in lipid rafts, includes ezrin, the chaperones HSP90 and CDC37, and PI3K, which accounts for drug resistance via activation of anti-apoptotic proteins. Apoptosis resistance

is not seen when the HA-CD44 interaction is blocked, which causes complex disassembly and inactivation of ERBB2 (291). The authors also unraveled activation of an ERBB2-PI3K/Akt- β -catenin axis, which contributes to COX2 expression and COX2-promoted suppression of caspase3 activation. The data argue for a feedback loop, whereby COX2 strengthens HA production and promotes prostaglandin E2 expression (292). An additional feedback loop proceeds via formation of the ERBB2/ERBB4-CD44 complex, which via ERK activation promotes HA production by HAS1, -2, and -3 phosphorylation/activation (293) (**Figure 4A**).

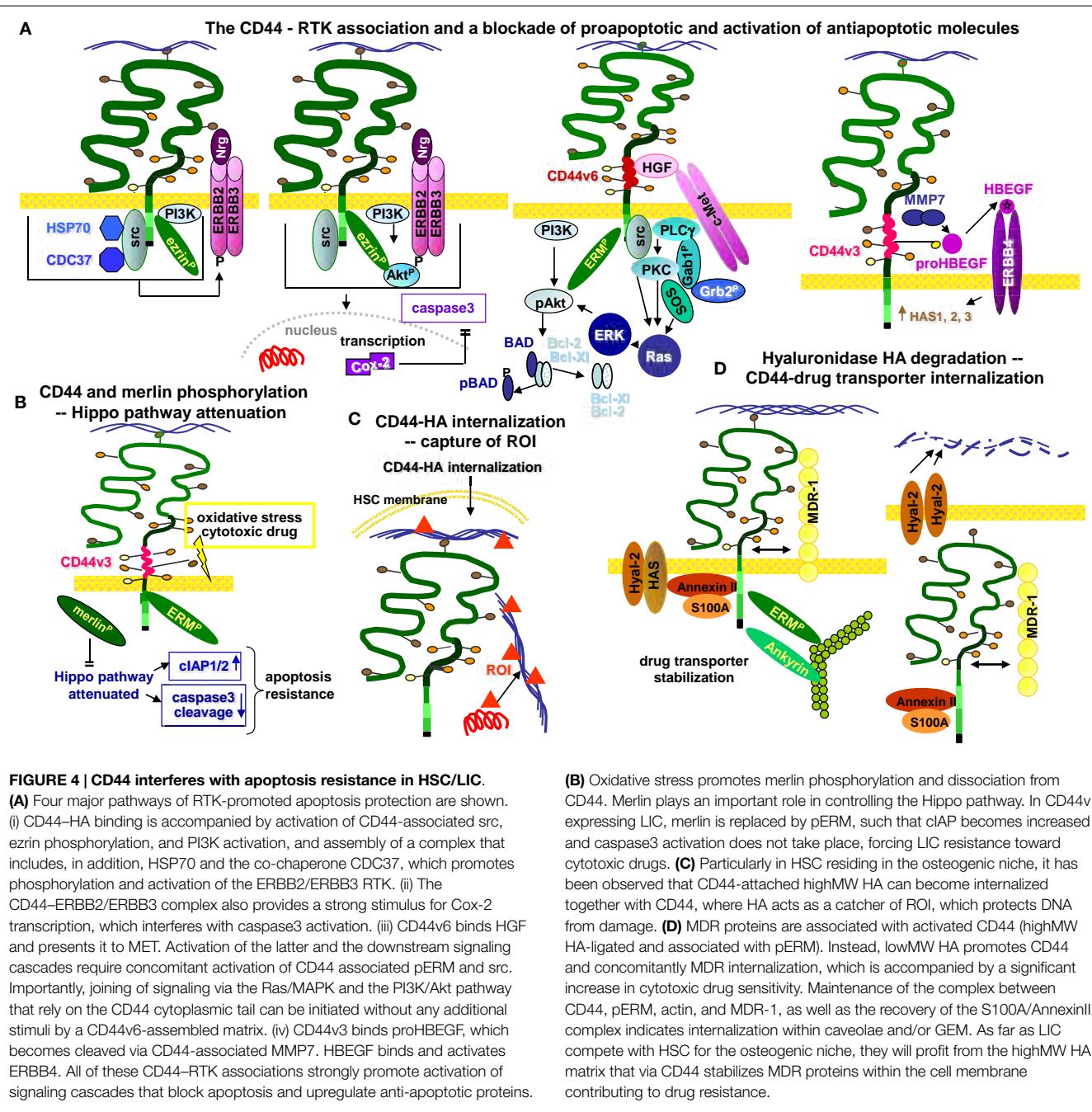
Another, well-known pathway of apoptosis resistance involves the CD44v association with MET, which is initiated by HGF binding to CD44v3 or CD44v6 (138, 294), and promotes MET phosphorylation. MET phosphorylation requires the cytoplasmic tail of CD44 and the interaction with ERM proteins for activation of the Ras-MAPK pathway (294). CD44v6 binding to the ECM also activates the PI3K-Akt pathway and Wnt/ β -catenin signaling (130, 295) and regulates MET transcription (86, 296). Similar observations account for insulin-like growth factor-1 receptor and PDGFR activation through HA-stimulated CD44 in transformed cells (133, 297, 298) (**Figure 4A**).

Importantly, CD44v6 promoted apoptosis resistance can also rely on the association of GEM-located CD44v6 with FAS. This association prevents trimerization of FAS upon ligand binding. Notably, apoptosis susceptibility strongly increases by a blockade of CD44v6 (299). Promoting FAS trimerization by a CD44v6 antibody blockade presents a new and interesting option in the therapy of CD44v6 expressing leukemia.

The interaction between CD44 and RTK can also proceed via proteases. CD44v3 binds the proform of the heparin binding epidermal growth factor (HB-EGF), which is cleaved by CD44-recruited MMP7. Cleaved HB-EGF binds and activates ERBB4, which signals for cell survival (300, 301). The interaction of CD44 with MMP9 leads to apoptosis protection independent of RTK. CD44 and MMP9 expression are interdependent (149), and in CLL patients with poor prognosis CD44, CD49, and MMP9 are physically associated (302, 303). CD44-associated MMP14 accounts for proMMP9 cleavage (191). Activated MMP9 can interfere with TGF β activation, whereby several mechanisms of TGF β -promoted apoptosis become silenced (304, 305).

An additional pathway of CD44-promoted resistance to reactive oxygen- and cytotoxic drug-induced stress under physiological and pathological conditions proceeds via the mammalian Hippo signaling pathway. In the resting state, CD44-associated merlin accounts for JNK, p53, and p21 upregulation, and YAP as well as ciAP1/2 downregulation, which jointly promote caspase3 activation and apoptosis. When CD44 becomes activated by HA binding, merlin is phosphorylated and dissociates from CD44. In the absence of merlin, CD44 directly regulates YAP expression via active RhoA. Thereby, the HIPPO pathway becomes blocked, which results in increased apoptosis resistance (306, 307). In a feedback loop, activated YAP binds to the promoter of RHAMM, thereby inducing RHAMM transcription (308, 309) (**Figure 4B**).

Besides via CD44-associating molecules, the direct interaction between CD44 and HA strongly affects apoptosis resistance. Exploring the effect of HA on the extent of DNA damage induced



by exogenous and endogenous oxidants revealed that CD44 in SC internalizes HA by endocytosis. One of the functions of the internalized HA is the protection of DNA from oxidants. The authors propose entrapment of iron ions. Thereby the Fenton's reaction, which produces secondary oxidative species becomes inhibited. Alternatively, though not mutually exclusive, HA directly scavenges primary and secondary ROI, which results in intracellular HA degradation (310). Palmitoylation and GEM recruitment of CD44 is a precondition for HA internalization (311). Thus, attenuating oxidant-induced damage can proceed through direct scavenging of oxidant molecules by HA (312) (**Figure 4C**).

Drug resistance can also be promoted through CD44v3-HA binding, which via the Oct4-Sox2-Nanog complex induces miR-302 transcription (74). The CD44-HA induced nuclear translocation of Nanog also leads to miR-21 production and upregulation of apoptosis inhibitors and MDR1 (77, 313).

Last, the interplay between CD44 and HA accounts for rapid drug elimination via drug transporters (288), which creates a major obstacle in leukemia and cancer therapy (314). Both CD44 and HA contribute to drug resistance. MDR genes are associated with CD44 and CD44 regulates expression of drug transporters. This likely is due to HA-activated CD44 binding to Gab1, which

promotes PI3K activation. Activated PI3K stimulates HA production as well as MDR transporter expression (315, 316). Alternatively, though not mutually exclusive, HA binding to CD44 up-regulates p300 expression and its acetyltransferase activity. This, in turn, promotes acetylating β-catenin and NFκB-p65. Activated β-catenin and NFAT act as cotranscription factors with NFκB in MDR1 transcription (317). The direct involvement of HA was demonstrated by replacing high MW by low MW HA. In the presence of high MW, HA activated CD44 is predominantly recovered in GEM and is associated with ERM and actin. MDR1 is associated with CD44 and the association stabilizes MDR1 expression. Instead, low MW HA does not stabilize the complex, but rather supports internalization such that all components of the complex including S100A and Annexin II are recovered in the cytoplasm (318). Whether the internalized complex becomes degraded or released as exosomes has not been explored. Independent of the answer to this question, the reduction of MDR1 in the cell membrane is accompanied by increased drug susceptibility (319). Notably, the three hyaluronan synthases are supposed to produce HA of different size. However, this as well as the major transcription factors engaged in HAS1, -2, and -3 transcription remain to be defined. Transcriptional regulation of hyaluronidases also awaits unraveling (320, 321). Taking into account that hyaluronidase as well as small HA oligosaccharides can improve drug efficacy (318), and that HA-CD44 cross-linking regulates expression of drug transporters (315, 316), filling this gap becomes demanding to improve therapeutic targeting of HA [review in Ref. (322, 323)] (**Figure 4D**).

The major importance of CD44 in apoptosis resistance relies (i) on the association of activated (HA-crosslinked) CD44/CD44v with RTK, which promote activation of anti-apoptotic signaling cascades, (ii) on interferences of CD44v6 with FAS trimerization, and (iii) the engagement of CD44 in regulating drug-resistance gene expression. The impact of CD44 is efficiently reinforced by high MW, but not low MW HA, where the latter may open a therapeutic window.

Open Questions

This review highlights the importance of CD44 in the crosstalk between HSC/LIC and the surrounding matrix. However, due to space constraints, this review does not cover the role of CD44 in all aspects of hematopoiesis and leukemia induction; this can be found within an excellent review which also focuses on signal transduction and transcription (324). Furthermore, while CD44-based therapeutic concepts have been highlighted in individual sections within this review, there is some excellent literature which describes CD44 antibody and vaccination-based therapeutic concepts that may be of further interest to the reader (325–332). However, there are a number of key issues/questions that need to be addressed before a major therapeutic breakthrough can be achieved:

1. More information on the active contribution of the niche is required as well as the contribution of the individual components. This includes the possible transfer of information, comprising miRNA, via exosomes and accounts for HSC and LIC.

2. There is insufficient information on the modulation of the osteogenic niche by LIC, to safely protect HSC, if LIC was to be targeted, and to reconstruct a destroyed niche for unimpaired hematopoiesis.
3. The generation of LIC is not well understood. Again, the possibility has to be taken into account that LIC are instructed by surrounding cells, preferably HSC or MSC, via exosomes. Furthermore, there is a paucity of knowledge on the decision for self-renewal versus differentiation, which to some degree also accounts for HSC.
4. Much progress has been made in homing, migration, and signal transduction, which could well become the first question to be comprehensively answered.

In summary, the specific therapeutic targeting of LSC is still very much a field in its infancy (323). However, there is justified hope that this may change in the near future.

Conclusion

Stem cells require a niche, which has been particularly well explored for HSC, where the central importance of the CD44-HA interaction, including more recently the cellular stroma elements, is amply demonstrated. The CD44-HA crosstalk promotes adhesion and via cytokines/chemokines harbored in the BM-Str, homing, and migration of HSC as well as HSC quiescence and resistance to low oxygen pressure. LIC share with HSC the requirement for the crosstalk with the stroma to promote adhesion, homing, and migration. Apoptosis resistance of LIC, though strikingly dependent on the CD44-HA crosstalk, proceeds differently to that of HSC predominantly via CD44v and the cooperation with RTK, proteases, and drug transporters.

The abundant array of HA-bound CD44-initiated activities relies on the cooperation of CD44 with multiple membrane molecules including integrins, chemokine receptors, RTK, and proteases as well as its transient association with cytoskeletal linker molecules and cytoplasmic signal transducers, which includes central stem cell fate regulators. The multitude of interactions is fostered by the GEM location of CD44, which also promotes the proximity to proteases. Proteases facilitate CD44 cleavage, where the CD44-ICD acts as a cotranscription factor. It has been suggested, but needs further approval, that CD44-ICD also regulates miR transcription/repression of CD44 cooperation partners, thereby creating an additional feedback loop.

In view of the most promising results in leukemia therapy by blocking CD44, awareness increased on possible selective differences between HSC and LIC in the crosstalk with the osteogenic niche. Several elegant and sophisticated studies clearly demonstrated the existence of differences not only between HSC and LIC but also between distinct leukemia. Progress in this field will greatly facilitate selective therapeutic interference with LIC homing and may allow for corrections of the LIC-distorted osteogenic niche. The latter as well as the interaction with the vascular niche and homing into the osteogenic niche is of particular interest in view of the HSC transfer being frequently a last chance for curative therapy. Further clarifying HA production and degradation may also open new avenues for a therapeutic dissection between the HSC- and the LIC-HA crosstalk. Last, not least, uncovering the

importance of miRNA and the role of exosomes in miRNA transfer will add optimizing the HSC–HA crosstalk in the osteogenic niche and will allow interfering with niche destructing activities of LIC.

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CD44 acts as a signaling platform controlling tumor progression and metastasis

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Members of the CD44 family of transmembrane glycoproteins emerge as major signal transduction control units. CD44 isoforms participate in several signaling pathways ranging from growth factor-induced signaling to Wnt-regulated pathways. The role of the CD44 family members in tumor progression and metastasis is most likely linked to the function of the various isoforms as signaling hubs. Increasing evidence suggests that these proteins are not solely cancer stem cell (CSC) markers but are directly involved in tumor and metastasis initiation. It is foreseeable that a link between the expression of CD44 isoforms in CSCs and their function as signaling regulators will be drawn in a near future.

Keywords: CD44, RTK, cancer stem cells, alternative splicing, EMT

The term CD44 designates a large family of transmembrane glycoproteins belonging to the class of cell adhesion molecules. CD44 family members are involved in physiological processes such as hematopoiesis, lymphocyte homing, or limb development [reviewed in Ref. (1)]. All CD44 isoforms are encoded by one single gene located on chromosome 11 in humans and chromosome 2 in mice [reviewed in Ref. (2)]. The exons 1–5 and 16–20 that encode the constant part of CD44 are included in all CD44 isoforms. Exons 6–15 that encode the variant exons v1–v10 are either completely excluded as in CD44s or are included in various combinations within the CD44 ectodomain, giving rise to the CD44 variant isoforms (CD44v). Of note, exon v1 is not expressed in human cells due to the presence of a stop codon. The heterogeneity of the CD44 family is further increased by several additional modifications including N- and O-glycosylations.

CD44 EXPRESSION

CD44s is expressed in nearly all tissues whereas the expression of CD44v isoforms is restricted to specific cell types [reviewed in Ref. (1) and table in Ref. (3)]. In human skin, the longest CD44 isoform containing the variants v2–v10 can be detected. Expression of CD44 variants on normal lymphohematopoietic cells is generally low. T lymphocytes activation by antigen or by mitogen leads, however, to transient expression of variant isoforms such as CD44v6 (4, 5). CD44v6 isoforms are also found in proliferative tissues such as the skin or the intestine (Figure 1).

Overexpression of various CD44 isoforms has been found in many types of human tumors. A comprehensive review was published by Naor et al. (6). In colorectal cancer, specific CD44 isoforms are expressed according to the progression of the disease (7). Isoforms containing the v5 exon could already be detected in early adenomas. The expression of v6-containing isoforms was

detected in early and advanced polyps as well as in invasive carcinomas where it correlated with the Duke stage (7). Interestingly, a cell-specific splicing was described in the intestine (8). Remarkably, the lgr5⁺ stem cells were shown to be CD44v4-v10⁺ whereas transit-amplifying cells exclusively expressed CD44s or smaller CD44v isoforms but not CD44v4-v10. Human cryptic foci corresponding to microadenomas retaining a stem cell program express CD44v4-v10 as well but not CD44s. In breast cancer, expression of CD44v6 was detected in intraductal carcinoma and was related to tumor invasion, metastasis, and pathological grade (9). CD44s and CD44v6 were shown to be up-regulated in pancreatic cancer adenocarcinoma (10). CD44s expression but not CD44v6 was associated with worse overall survival. In head and neck cancer patients, high expression of CD44v6 prompted the development of a CD44v6 antibody for clinical trial phase I [reviewed in Ref. (2)].

CD44 ACTS AS A SIGNALING HUB

In some cases, the role of CD44 in tumor progression could be linked to its function as the main receptor for hyaluronan (HA), a major component of the extracellular matrix (ECM) [reviewed in Ref. (11)]. This was, for example, the case in breast cancer (12). The question remains whether other molecular functions of CD44 drive its involvement in specific types of cancers. Accumulating evidence demonstrate that CD44 acts as a signaling hub controlling cell surface receptors of very diverse structure and function, e.g., CD44v6 isoforms act as co-receptors for RTKs such as Met, Ron, or VEGFR-2 [reviewed in Ref. (11)]. The function of CD44v6 for these RTKs is a twofold. On one hand, the CD44v6 ectodomain drives the activation process of the RTK. On the other hand, the cytoplasmic domain recruits ezrin–radixin–moesin (ERM) proteins together with the cytoskeleton in order to promote signaling from the RTK. CD44v6 and Met could be found in a complex

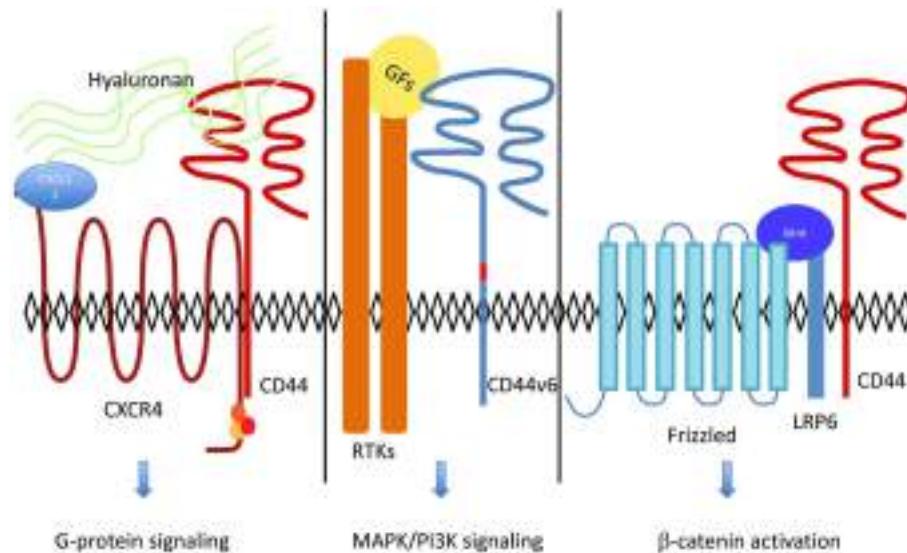


FIGURE 1 | CD44 acts as a co-receptor for several cell surface receptors including RTKs, G-protein coupled receptors, and LRP6. Hyaluronan binding to CD44 increases CXCL12-induced CXCR4-G Protein signaling (18). RTKs activation by their ligands [growth factors

(GFs) such as HGF or VEGF] and downstream signaling are dependent on CD44v6 [reviewed in Ref. (11)]. In the Wnt- β -catenin pathway, LRP6 recruitment of CD44 leads to β -catenin activation and translocation to the nucleus (3).

with the Met-ligand HGF (13). Furthermore, Met internalization was shown to depend on CD44v6 (14). Intriguingly, the function of the CD44v6 isoform for VEGFR-2 is very similar to the CD44v6/Met pair. VEGF-induced VEGFR-2 activation depends on CD44v6 and in addition VEGFR-2-induced signaling requires the link of CD44v6 to ERM proteins and to the cytoskeleton (15). Moreover, CD44v6 and VEGFR-2 could be found in a complex, which was not inducible by VEGF. Most strikingly, CD44v6-dependent Met (16) and VEGFR-2 activation (15) could be blocked by small peptides containing a CD44v6 exon sequence; these peptides inhibited the angiogenesis of pancreatic tumors (15).

A participation of various CD44 isoforms in the activation of other RTKs (EGFR, PDGFR, or FGFR) was reviewed in Ref. (11). For example, CD44v3 heparan-sulfated isoforms were shown to act as a recruitment platform for metalloproteinases like MMP-7 (or matrilysin) involved in the maturation of HB-EGF and subsequent activation of ErbB4 (17).

A completely different receptor, namely, CXCR4 also appears to be dependent of CD44 (18). This time the binding of HA to CD44 is instrumental for this co-operation. High-molecular weight HA binding to CD44 augmented the activation of CXCR4 by its ligand CXCL12 whereas small HA fragments completely abrogated the CXCL12-induced CXCR4 signaling. CD44 and CXCR4 were found in a CXCL12-dependent complex regulated by HA. Interestingly, this interaction appears to be crucial for angiogenesis.

More recently, the Wnt-target gene CD44 (19) was shown to regulate Wnt signaling (3). Overexpression or downregulation of various CD44 isoforms modulated positively or negatively, respectively, Wnt activity. Downregulation of CD44 expression inhibited activation of β -catenin and subsequent translocation to the nucleus. The role of CD44 in Wnt signaling was independent of its binding to HA. In contrast, the binding of the CD44

cytoplasmic domain to ERMs was instrumental. Epistasis experiments revealed a function of CD44 at the level of LRP6 and both LRP6 and CD44 could be found in a Wnt-inducible complex. The function of CD44 for LRP6 is dual. On one hand, CD44 controls LRP6 activation, a Wnt-dependent event. On the other hand, CD44 is involved in LRP6 maturation, a Wnt-independent step. Indeed, downregulation of CD44 expression lead to a lack of LRP6 membrane expression and to an inhibition of Wnt-induced LRP6 phosphorylation. Downregulation of CD44 in the central nervous system of *Xenopus laevis* embryos impaired expression of Wnt-regulated genes such as eng-2 and tcf-4. CD44 is therefore able to act as a Wnt-target gene as well as a Wnt regulator.

ALTERNATIVE SPLICING OF CD44 ISOFORMS IN TUMOR PROGRESSION

Since CD44s and CD44v isoforms are involved in tumor progression and metastasis alternative splicing of CD44 seems to be a decisive event controlling the progression of cancer. Several years ago, a mini-gene construct was used to investigate exon v5 alternative splicing and the relevance of signal transduction for this process was shown (20). The nuclear RNA-binding protein Sam68 was shown as a decisive factor controlling CD44 splicing (21). This splice regulator was under the direct control of Erk and consequently under control of Ras confirming the involvement of growth factor signaling in the regulation of CD44 alternative splicing. Moreover, a positive feedback loop in which Ras signaling-induced CD44v6 splicing was unraveled. In turn, CD44v6 promoted late Ras signaling, which was shown to be important for cell cycle progression (22).

The regulation of CD44 alternative splicing during tumor progression is still not completely unraveled. Conversely, the steps in tumor progression regulated by alternative splicing of CD44

still need to be defined. Some indications came from studies on epithelial–mesenchymal transition (EMT). EMT is a process by which epithelial cells loose their polarity, gain invasive properties, and acquire mesenchymal features. On one hand, EMT was shown to induce a CD44⁺ phenotype (23). On the other hand, a shift from CD44v to CD44s was essential for cells to undergo EMT as well as for the formation of breast tumors. In this case, a decreased expression of the splicing factor epithelial splicing regulator 1 (ESPR1), which promotes the switch to CD44v isoforms was critical for EMT. In other studies, the heterogeneous nuclear ribonucleoprotein M (hnRNPM) was identified as an essential splicing regulator involved in TGFβ-induced EMT (24). hnRNPM-mediated CD44 exon skipping was induced through inhibition of ESPR1 function and was essential for breast cancer metastasis.

In contrast to the above described studies, colonization of the lung by 4T1 mouse breast cancer cells was shown to be dependent on the switch from CD44s to CD44v isoforms (25). This time the knockdown of the ESPR1 protein led to the reduced cell surface expression of the Na⁺-independent cystine transporter xCT and suppressed lung colonization.

In the same line, the activation of the Wnt pathway, a central player in EMT, induced CD44v6 expression in colorectal cancer cells [reviewed in Ref. (26)] as well as in breast cancer cells (27).

FUNCTION OF CD44 ON CANCER STEM CELLS

A link between EMT and stemcellness was demonstrated using immortalized mammary epithelial cells (23). EMT was shown to generate cells with many of the properties of self-renewing stem cells. These cancer stem cells (CSCs) that have the ability to seed a tumor were shown to be CD44⁺. CD44 is also expressed on several other types of CSCs including pancreatic and colorectal CSCs [reviewed in Ref. (28)]. However, little is known on the molecular function of CD44 in these CSCs. A link between the presence of specific CD44 isoforms on CSCs and their function as co-receptors might exist. Several years ago, CD44 and EpCAM were described as robust markers of colorectal CSCs (29). More recently, the CD44v4-v10 isoform was detected on lgr5⁺ stem cells in the intestinal crypts. The presence of this CD44 isoform on colorectal cancer cells was linked to tumor progression in Apc^{Min/+} mice. The function of CD44 in colorectal cancer might be due to its role as a modulator of Wnt signaling (3) or as a Wnt-target gene since one partner of CD44v6, namely the Met RTK, is also over-expressed in colorectal cancer (30). Met overexpression could be detected already in dysplastic aberrant crypt foci, one of the earliest lesions in colorectal cancer. Similarly to CD44, Met expression seems to be controlled by Wnt. Therefore, collaboration between the Wnt-target genes Met and CD44v6 might be required for the progression of colorectal cancer. This idea was further strengthened by the finding that the metastatic potential of colorectal spheres orthotopically injected in the mouse cecum was abrogated by depletion of CD44v6 and Met (31). Furthermore, in colorectal CSCs, the expression of CD44 is controlled by several cytokines including HGF by activating the Wnt pathway thereby promoting migration and metastasis (31).

Interestingly, circulating primary luminal breast cancer cells containing a population of metastasis-initiating cells (MICs) express among other markers Met and CD44 (32). However,

whether any interplay between Met and CD44v6 is necessary for the metastatic potential of these MICs is not yet clear.

CD44 was suggested as a marker on acute myeloid leukemia (AML) leukemic stem cells (LSCs) [reviewed in Ref. (28)]. A mAb (H90) mediating CD44 ligation inhibited AML–LSCs homing to the bone marrow and their engraftment. The same antibody was shown to abrogate adhesion of AML CD34⁺CD38[−] cells to HA suggesting that CD44 binding to HA is involved in the homing process and growth in the bone marrow. Moreover, the interaction between CXCR4 on leukemic cells and CXCL12 in the niche is needed for the homing and growth of LSCs (33). Taken together, these data strongly suggest that the molecular function of CD44 and HA in CXCL12-induced signaling (18) might be involved in AML.

OUTLOOK

For long, the role of CD44 in tumor progression and metastasis remained unclear. Many lines of evidence indicate that CD44 organizes a signaling platform at the cell surface by acting as a co-receptor for various types of cell surface receptors (Figure 1). In addition, large amount of data show that CD44 is a bone fide CSC marker. Understanding whether these two aspects are linked will require a better knowledge of the functions of CD44 isoforms at the molecular level and the systematic identification of specific CD44 isoforms expressed on CSCs. A CD44-based therapy targeting CSCs will certainly benefit from specific tools blocking the co-receptor function of CD44 for cell surface receptors.

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