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Spatial and Temporal Sequence of Events in Cell Adhesion: From Molecular Recognition to Focal Adhesion Assembly

Miriam Cohen,[a] Derk Joester,[b] Benjamin Geiger,*[a] and Lia Addadi*[b]

A new concept that attributes a pivotal role to the pericellular coat in the regulation of the early stages of cell adhesion is presented. Quick, adaptable, and transient adhesion through multiple cooperative weak interactions provides the cell with an additional level of modulation in the decision-making process that precedes the commitment to adhesion at a particular site. Hyaluronan emerges as a modulator of cell adhesion in certain cells, mediating binding or repulsion through its polyelectrolyte character, in addition to its chirality and molecular-recognition properties. The biophysical properties of hyaluronan as well as its ultrastructural organization are analyzed in relation to this proposed function.

The Biological Significance of Cell Adhesion

The adhesive interaction of cells with external surfaces is an ancient biological phenomenon and characteristic of essentially all forms of life, from unicellular to the most complex multicellular organisms (metazoa). In a variety of unicellular organisms, transient adhesion to external surfaces is essential for such processes as locomotion toward food sources or away from repellents, attachment to prey, or binding to another cell during mating.[1] The development of metazoan life, some 600 million years ago, is characterized by the dramatic diversification of cell types forming multicellular organisms and the formation of stable tissues and organs. Concomitantly, new and effective strategies for adhesion emerged that enable individual cells to form higher-order structures. In the slime mold *Dictyostelium discoideum*, for example, the free-living amoeboid cells can adhere to a variety of surfaces and actively migrate as long as the environmental conditions are favorable. When confronting starvation, these cells undergo massive aggregation, leading to the formation of a multicellular organism, where cell–cell adhesion and communication take place.[2]

Beginning in the 1960s, ultrastructural studies[3] and subsequent molecular analyses have revealed a rich variety of adhesive structures between neighboring cells or between cells and the extracellular matrix. These, among others, can be linked to different cytoskeletal networks, form intercellular channels, compartmentalize membrane domains, and mediate signaling events. Numerous genes concertedly participate in the formation, maintenance, and regulation of such adhesive interactions.

Molecular Diversity of Cell Adhesions

To illustrate the structural and molecular complexity of cell adhesion let us consider two examples: leukocytes and epithelial cells. The former are short-lived single cells, whose function in protecting the organism from invaders depends on a highly complex adhesive machinery.[4–8] These adhesions involve both protein–protein and protein–carbohydrate interactions, which are regulated by specific signaling factors (chemokines) and their receptors (Figure 1). Epithelial cells, on the other hand, form stable adhesions with their neighbors and with the underlying basement membrane. These include tight junctions that block diffusion of molecules across the epithelial layer, adherens junctions that are associated with the contractile actin cytoskeleton, and desmosomes—linked to the cytokeratin network.[9] Gap junctions are dot-like adhesions forming intercellular channels that allow passage of small molecules from one cell to the next.[10] Attachments to the basement membrane consist of focal adhesions (FA) and hemidesmosomes, each comprised of tens of different proteins, including links to the actin and intermediate filament systems, respectively (Figure 2).[11,12]

How are these complex, multimolecular adhesive systems regulated in space and time? Does each system function as an independent unit or are they coordinated or even interdependent? How can the cell down-regulate its adhesive interaction to allow, for example, cell migration or division?

In this article we will consider the dynamic molecular events involved in the development of cell adhesions, across a wide range of temporal and spatial scales. While first cell contact and recognition occur on a subsecond timescale, full cell spreading takes tens of minutes to hours. During that time, the distance between the cell membrane and the external surface decreases from micrometers to 10–20 nm. Concomitantly,

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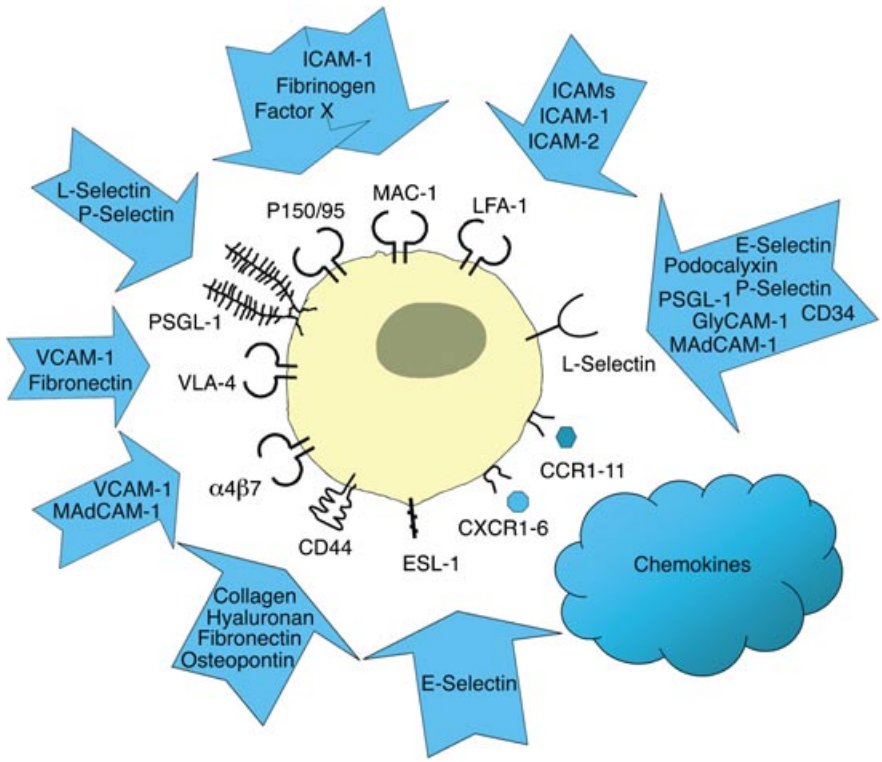

Figure 1. *Diversity and complexity of molecular mechanisms underlying leukocyte adhesion. Leukocytes express a large variety of adhesion molecules, which are important in the response of the cell to inflammation.* Recruitment *of leukocytes from the blood stream to inflammation sites is mainly mediated by interaction of L-selectin on the leukocyte membrane with E-selectin or P-selectin on endothelial cells through the lectin domain.[4] E-selectin also interacts with E-selectin ligand-1 (ESL-1) and P-selectin interacts with P-selectin glycoprotein ligand-1 (PGSL-1) on the leukocytes membrane. In addition, PSGL-1 can interact with L-selectin, forming leukocyte–leukocyte* adhesions.[4] *Inflammation sites are enriched with hyaluronan; CD44, a transmembrane glycoprotein receptor, binds hyaluronan, collagen, fibronectin, and osteopontin, contributing to leukocyte recruitment and rolling.[5]* Leukocyte rolling *involves the interaction of L-selectin with CD34, glycosylation-dependent cell adhesion molecule-1 (GlyCAM-1), mucosal addressin cell adhesion molecule-1 (MAdCAM-1), and podocalyxin.[6]* Rolling arrest *of leukocytes is mediated by interaction of integrin receptors, transmembrane heterodimers of α and β subunits (VLA-4, $\alpha4\beta7$, LFA-1, P150/90, and Mac-1) with intercellular adhesion molecule (ICAM) and vascular-cell adhesions molecule-1 (VCAM-1).[7] Integrin activation involves chemokines, 8–12 kDa heparin-binding proteins with conserved cystein motifs (mainly CXC and CC). Leukocytes express six receptors for CXC chemokines (CXCR1–6) and 11 receptors for CC chemokines (CCR1–11).[8]*

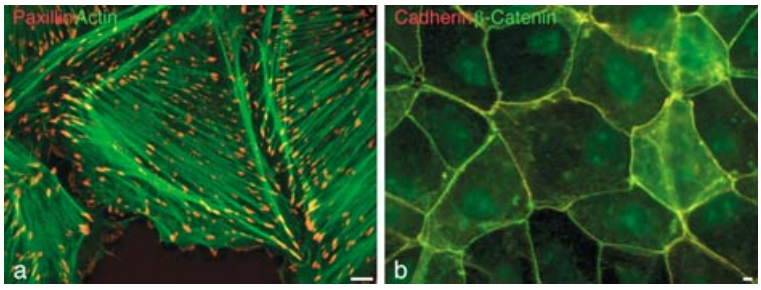

Figure 2. *Diverse adhesive mechanisms in endothelial cells. Immunofluorescent micrographs of a) actin-associated focal adhesions and b) cadherin-β-catenin-containing adherens junctions. a) Pig aortic endothelial cells, fluorescently immunolabeled for paxillin (red), one of the many focal adhesion components,[12] and actin filaments (green). Focal adhesions mediate cell attachment to the extracellular matrix.[11] b) Bovine capillary endothelial cells, immunolabeled for the Ca^{2+}-dependent cell–cell adhesion molecule cadherin (red) and the plaque protein β-catenin (green), forming adherens junctions[9] (micrograph kindly provided by Noam Erez). Red-green superposition appears yellow. Scale bar 5 μm.*

The Elementary Modules of Cell Adhesions

Despite the enormous heterogeneity of adhesion systems, there are some common and distinctive features that characterize the molecular interactions frequently found in these sites. Adhesion receptors can mediate direct protein–protein interactions with the external surface. Such receptors are usually single- or multiple-chain transmembrane proteins with an extracellular domain involved in binding to the external surface and an intracellular domain that can interact with the cytoskeleton. Typical examples for such receptors are members of the integrin family, which mediate adhesion to the extracellular matrix or to other cells,[13] and cadherins, which form Ca^{2+}-dependent cell–cell junctions.[14] In such systems, the characteristic spacing between the plasma membrane and the "adhesive ligand" on the external surface is of the order of 15–20 nm.

Another common mechanism involves interactions between lectin-like protein receptors and their carbohydrate ligands on the external surface. Examples for such receptors include among others different selectins, the hyaluronan receptor CD44, and galectins.[4,15] Their ligands are various glycoproteins and glycosaminoglycans.[16] These adhesions can be formed directly with ligands on the external surface, or indirectly, through a carbohydrate-rich membrane-bound (pericellular) "coat".[17] The membrane-to-surface spacing of such adhesions may vary from a few tens of nanometers to micrometers, depending on the carbohydrates involved and on whether the interaction with the surface is direct or indirect. Moreover, some carbohydrate-specific adhesion receptors, such as CD44, can themselves be glycosylated and serve as ligands for other lectins.[18] Adhesive surface lectins, (e.g. galectin-8) can also be secreted by cells and competitively block, rather than promote, adhesion.[19]

The interactions mediated by adhesive receptors and their ligands can be regulated by a variety of external and internal factors. Cadherins, for example, are activated by ex-

the interface area between the cell and the surface grows from a few to thousands of μm^2 (Figure 3).

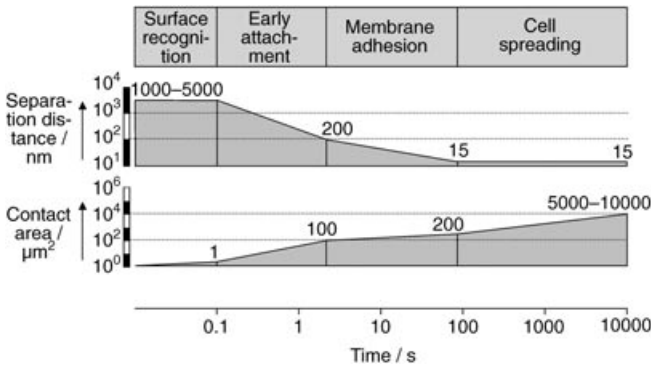

Figure 3. *Evolution of cell–substrate adhesions in terms of separation distance and contact area as a function of time. Initial* cell–surface recognition *occurs within less than a second. At this stage the cell membrane is 1–5 μm from the surface. Within seconds,* early attachment *ensues, anchoring the cell to the substrate. Reorganization of the pericellular coat enables the cell membrane to approach the surface. At this stage the cells have not yet flattened, but the contact area increases to about 100 μm². Transition to the* membrane adhesion *stage takes on the order of minutes. Integrins begin to interact with RGD epitopes on the surface, initiating the formation of focal adhesions where the separation distance decreases to 15 nm. At the last stage,* cell spreading*, the cell contact area increases by two orders of magnitude within a few hours.*

tracellular calcium ions, which affect the conformation of the extracellular domain and promote the formation of an "adhesive zipper". Integrins can undergo an activation when changes in the relative position of the α and β chains lead to an exposure of the binding domain.[20] Another important factor in the regulation of adhesive interactions is the cytoskeleton, which can affect adhesion by at least two distinct mechanisms. Tethering of adhesion receptors to the cytoskeleton can greatly increase the avidity and stabilize multivalent molecular interactions. Moreover, application of mechanical forces to adhesion sites (characteristic for cytoskeleton-associated adhesions) stimulates their growth.[21]

Molecular Events Associated with the Formation of new Adhesions

Unraveling the basic processes of cell adhesion requires an understanding of how multiple adhesive mechanisms are regulated and coordinated in time and space, such that the adhesion is selective, efficient, and dynamic (reversible). Herein we shall examine conceptually an approach to solve this problem, as it has evolved in certain cells.

Consider a cell, suspended in aqueous medium, approaching a surface. Such a surface will display varied, repetitive, and sometimes periodic patterns of charged groups, hydrogen-bond donors and acceptors, and lipophilic patches. Counter ions and bound water molecules of varying mobility complete the picture. Individually, these form what we shall refer to as *molecular binding sites* (as opposed to protein, transmembrane receptors) that can interact with molecularly compatible partners on the surface. Generally, they show little specificity in binding; for example, there is no selectivity for a particular charged group or even for the chemical nature of a charged group. Occasionally they might, however, be highly specific to certain molecular counterparts, such that stereoselective or even enantioselective interactions (see below) are observed. Individually, the energy involved in each of these interactions is relatively small, of the order of a few kcal mol^{-1} or a few k_BT per interaction. Extensive cooperativity may, however, build up to substantial interaction energies. In three-dimensional arrangements, multiple molecular binding sites constitute ligands (*epitopes)* for highly oriented, localized, and (stereo-) chemically specific recognition and binding by matching *receptors* on the cell (e.g. integrin-RGD). Recognition in this sense involves binding between matching (e.g. electrostatic, polarity) topographies, and is not limited in complexity to a single receptor–epitope pair. Rather, it can involve any number of simultaneous contacts in a multireceptor cluster. Such receptor–epitope clusters can contribute up to several tens of kcal mol^{-1} in binding energy.[22,23]

Attractive interactions (i.e. electrostatic or hydrophobic) both between complementary molecular binding sites and between receptors and epitopes operate on a very short range, and may occur only at distances not much larger than 5 Å in aqueous salt solutions.[24] Any binding events between cell-based binding sites or receptors and surface elements thus require prior removal of water, solutes, and membrane constituents that might mask interactions between the cell and the substrate.

Adhesive Interactions as a Multistep Process: The Concept

Time-wise, the establishment of receptor–epitope based complexes such as integrin–RGD contacts is observed to occur within a framework of minutes after the first molecular interactions have been established.[25] This lag depends on the surface densities of both receptor (ca. 5×10^3 μm^{-2})[26] and epitope, which determine the frequency of mutual encounters. As complex three-dimensional interactions require an appropriate relative orientation between the interacting partners, only a negligible fraction of random encounters would develop into a stable interaction. Binding of pericellular components to the surface, in contrast, may occur within less than a second.

Given these premises, it is reasonable to assume that rapid and transient interactions must ensue first. The transition of a cell in suspension to interacting with a surface could then be based on a subtle interplay of recognition, cooperativity, and kinetic parameters. A great number of simultaneous but rather weak interactions could conceivably lead to a transient bound state, where the multiplicity and cooperativity of weak interactions provide the necessary binding strength. If the arrangement is dynamic, it can both adapt itself to three-dimensionally structured surfaces and quickly accommodate subsequent changes in organization. Such is the interaction between hyaluronan and substrate that we shall consider below.

The adhesion model that derives from the above assumption would then include at least three consequent steps:

1) Recognition and establishment of contacts between pericellular components and complementary binding domains

on the substrate. These contacts involve instantaneous, multiple, cooperative, and sometimes stereoselective molecular recognition.

2) Pericellular component-mediated contacts trigger the approach of the membrane-bound receptors to the substrate.
3) Interactions between integrins in the membrane and their binding domains (e.g. fibronectin) on the surface initiate focal contact development and maturation, including linking to the cytoskeleton.

Although the existence of pericellular components is well documented, their direct participation in cell adhesion events has been firmly established only for few systems, notably selectin–carbohydrate interactions in blood cells.

We report below evidence for and characterization of similar processes for epithelial and chondrocyte (cartilage-forming) cells. This evidence puts the process in a new perspective and suggests that such events might be much more widely spread than suspected so far, and that they might regulate the "decision-making process" of cells vis-à-vis the establishment of stable contacts with substrates.

Adhesive Interactions as a Multistep Process: Experimental Evidence

The possibility of very fast and dense interactions between substrate and some component present on the cell membrane was forcefully brought to our attention in the attachment of certain epithelial cells to one crystal surface type (but not the other) of calcium tartrate tetrahydrate crystals.[27] It was subsequently proven to be operative also in the adhesion of the same cells to more conventional substrates, such as glass and tissue culture dishes. The stereoselectivity of the interaction, manifested in the fast and dense attachment of cells to calcium-(*R*,*R*)-tartrate, but not calcium-(*S*,*S*)-tartrate crystals (the molecular and structural mirror image), was the give-away evidence for the identity of the component. This had to be a chiral cell-associated biopolymer, presumably a polysaccharide, that was eventually identified as being hyaluronan.[26,28] Not only did hyaluronan adsorb selectively on the crystal that the cells attach to, and not to the mirror image crystal, but its removal by hyaluronidase hydrolysis substantially reduced cell attachment to the crystal surfaces and to glass and cell culture substrates.[26]

The glycosaminoglycan hyaluronan[29–31] is a high molecular weight (up to 10×10^6 Da), polydisperse linear polysaccharide composed of several thousand repeat units (Figure 4a).[29] In the absence of proteins and at physiological conditions, hyaluronan behaves as a weak polyelectrolyte.[30]

Recognition and immediate establishment of extensive cooperative contacts between hyaluronan and complementary binding domains on the substrate requires that a continuous and relatively thick hyaluronan coat cover the whole surface of the cell in suspension (Figure 5a, b). What are the density and the thickness of the putative hyaluronan coat?

We have demonstrated that the hyaluronan pericellular layer on A6 epithelial cells has a thickness of approximately 2 μm,

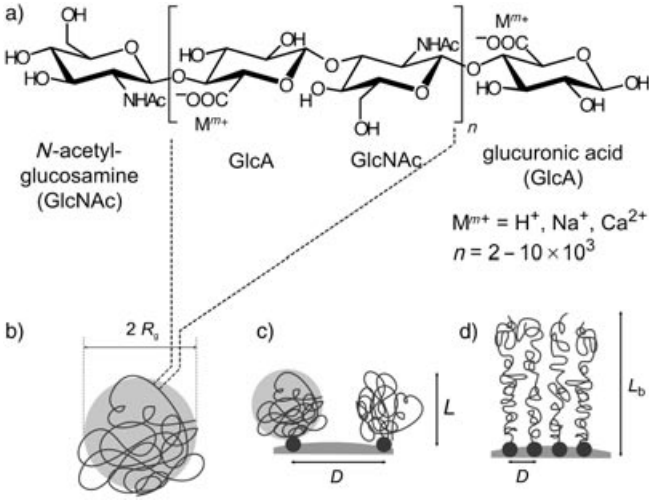

Figure 4. *a) Chemical structure of salts of hyaluronic acid (hyaluronan). The polymer chain can contain more than 10^4 disaccharide repeat units, which consist of β-1,3-glucuronic acid and β-1,4-*N*-acetylglucosamine. The acid functions of glucuronic acid are spaced at roughly 1 nm along the chain. b) Under physiological conditions hyaluronan of 2×10^6 Da molecular weight (5000 repeat units, 5 μm total length) behaves as a weak polyelectrolyte at the high-salt limit, basically indistinguishable from a nonassociating neutral polymer.[30] It forms random coils with a characteristic radius of gyration (R_g) of 180 nm. It is considered a semi-stiff polymer, with a persistence length of 4–8 nm[31] (DNA with a persistence length of 50 nm is considered stiff). The chains overlap and entangle at low concentrations (overlap at $c > c^* = 0.59\ mg\,mL^{-1}$; entanglement at $c > c_e = 2.4\ mg\,mL^{-1}$).[30] Hyaluronan from bacterial, animal, or human sources is found to be a strong gel former at concentrations ranging from 0.1 $mg\,mL^{-1}$ (vitreous of the eye) to 4 $mg\,mL^{-1}$ (synovial fluid).[29] Reversible gel formation seems to depend on the presence of proteins or multivalent cations. c) If the average distance D between binding sites of hyaluronan (solid black circles) on the cell surface is greater than the coil diameter ($2R_g$), a so-called mushroom type surface will form, where the layer thickness L is on the order of magnitude of hundreds of nanometers. d) If D becomes smaller than the coil diameter, the chains are forced to stretch and a dense brush may form. In a brush the roughly parallel chains are bound at one end. The other end is free and usually located at the periphery of the brush. When grafted onto a flat surface, brushes are continuous, their density is high and constant throughout the brush and drops off steeply at the edge. The thickness of a hyaluronan brush L_b can potentially reach micrometer scale.*

while it reaches up to 5 μm thickness around chondrocytes in suspension. Furthermore, the 2 μm thick pericellular coat around the epithelial cells establishes a "rigid" nondeformable contact with glass substrates, which is strong enough to resist a shear force of 6.5 dyn cm^{-2} (0.65 pN μm^{-2}), applied by flow.[32] Conversely, the 5 μm thick pericellular coat of chondrocytes establishes "soft" contacts, which react to shear by sliding on the glass surface, leaving a hyaluronan "trail" behind the cell.[32] We suggest that the hyaluronan coat consists of one layer of densely packed hyaluronan molecules, directly anchored to the membrane of epithelial cells to form a brush (Figure 5c, e). In contrast, chondrocytes have multiple layers of entangled and cross-linked hyaluronan molecules (Figure 5d, f). Other proteins and glycosaminoglycans such as aggrecan and heparan sulfate most probably contribute to the integrity and to the properties of the coat.

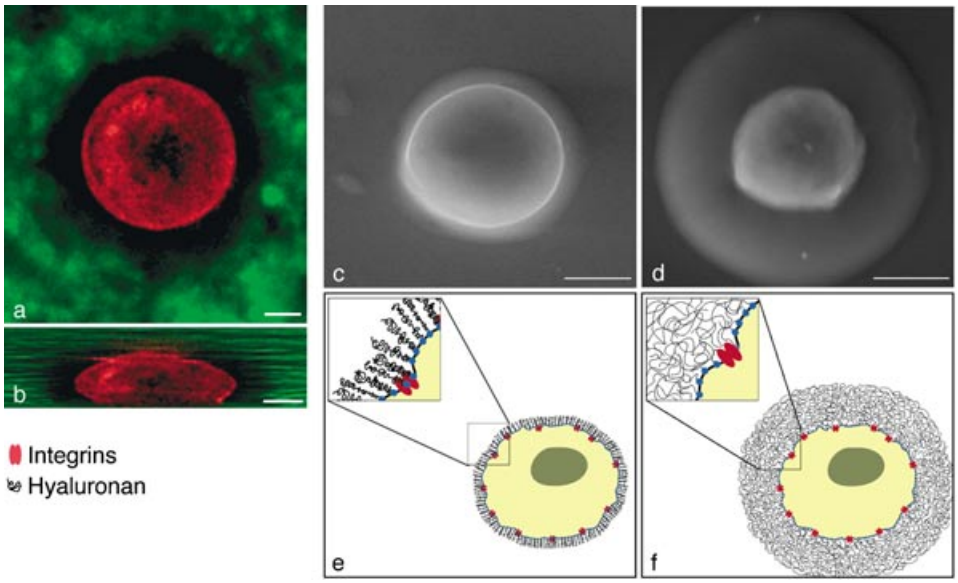

Figure 5. *The hyaluronan pericellular coat. a) Fluorescence micrograph (xy section) of a particle exclusion assay featuring a 5 μm wide excluded zone (dark) around rhodamine-labeled chondrocytes (red) immersed in FITC-labeled silica beads (green). b) An xz section of the cell in a) was generated by image reconstruction (0.5 μm resolution). An excluded zone is visible on top of the cell, confirming that the pericellular coat completely envelops the cells. c) and d) Environmental scanning electron micrographs of the hyaluronan pericellular coat. The coat thickness around epithelial cells is 2.2 ± 0.4 μm (c), around chondrocytes it reaches 4.4 ± 0.7 μm (d). e) Schematic representation of a hyaluronan brush anchored to the cell membrane of the epithelial cell. f) Schematic representation of the soft, thick layer of entangled hyaluronan around chondrocytes. The first layer in contact with the cell membrane may also be a brush. Scale bar is 5 μm.[32] FITC = fluorescein isothiocyanate.*

Pericellular Hyaluronan Regulation of Cell Adhesion: Biophysical Considerations

The following theoretical considerations support the above model: hyaluronan deposited on a surface without space constraints forms typically a layer of 200 nm thickness, on the order of magnitude of the characteristic molecular radius (radius of gyration R_g, Figure 4b). However, if there is a sufficient number of membrane binding sites (e.g. CD44) for hyaluronan such that the distance between individual chains bound on the surface is less than the radius of gyration (ca. 180 nm for a 2 MDa chain), the chains could be forced to stretch out and form an "Alexander-de-Gennes" polymer brush (Figure 4c, d).[33,34] This brush is in fact in an equilibrium state in which the osmotic pressure exerted by the tethered polymer in solution drives the stretching of the chain. This is opposed by the elastic energy of the polymer chain, which, for entropic reasons, prefers the coiled state. The brush thickness is primarily a function of molecular weight and grafting density.[33–36] The molecular weight distribution of hyaluronan in the pericellular coat of chondrocytes or A6 epithelial cells is not known. It seems reasonable, however, to assume an average weight of 2×10^6 Da (5 μm total length). With a chain density where the distance between chains is between 100 and 10 nm (equivalent to 10^2–10^4 binding sites per μm^2), the brush thickness could reach 2 μm.

The presence of such a thick layer of hyaluronan must influence the subsequent fate of the adhesion and of any other signaling process to the cell. In particular, the layer must be modified or removed to make way for receptor–integrin contacts. Any of at least three distinct processes, or combinations thereof, may conceivably foster the evolution of contacts to the second stage. Hyaluronan can be removed from the contact site by: 1) directed (lateral) diffusion or active transport of the hyaluronan and the attached membrane receptors, 2) hyaluronidase-induced hydrolysis or other degradative removal, or 3) collapse of the hyaluronan brush through interaction with the surface or by cell-induced changes of the effective hyaluronan receptor density (Figure 6).

The density and affinity of the molecular contacts will have a determining effect on the process. It may be expected that establishment of a tight network of high affinity and high density contacts will lead to rapid attachment, which may be too strong and essentially irreversible (which is incompatible, for example, with cell motility). This was observed on the {011} faces of (*R*,*R*)-calcium tartrate crystals, where the cells attached but did not spread further.

The situation of high affinity but low density receptor contacts is well represented by the integrin interaction, which is consequently slow but stable, once formed. Alone, the time and space scales of these interactions are not sufficient to warrant adhesion. This is demonstrated by the inability of hyaluronidase-treated cells to develop adhesion despite the presence of integrins on their surface. These contacts have to be preceded by the establishment of low-affinity, high-density transient contacts, such as those of hyaluronan. On the other hand, low-affinity and/or low-density contacts will not be efficient. The last case is represented by the inability of hyaluronan-coated cells to attach to hyaluronan-coated surfaces, on the {101} faces of calcium-(*R*,*R*)-tartrate, or on any face of calcium-(*S*,*S*)-tartrate.[28]

Concluding Remarks

Herein we have developed the concept of a pivotal involvement of the pericellular coat in the early stages of cell adhesion. Quick, adaptable, and transient adhesion through multiple cooperative weak interactions provide the cell with an additional level of modulation in the decision-making process that precedes the commitment to adhesion at a particular site. Hyaluronan emerges thus as a mediator and modulator of cell adhesion, through its properties of electrostatic attraction or

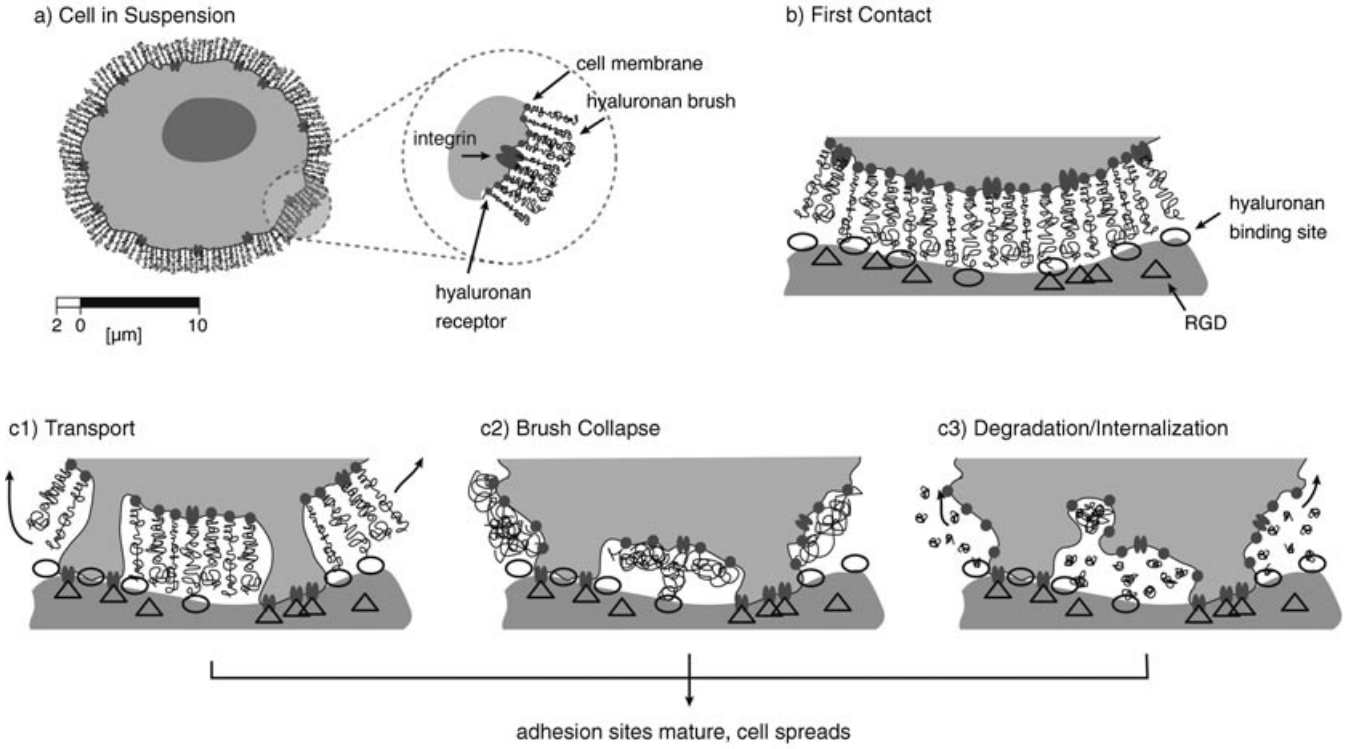

Figure 6. *Tentative phases leading to adhesion of cells covered with a hyaluronan brush of several microns thickness. The drawing is roughly to scale for epithelial cells (10 μm radius, 1–2 μm pericellular coat). a) Cell in suspension approaches surface. b) Early attachment: Hyaluronan establishes contact to molecular binding sites on the surface (circles). The cell is thus bound, but its membrane is still several microns away from the surface. From this stage, any one (or a combination) of three following processes could lead to the next phase: c1) Receptor-bound hyaluronan diffuses or is actively pushed/pulled away from the developing adhesion site. The underlying membrane is exposed, a protrusion can be directed towards the surface, and integrins (double ovals) can now bind to RGD sites (triangles) on the surface. c2) Either by binding to the surface or by cell-induced changes in the medium the brush collapses, the cell is drawn to the surface, and integrin-mediated binding becomes possible. c3) The hyaluronan is either internalized or degraded by hyaluronidases. The cell can thus get closer to the surface and integrins can bind to RGD domains (triangles). Once Integrin-RGD contacts have been established, adhesion sites mature and the cell spreads.*

repulsion due to its polyelectrolyte character, in addition to its chirality and molecular recognition properties.

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Keywords: carbohydrates · cell adhesion · hyaluronan · pericellular coat · polyelectrolyte brush

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