ORIGINAL RESEARCH

Hyaluronan oligosaccharides are potential stimulators to angiogenesis via RHAMM mediated signal pathway in wound healing

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Abstract

Purpose: To determine if oligosaccharides of hyaluronan (o-HA) promotes wound recovery by accelerating angiogenesis and to study the mechanisms by which o-HA stimulates endothelial cell (EC) proliferation.

Methods: Using hyaluronidase digestion, we prepared a mixture of hyaluronan (HA) fragments sizesd 2 to 10 disaccharides units, and studied their effects on EC growth and migration in mimicking wound recovery *in vitro*. The effects of o-HA on EC growth *in vitro* were studied by counting cell numbers. The roles of 2 hyaluronan receptors on EC cells, CD44 and RHAMM (Receptor for HA-Mediated Motility), were studied in initiating signaling cascades, using immunoblot assay. Signal transduction was determined by blocking antibodies to CD44 and RHAMM. An *in vitro* wound healing model was prepared by scratching the cellular layer of cultured EC, and movement of cells into the denuded area was quantified.

Results: o-HA was a strong stimulator to EC proliferation at low concentration 10μg/ml compared with native high molecular weight HA (n-HA) (P<0.01). Signal transduction may be initiated by o-HA via RHAMM receptor on EC membrane, but not CD44. In the *in vitro* model, the lesion area was nearly completely recovered when the EC layer was exposed to o-HA 40hrs post-injury, whereas the wound area remained half recovered pretreated with native undegraded large HA and control medium.(P <0.05 from 24 to 40hrs).

Conclusion: Hyaluronan oligosaccharides may play a role in wound healing by increasing angiogenesis. o-HA-RHAMM binding dependent signal transduction pathway may be important in the regulation of angiogenesis associated with EC proliferation.

List of Abbreviations

GAG	glycosaminoglycan
HA	hyaluronan
o-HA	oligosaccharides of HA
n-HA	native HA
EC	endothelial cell
PIEC	porcine vascular endothelial cell
RHAMM	receptor for HA-mediated motility
MAPK	mitogen-activated protein kinase
ECM	extracellular matrix
GAPDH	glyceraldehydes-3-phosphate dehydrogenase

Wound healing or tissue injury repair is a problem. Angiogenesis, the formation of new blood vessels, is an essential feature of tissue development and wound healing, and is a dynamic process that is highly regulated by signals from the surrounding extracellular matrix (ECM).[1] Tissues or organs survive because of the appropriate development of blood vessels that

supply oxygen and nutrients and remove metabolic by-products. Angiogenesis has drawn increasing attention in recent years for its biological functions in a variety of pathological processes. Excessive vascularization occurs in rheumatoid arthritis[2], diabetic retinopathy[3], psoriasis[4], and neoplasia.[5] Angiogenesis is tightly controlled in remodeling tissues such as wounds. Numerous factors associated with angiogenesis have been studied. The best known stimulators of angiogenesis include fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF). Since these factors are proteins they may be digested in wound healing treatments, resulting in loss of biological activity. Currently, the only growth factor approved for clinical use is PDGF.[6] Although growth factors are critical to angiogenesis [7], little is known about their roles in wound healing. In addition to known growth factors, such as VEGF and PDGF, other angiogenic constituents need to be studied and developed. Hyaluronan (HA), a member of the glycosaminoglycan (GAG) family, is a non-protein extracellular molecule. HA is closely related to the tissue repair process and has been used in clinical treatment. Of the biological activities of HA, the most interesting finding may be its degradative fragments that are able to stimulate angiogenesis.[8] Little is known about such HA oligosaccharides on their role in regulating angiogenesis and as a treatment for wound healing.

Structurally, hyaluronan is a polymer of glucoronic acid alternating with *N*-acetyl glucosamine, and is of huge molecular weight (~10^7 Da). Unlike other GAGs, HA contains no sulfate groups or epimerized uronic acid residues. Physiologically, HA occupies a large hydrodynamic domain that greatly influences the hydration and physical properties of tissues. It usually interacts with other ECM macromolecules, including the proteoglycans aggrecan and versican that are essential to the structure and assembly of several tissues. Hyaluronan is associated with the tissue repair process.[9,10] Native high molecular hyaluronan is an integral part of the extracellular matrix where it provides primarily a structural role. However, hyaluronan oligosaccharides (the fragments digested from large molecular hyaluronan) are different functionally from their parental native non-degradation state. The most important biological function of hyaluronan oligosaccharide (o-HA) may be that it promotes angiogenesis, *in vivo* and *in vitro* models, to benefit the healing of acute and chronic wounds.[11,12] Recent studies have focused on the role of o-HA in stimulating angiogenesis associated with pathological and physiological processes. The biological active o-HA is usually reported to be in molecular sizes between 3 and 10 disaccharides units [13] that are not easy to digest further.

We examined the effects of defined length o-HA mixtures (2-10 disaccharides or 4-20 mers) on wound recovery using an *in vitro* model in which the endothelial cell layer was artificially wounded and the recovery was determined. We also assessed the effect of o-HA on invasive properties of endothelial cells in an *in vitro* angiogenesis model. We tried to determine which cellular signal pathway was involved in the cell proliferation when subject to the effect of o-HA. The biological activities of o-HA/HA are believed to be initiated through cell surface receptors (like CD44 and receptor for HA-mediated motility, RHAMM), resulting in signal transduction activation and ultimately mitogenesis.[11] Thus, we examined the binding of o-HA to CD44 and RHAMM on triggering cell signal transduction.

Methods

Media and Reagents.

Human umbilical cord HA, bovine testicular hyaluronidase, and all other laboratory reagents unless specified otherwise, were obtained from Aldrich-Sigma. Monoclonal or polyclonal antibodies against *p44/42* MAPK (mitogen-activated protein kinase), phospho-Src *(p-Src)*, *c-myc*, GAPDH (glyceraldehydes-3-phosphate dehydrogenase, for internal standards in Western blotting assay), and HRP-conjugated rabbit anti-mouse or goat anti-rabbit IgG

were obtained from Cell Signal Technology (Beverly, MA). Mouse neutralizing monoclonal antibody to CD44 (clone: 515) was obtained from BD Biosciences Pharmingen (San Diego, CA), and rabbit antibody to RHAMM (N-20, sc-16169,) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse $IgG_1\kappa$ (clone 103.7) and rabbit IgG were served as isotype controls for antibodies against CD44 and RHAMM, respectively. ECL (enhanced chemiluminescence) kits were from Super Signal® West Pico (Pierce Biotechnology, Inc. Rockford, IL). Porcine iliac vascular endothelial cell line (PIEC) was a gift from Dr. Zhang L (Department of Radiation Oncology, University of Rochester Medical Center, US) and cultured routinely in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 100U/ml penicillin-streptomycin. All reagents, media, and buffers were checked by Limulus amebocyte lysate tests.

Preparation of the HA oligosaccharides

HA oligosaccharides were prepared as described previously.[14] Native high-molecular hyaluronan (n-HA) was digested by hyaluronidase and fragments from 2-10 disoligosaccharides units (4-20 mers) were pooled. Endotoxin activity was checked by *Limulus* amebocyte lysate assay and the endotoxin content in prepared HA oligosaccharide was assayed to 0.06 U/ml for cell culture.

EC Proliferation Studies

PIEC was grown at a concentration of 2×10^4/ml in complete medium containing 10% FCS, in triplicate 24-well plates. After attachment, medium was replaced with serum poor medium, containing 2.5% FCS in which the cells grew at a reduced rate. Both dose-dependent and time-dependent proliferation studies of o-HA on PIEC growth were carried out. First, a series concentrations of o-HA (0, 1, 3, 5, 10, 20，50，100μg/ml) were added to the culture of EC. Second, after choosing the appropriate concentration of o-HA at which EC growth was promoted the most, the corresponding o-HA was incubated with EC for periods of times (0, 6, 12, 24, 48, 72，96hr). Control wells contained appropriate concentrations of n-HA and vehicle (DMEM) (the same concentrations as o-HA), respectively. Trypan blue exclusion studies confirmed that o-HA was not cytotoxic to cells (data not show). After various times of culture, the cellular proliferation was quantified by counting the cell number on a Vi-Cell XR Coulter counter (Coulter Electronics, Hialeah, FL). Triplicate experiments were repeated at least five times.

Wound Recovery Assay

The EC injury model has been described in detail elsewhere[15] and was modified as follows. Cells cultured on slides in culture wells were grown to 75% confluence in RPMI 1640 containing 10% FCS for 24 hrs. Cells were then washed in PBS, and the cellular layer was wounded using a mechanical wounder, rinsed again in PBS to remove loose and dislodged cells. Different amounts of o-HA (1~20 μg/ml) and controls were added to the slides and the wells were incubated at 37°C in RPMI 1640 containing 2.5% FCS for injury recovery observation. The mechanical wounder produced 500μm lesion wide across the cell monolayer. Movement of cells into the denuded area was quantified using an Image-Pro® plus version 5.0 computerized image analysis system (Media Cybernetics, Georgia). For each slide, 10 fields of view were examined at random. The lesion area in each field of view was measured and using the data from time 0 (T_0), the wound area (T_t, 40hrs post-injury) was then converted to give mean % recovery from 3 identically treated slides. The equation is: % r = [1-(wound area at T_t/wound area at T_0)]×100. The experiments were repeated at least three times.

EC Migration assays

The migration assay was performed in 35mm Corning well (Corning Costar, Cambridge, MA) as previously

described.[16] Briefly, 1.5% agarose was dissolved in the culture medium free of FCS to form a gel in the wells. Half of the agarose cylinder was then cut off from each well. EC cells were added into the empty part of the wells and cultured until confluence. Each well was added with the same concentration of cells at 1×10^5/well. The remaining agarose gel filling the other half of the well was removed to allow cell migration towards the resulting free space, and appropriate o-HA (the concentration was adjusted to the one that promoted wound healing) was added. Controls were performed in parallel. Then, a transparent graph paper was stuck to the bottom of the culture plates to allow the measurement of the cell migration into the opposite half vacant part of the culture well. The number of cells migrating into the agarose cut off area was also counted using the Image-Pro® plus version 5.0 computerized image analysis systems. The area of the cells migrating into the vacant well was calculated by computer. Then the data was divided by the area of the well and presented as percentage of the area (%). The experiments were repeated at least three times and data was shown as the migration distance towards controls.

Cell signal transduction pathway study

Expression of CD44 and RHAMM on PIEC cells membrane

To identify the expression of CD44 and RHAMM on PIEC membrane, an indirect immunofluorescent stain assay was performed. Anti-CD44 and anti-RHAMM were used as the first antibodies and FITC-labeled anti-mouse or anti-rabbit IgG as the second antibodies. NIH-3T3 cells were used as controls and were stained with the same antibodies. The expression was observed under the fluorescent microscope routinely.

Immunoblotting with antiphosphotyrosine antibody

EC (80% confluence) was cultured in 24-well plates in complete medium. After attachment, the medium was replaced with serum free medium containing o-HA, n-HA and control (medium only). The concentrations of o-HA or n-HA for the signal transduction study were based on preliminary experiments that showed o-HA at concentration 1μg/ml, 5min was sufficient to cause signal molecule expression (data not shown). Total cell lysates were collected and equal quantities of protein were separated by 12% SDS-PAGE and blotted onto a PVDF membrane using a Bio-Rad electroblotting system (Mini Tran-Blot®). The membranes were blocked overnight at 4°C and the blots were probed with primary antibodies as follows: mitogen-activated protein kinase (*p44/42* MAPK) (1:1000), phospho-Src (*p-Src*)(1:1000), and *c-myc* (1:1000). Anti-GAPDH (1:5000) antibody was used for internal standards. After washing in TBS-Tween, filters were stained with either goat anti-rabbit or rabbit anti-mouse horseradish peroxidase-conjugated secondary antibodies. The blots were developed using a chemiluminescent system (Pierce Biotechnology, Inc. Rockford, IL). Chemiluminescence was photographed using a Kodak Medical X-ray Processor (Kodak, Rochester, NY).

Immunoblotting with anti-CD44 and anti-RHAMM antibodies

To determine which receptor was involved in o-HA response in signal transduction, a blocking experiment was performed. EC cultures (80% confluence) were pretreated with mouse anti-CD44 monoclonal antibody (80μg/ml), rabbit anti-RHAMM antibody (80μg/ml), or normal mouse and rabbit IgG (80μg/ml) for isotype controls at 37°C for 12hr. The cells were then stimulated with o-HA or n-HA at 1μg/ml for 5 min. Following stimulation, the cells were extracted in SDS sample buffer, and an equal aliquot from each sample was analyzed by anti-phosphotyrosine antibody blotting as described above.

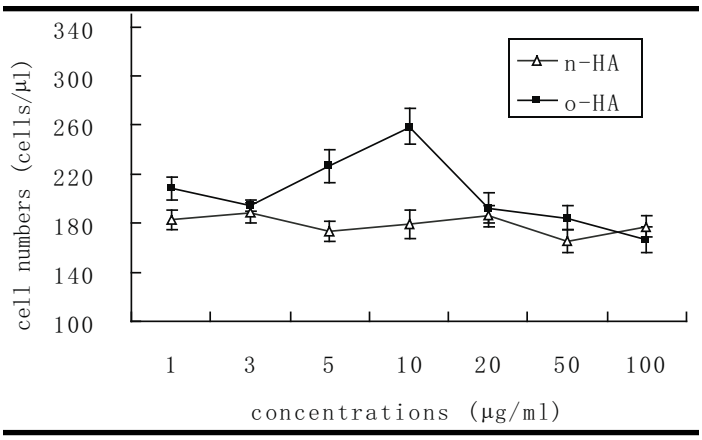

FIGURE 1. Effects of o-HA on EC proliferation. *p<0.01

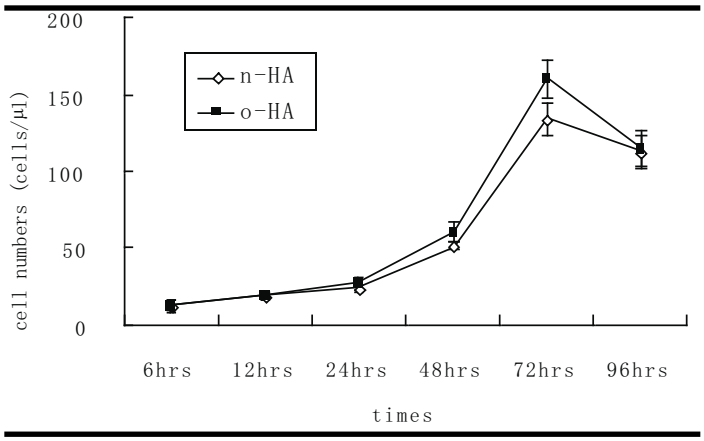

FIGURE 2. Different times of EC exposure to o-HA stimulation. *p<0.05

Statistical Analysis

Data are presented as mean ± SD. Student's t test was used to compare the two groups. P<0.05 was considered statistically significant.

Results

Stimulation of endothelial cell proliferation by HA oligosaccharides

Fractions containing HA fragments with sizes from 4 to 20-mer were pooled by distinct oligosaccharide species. A dose-dependent cell growth assay was performed by choosing o-HA concentrations from 1 to 100 μg/ml. Compare with n-HA, o-HA caused an enhanced increase of PIEC proliferation, which was the most at 10μg/ml (Fig. 1. o-HA vs. control, P <0.01). Increasing the amount of o-HA exposure caused no further increase in cell growth. Cell proliferation was not different with o-HA at other concentrations. In the time-dependent cell growth study, the EC proliferation was different at 12hrs and reached a peak at 72hrs (Fig. 2. o-HA vs. control, P <0.05). In contrast, n-HA had no effect under the same conditions, even at concentrations as high as 20~100 μg/ml.

Effects of o-HA on wound recovery in vitro

Various amounts of o-HA and n-HA were added to the wounded EC culture at different points of time, and the lesion closing rates were measured. The rates of wound closure in the different treatments were shown in Table 1. The results showed that the lesions began to close at 12 hrs after the addition of exogenous o-HA, which was apparently in parallel with the study of EC proliferation described above. Cell movement resulting in wound closure was almost complete in cells treated with o-HA after 40 hrs of treatment, whereas n-HA and control medium had no effects (Fig.3). The concentrations of exogenous o-HA added were from 1μg/ml to 20 μg/ml, and the wound recovered the most significantly at concentration of o-HA at 10μg/ml. As shown in Fig. 3 I, the lesion area was almost completely recovered by EC layer exposed to o-HA 40hrs post-injury under eye's view, whereas in control groups, the wound area remained half recovered pretreated with n-HA and control medium (Fig. 3C).

TABLE 1. The recovery rates of wounded area in EC layer (%)

	0 hr	24 hr	40 hr
Control	0	9.27	15.56
n-HA	0	7.17	14.12
o-HA	0	23.37*	30.80*

The movement of EC cells into the denuded area was quantified using an Image-Pro® plus version 5.0 computerized image analysis systems. Differences were observed with o-HA compared with control and n-HA at 24 hrs and 40 hrs (*P <0.05).

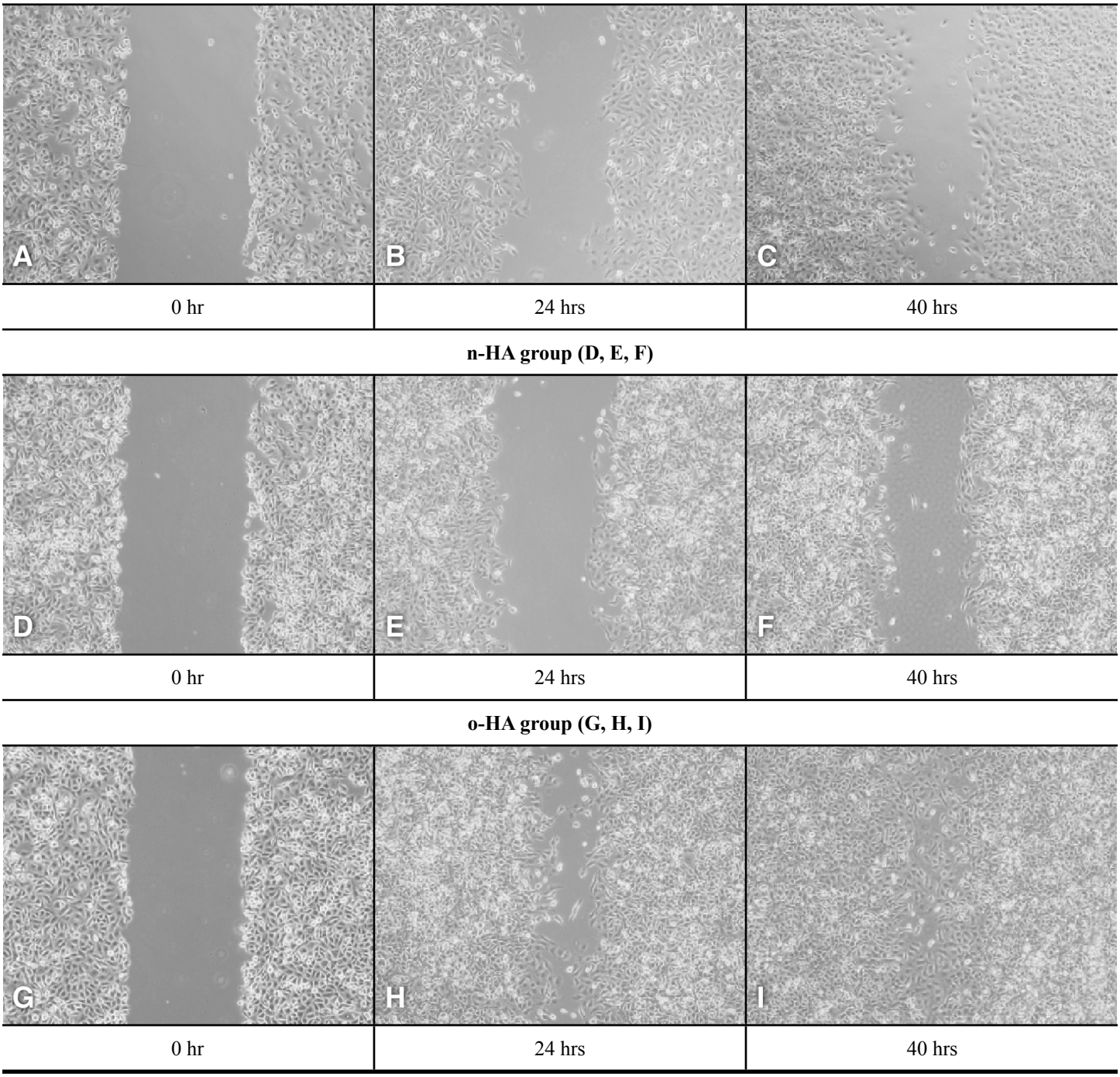

FIGURE 3. Wound healing study. Lesion closing begun at 12 hrs: half closure was observed at about 24hrs within o-HA group. Magnification was ×10.

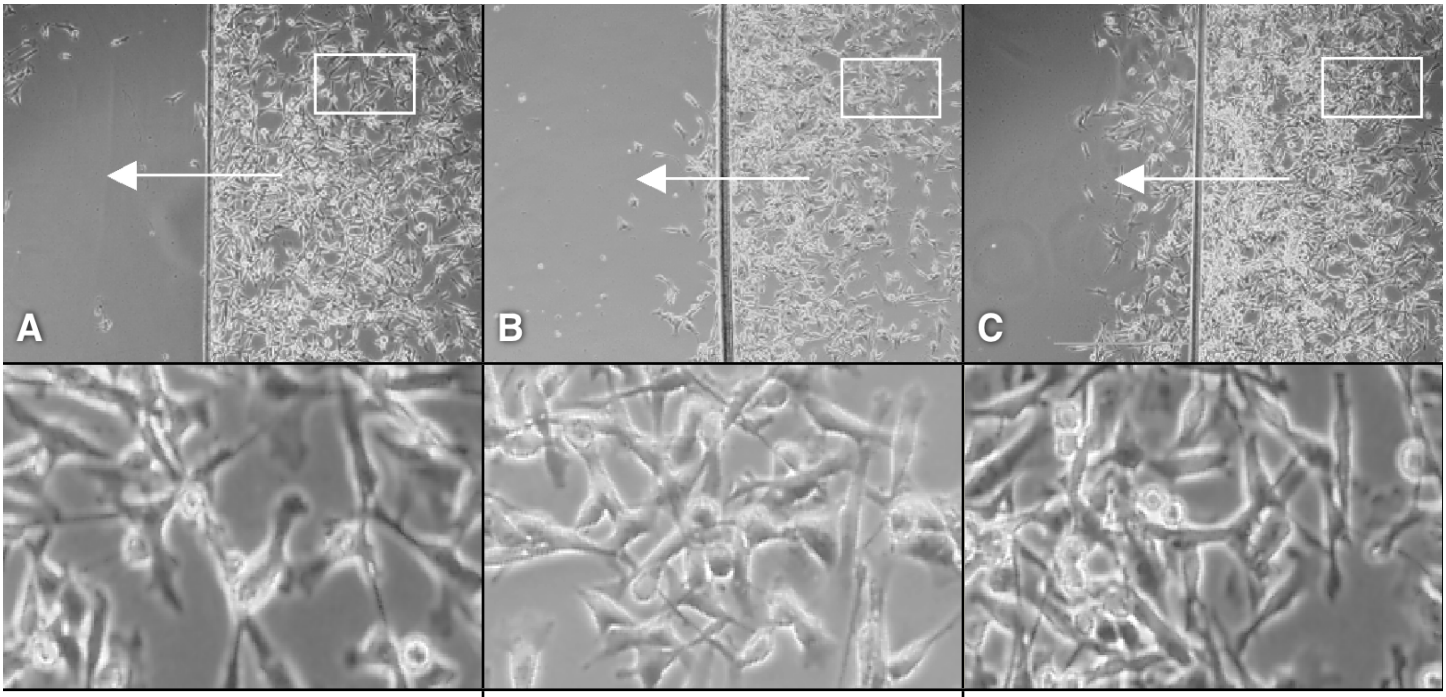

FIGURE 4. EC migration study. Stimulating to PIEC cells by o-HA caused an enhanced migration towards to the cell-free area (C), and the cell movement in n-HA group was not as significant as o-HA group (B). Magnification: ×40.

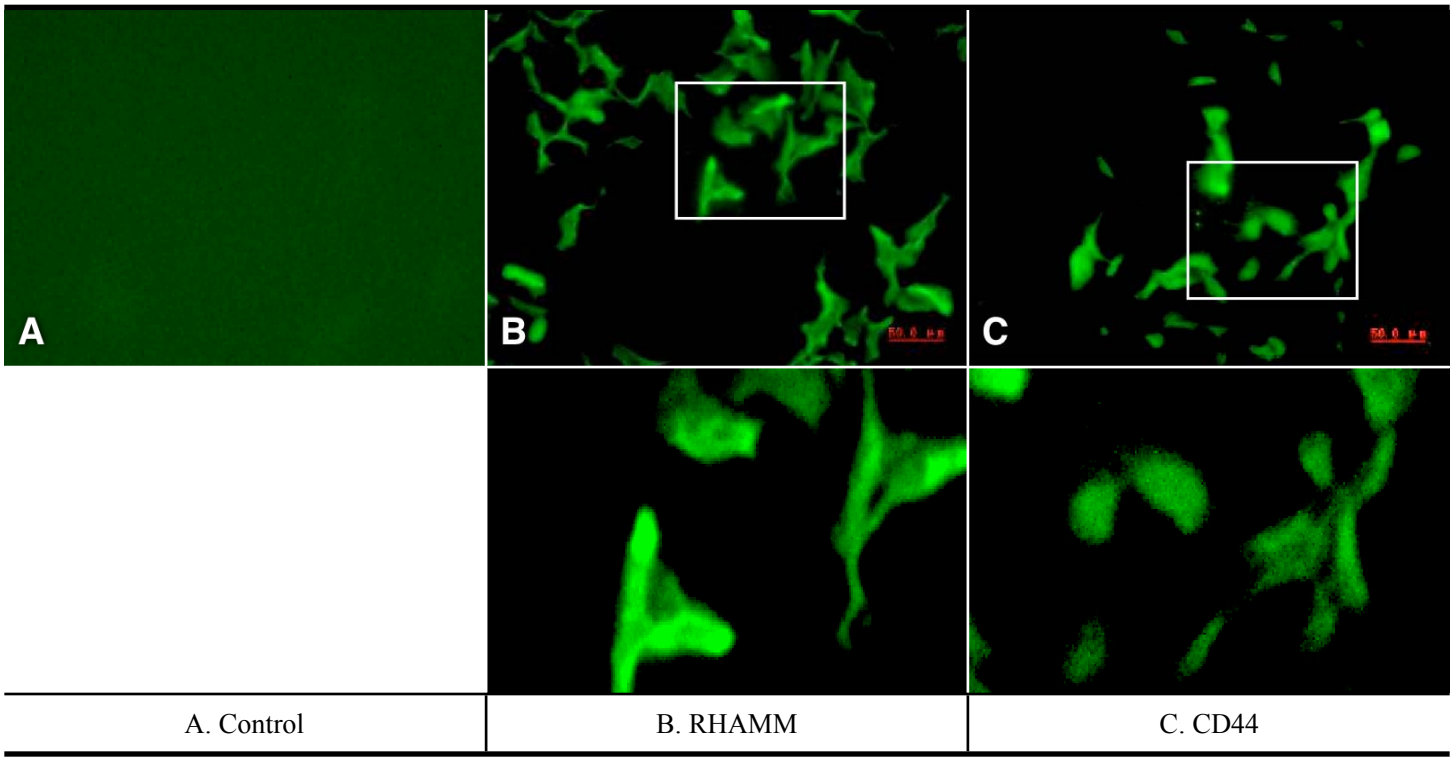

FIGURE 5. Immunofluorescent staining of CD44 and RHAMM on PIEC membrane. Both HA receptors, CD44 and RHAMM, were found highly expressed on PIEC membranes (B, C). Magnification was ×40.

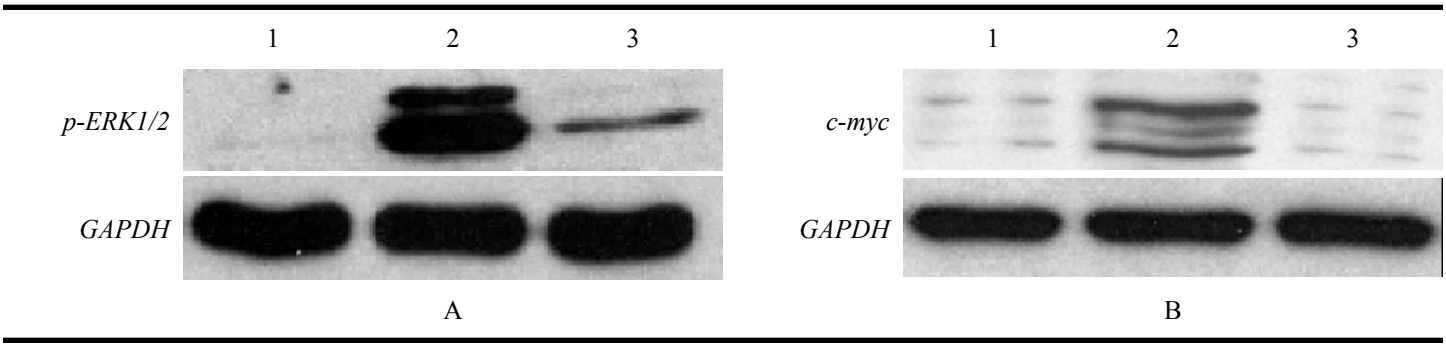

FIGURE 6. Involvement of MAPK activation in o-HA-induced signaling transduction. All experiments were carried out at least 3 times and a representative example is shown. 1: control; 2: o-HA; 3: n-HA

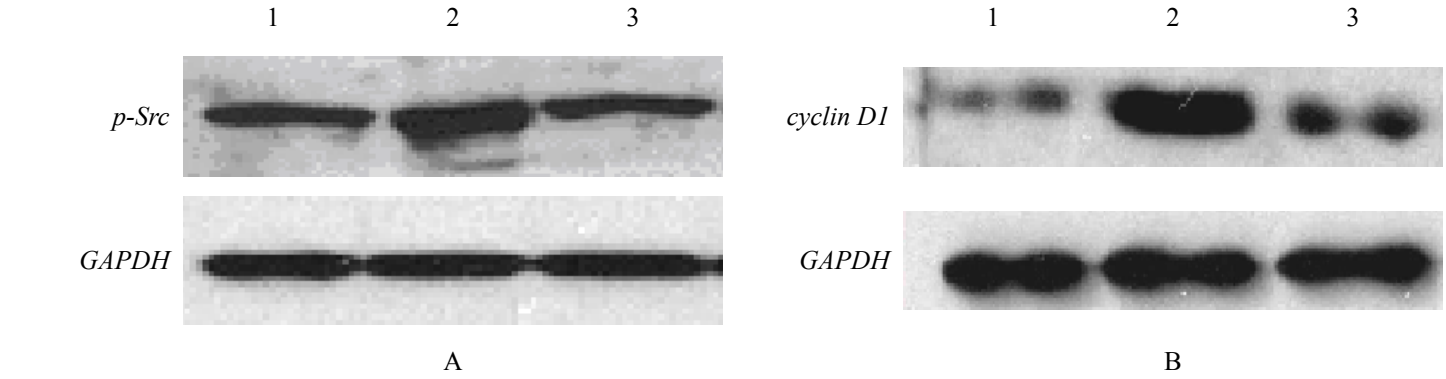

FIGURE 7. o-HA induces activation of *Src* and cell cycle protein *cyclin D1*, representative example. 1: control; 2: o-HA; 3: n-HA

o-HA induces EC migration in culture

The concentration of o-HA was chosen to be 10 μg/ml, which was necessary to stimulate EC proliferation and wound recovery. Lower concentrations of o-HA (~1μg/ml) did not show any effect. EC exposed to o-HA migrated about 2-fold farther than in the n-HA group after 24hrs treatment (Fig. 4). Cell numbers migrating into the vacant area were also counted, as a percentage, in which o-HA was 11.43% and n-HA was 4.45%, 24 hrs post-treatment, respectively. The lengths of cell movement from the cut line were measured, as o-HA 464μm and n-HA 245μm, respectively.

o-HA stimulates signal transduction

Both CD44 and EHAMM were expressed highly on PIEC membranes compared with control cells (Fig. 5). A cascade of main signal pathway was selected for cell activation study: *p-Src, c-myc*, and *p42/44* (extracellular signal-regulated kinase, ERK1/2). o-HA promotes EC signal transduction as reported previously[17] (Fig. 6-7).

In addition, an increased cell cycle was observed as Cyclin D1 expression was higher in o-HA treated cells (Fig.7 B). Opposing the stimulating effects of o-HA (10μg/ml) on PIEC proliferation and movement, the concentration of o-HA in activating cell signal cascades was only 1μg/ml (Fig. 6-7), which is consistent with earlier reports.[15,20] Compared with o-HA, n-HA and control groups had little or no effect on EC signal transduction activation.

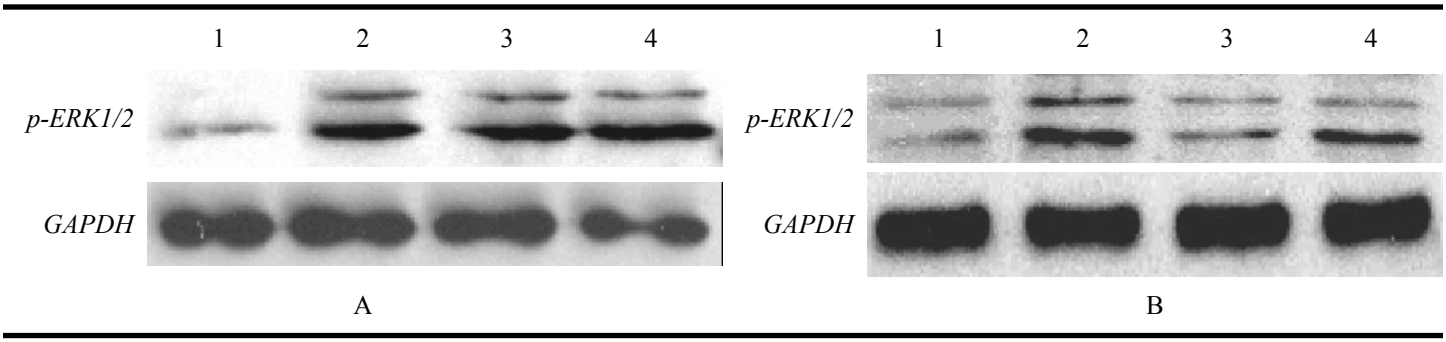

FIGURE 8. Down-regulation of MAPK using blocking antibodies RHAMM and CD44. representative example. (in a) 1: control cells lysate (1 μg/ml); 2: o-HA (1 μg/ml) only; 3: anti-CD44 mAb (80μg/ml) plus o-HA; and 4: isoform anti-IgG1k (80μg/ml) plus o-HA. (in b) 1: control cell lysate (1 μg/ml); 2: o-HA (1 μg/ml) only; 3: anti-RHAMM antibody (80μg/ml, 12hrs) plus o-HA; and 4: rabbit IgG (80μg/ml) plus o-HA

RHAMM may function as major receptor for o-HA triggering signal-regulated kinase in PIEC

The upstream of the MAP kinase was not decreased after blocking CD44 binding site by a neutralizing antibody (Fig. 8A). However, the expression of *p-ERK1/2* was inhibited after the blockage of RHAMM using polyclonal antibodies, supporting that the stimulating effect of o-HA on PIEC signal transduction might be mediated at least through o-HA/RHAMM pathway (Fig.8B).

Discussion

In 1985, West DC[8] first demonstrated that hyaluronan fragments in defined lengths (less than 20 disaccharides) stimulated angiogenesis. In this study, we prepared a defined mixture of o-HA by 2-10 disaccharides using the digestion method.[14] The angiogenic activities of o-HA were tested on EC growth and migration in culture. The results suggest that oligomers of HA at 10μg/ml concentration cause proliferative effects on EC proliferation and movement, compared with native HA (n-HA) molecules (Figs.1, 2, 4). Endothelial cells (EC) have a critical role during the wound-healing process because cell growth is associated with capillary tubule formation. Therefore, EC is an appropriate and relevant model for the study of the action of o-HA on vascular formation. The stimulus to PIEC growth by o-HA in this study suggests that oligosaccharides of HA (o-HA) may be involved in angiogenesis and implies a potential role in wound repair.

Then, we studied the effects of o-HA on wound recovery in an *in vitro* tissue injury model. This model partially mirrored the pathological response to skin injury.[15] o-HA added to the wound culture cellular layer caused an enhanced lesion 40 hrs post-treatment, whereas n-HA showed no effect (Fig. 3).

The EC signal transduction activation by o-HA interaction with CD44/RHAMM implies a functional role for o-HA in wound recovery treatment. We demonstrated that both CD44 and RHAMM were expressed on PIEC membranes, and also showed that o-HA caused up-regulation in EC signal transduction (Fig. 6-7).Signaling transduction was initiated by RHAMM, but not CD44, (Fig. 8). PIEC pre-exposed to neutralizing antibody to CD44 showed no reduction in MAP kinase activation in PIEC, whereas antibodies to RHAMM showed down-regulation of *p-ERK1/2* expression, suggesting that RHAMM might be the functional HA receptor.

Wound healing is a complex process that involves cells, chemical signals, and the matrices cells use, which in combination leads to eventual repair. Angiogenesis is closely related to wound healing that promotes the formation of granulation. This early aspect of neovascularization depends on chemotactic factors supplied by neighboring cells and matrix. Hyaluronan

(HA) is a major extracellular glycosaminoglycan and is found in most types of extracellular matrix. HA appears to have distinct biological functions depending on the circumstances under which it is produced. In the physiological state, HA exists as a high molecular weight polymer in excess of 10^6 Da., promotes cell quiescence and supports tissue integrity. However, after tissue injury, HA fragments of lower molecular mass accumulate, as a result of excessive digestion by hyaluronidase. Generation of HA fragments under conditions of tissue injury may signal to the host that normal homeostasis has been profoundly disturbed. More importantly, oligosaccharides of HA produced by hyaluronidase can induce wound angiogenesis.[21] We demonstrated that o-HA in a defined length of 2-10 disaccharides promote EC proliferation and migration *in vitro*. The results confirmed previous findings and suggest a possible role for o-HA in angiogenesis formation. Results from the *in vitro* wound healing model further proved the angiogenic activities of o-HA. As reported previously, the most angiogenic activities of o-HA were observed between 4-25 disaccharides in length.[8] Single elute of o-HA species or mono-saccharide had less or no effect on EC growth or chick chorioallantoic membrane angiogenesis. The o-HA used in this study was a mixture of HA oligosaccharides (2-10 disaccharides), which was consistent with other reports.[20]

There are few reports of how o-HA interacts with EC during wound angiogenesis process. Slevin et al [15] reported that o-HA induced tyrosine phosphorylation through CD44 receptor in BAEC (bovine aortic endothelial cells). It seems that o-HA induces vascular EC proliferation via ERK activation, mainly through the CD44 or RHAMM receptors. In this study, the main functional role of HA receptors on EC cells seems to be RHAMM, rather than CD44. These differences may have resulted from different cell lines used, different reagents added and even different conditions in various laboratories. As described by Savani et al [18], both of the receptors CD44 and RHAMM were detected on vascular endothelium *in situ* and on the surface of cultured EC. Anti-CD44 antibody inhibited the adhesion of HUVEC (human umbilical vein EC) to immobilized HA; anti-RHAMM antibody did not block the binding of HUVEC to HA-coated surfaces, indicating that the two receptors for HA have different binding abilities to HA or o-HA. They also reported that anti-CD44 antibody inhibited EC proliferation, whereas anti-RHAMM did not. However, Lokeshwar et al [19] demonstrated that RHAMM, but not CD44, bound HA in all three EC used, and that RHAMM was the functional HA receptor in primary human EC. These controversial findings provided the conclusion that heterogeneity exists among primary human EC of different vascular origins, with respect to functional HA receptor expression and function. It seems that RHAMM and CD44 can perform separate functions in regulating cell signaling. Whether our results are the final demonstration for the RHAMM function, as receptor for o-HA in EC proliferation or mitogenesis, needs to be determined by further detailed experiments.

Angiogenesis has become a focus for study and several investigators are trying to identify new mediators or stimulators to angiogenesis.[22] Over the past two decades, several growth factors have been implicated as possible stimulators of angiogenesis during wound repair, including transforming growth factor beta-1, tumor necrosis factor α, fibroblastic growth factor (FGF), platelet-derived growth factor (PDGF), and most importantly, vascular endothelial growth factor (VEGF). Of these, FGF, PDGF, and VEGF have been shown to be directly mitogenic to endothelial cells. The only growth factor currently approved for clinical use is PDGF.[6] There are limitations in the application of growth factors in the clinical treatments of wound healing: the growth factors are proteins that might be digested by proteases when applied directly on injured tissue. Oligosaccharides of HA are final digested products that contain no proteins and may be advantage over protein growth factors. The findings that o-HA is able to promote EC growth may highlight

the clinical and experimental importance of determining the role of o-HA in angiogenesis and wound treatment in future.

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