DIFFERENCES IN HYALURONIC ACID-MEDIATED FUNCTIONS AND SIGNALING IN ARTERIAL, MICROVESSEL AND VEIN-DERIVED HUMAN ENDOTHELIAL CELLS

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Abbreviations used: EC: Endothelial cell; HA: Hyaluronic acid; HPAEC: Human pulmonary artery ECs; HMVEC-L: Human lung microvessel ECs; HUVEC: Human umbilical vein ECs; PBS: Phosphate buffered saline.
SUMMARY:

Hyaluronic acid (HA), a nonsulfated glycosaminoglycan, regulates cell adhesion, and migration. Small HA fragments (3-25 disaccharide units) induce neovascularization. We investigated the effect of HA and a HA fragment (10-15 disaccharide units, F1) on primary human endothelial cells (ECs). Human pulmonary ECs (HPAEC) and lung microvessel ECs (HMVEC-L) bound HA ($K_d \sim 1$ nM and 2.3 nM, respectively), and expressed 17,780 and 16,690 HA binding sites, respectively. Both ECs showed HA-mediated cell adhesion, however, HMVEC-L was 1.5-fold better. Human umbilical vein ECs (HUVEC) neither bound HA nor showed HA-mediated adhesion. All three ECs expressed CD44 (~ 110 kDa). The expression of RHAMM (~ 80 kDa) was the highest in HMVEC-L, followed by HPAEC and HUVEC. RHAMM, not CD44, bound HA in all three ECs. F1 was better than HA and stimulated a 2.5-fold and 1.8-fold mitogenic response in HMVEC-L and HPAEC, respectively. Both HA and F1 induced tyrosine phosphorylation of p125$^{FAK}$, paxillin and p42/44 ERK in HMVEC-L and HPAEC, which was blocked by an anti-RHAMM antibody. These results demonstrate that RHAMM is the functional HA receptor in primary human ECs. Heterogeneity exists among primary human ECs of different vascular origins, with respect to functional HA receptor expression and function.
INTRODUCTION

The integrity of vascular endothelium is crucial in several important processes, including maintenance of blood flow, blood vessel permeability and tone. An injury to the vascular endothelium marks the beginning of diseases such as atherosclerosis, and the endothelium regeneration signals disease regression (1). Microvessel endothelium is the key element in angiogenesis that involves sprouting of preexisting microvessels. Angiogenesis is necessary for wound healing, collateral blood vessel formation in ischemia, tumor growth and metastasis (2,3). Endothelial cell (EC) functions such as, proliferation, migration and capillary formation regulate the integrity of the normal vascular endothelium and control angiogenesis in microvasculature (4). A large number of cytokines, including the major ones such as basic fibroblast growth factor and vascular endothelial growth factor, stimulate EC functions. In addition to cytokines, extracellular matrix components regulate vascular and microvessel endothelial behavior and play a role in vessel wall diseases and angiogenesis (5).

Hyaluronic acid (HA), a non-sulfated glycosaminoglycan, is known to accumulate in the vessel wall intima during early stages of atherosclerotic plaque formation. The concentration of HA is low in the advanced lesions, but HA levels rise once again during plaque regression (6-8). HA concentration is also elevated in tumor tissues in cancers of the prostate, bladder, colon, liver, etc. (8-11). In addition to its structural role, that is keeping tissues hydrated and maintaining osmotic balance, HA regulates cellular processes such as, adhesion migration and proliferation (12-14). Different lengths of the HA polymers that are made up of repeated disaccharide units, D-glucuronic acid and N-acetyl-D-glucosamine, affect different cellular processes (12,13). For example, the high molecular mass HA (~ $10^6$ Da) is anti-angiogenic, whereas, HA fragments of 3-25 disaccharide units induce angiogenesis (15-17). We have previously isolated small HA fragments (10-15 disaccharide units) from the urine of bladder cancer patients and have shown that these fragments induce the proliferation of human
microvessel ECs (11). It has also shown been shown that angiogenic HA fragments induce EC migration and capillary formation (18,19). HA fragments are generated when hyaluronidase, an endoglycosidase degrades the HA polymer (20-22). We have previously shown that hyaluronidase levels are elevated in cancers of the prostate and bladder (23-25). Furthermore, elevated HA and hyaluronidase levels serve as sensitive and accurate non-invasive urine markers for bladder cancer detection and evaluation of its grade (26).

The biological functions of HA are mediated by cell surface HA receptors or HA binding proteins (27,28). At present several HA receptors have been described in ECs (29-32). For example, the HA receptor expressed on liver ECs is crucial for the clearance of HA from blood (30). Another well characterized HA receptor on ECs is CD44. CD44 denotes a family of glycoproteins that are expressed on a variety of cells and tissues (33,34). The distinct feature of the CD44 family is alternative splicing that gives rise to numerous isoforms. Out of the 19 exons present in CD44 mRNA, most often the exons between 5 and 15 are alternatively spliced (33,34). We have previously shown that a chemically transformed bovine EC line 7372A expresses an isoform of CD44 (ex14/v10) that appears to be required for HA-mediated EC proliferation (14). Human umbilical vein ECs (HUVEC), however, do not express CD44 in vivo but begin to express different CD44 isoforms in culture and when activated with basic fibroblast growth factor (35).

Besides CD44, another well studied HA receptor is RHAMM (receptor for HA-mediated motility) (36). Although, RHAMM expression has not been described in ECs, it is expressed in many cell types including fibroblasts, smooth muscle cells, T-cells, macrophages and cancer cells (37). RHAMM is probably expressed both on the cell surface and intracellularly (36,38,39). A critical regulator of cell motility, RHAMM causes cellular transformation when over-expressed (40). RHAMM is known to induce HA-mediated tyrosine phosphorylation of focal adhesion
kinase, p125FAK (41). It is also required for the activation of ERK (extracellular kinase) by platelet-derived growth factor (PDGF) and ras (42).

In this study we examined HA-binding, HA-mediated cell adhesion and proliferation in primary human ECs. Since ECs from different tissues and vascular origins differ in their structural architecture and functional responses to environmental stimuli, we used human pulmonary artery, lung microvessel and umbilical vein-derived ECs to perform the experiments described in this study. We also investigated the expression of HA receptors and HA-induced signal transduction events in these ECs.

EXPERIMENTAL PROCEDURES

Primary human EC cultures: Human pulmonary artery ECs (HPAEC), human lung microvessel ECs (HMVEC-L) and human umbilical vein-derived ECs (HUVEC) were obtained from Clonetics/Bio Whittaker Inc., Walkersville, MD. HPAEC, HMVEC-L and HUVEC lines were grown in “Endothelial cell growth medium (EGM)” which was purchased from Clonetics/Bio Whittaker Inc. All of the experiments described in this study were performed on cells between 2-3 passages. The company guarantees the EC cultures for 15 population doublings or 3 passages in culture.

Reagents: Human umbilical cord HA was obtained from ICN Biomedicals. Horseradish peroxidase conjugated recombinant anti-phosphotyrosine RC20 antibody and mouse anti-FAK, anti-paxillin and anti-pan ERK (the antibody that recognizes both ERK1 and ERK2 and was generated against an ERK2 sequence) antibodies were obtained from Transduction Laboratories, Lexington KY. A rat anti-CD44 antibody that recognizes a common epitope on all CD44 isoforms was obtained from Zymed Inc. A rabbit anti-RHAMM antibody R3.6 was kindly provided by Dr. Eva Turley, Hospital for sick children, Toronto, Canada. Goat anti-rabbit IgG
Sepharose, goat anti-mouse IgG Sepharose, and alkaline phosphatase conjugated goat anti-rabbit IgG and anti-biotin IgG were obtained from Sigma Chemical Co., St. Louis, MO.

\[^{3}\text{H}\]-HA binding: \[^{3}\text{H}\]-HA was prepared as described previously (21). The specific activity of \[^{3}\text{H}\]-HA was 1.0 x 10\(^4\) cpm/\(\mu\)g and it had a molecular mass of \(~10^6\) Dalton. ECs were grown on 6-well plates and at \(~80\%\) confluence the cultures were washed three times in phosphate buffered saline (PBS). The ECs were then incubated with various concentrations (0.15 to 3 \(\mu\)g/ml) of \[^{3}\text{H}\]-HA in PBS + 0.2% bovine serum albumin (binding buffer). Following incubation at 4\(^0\) C for 4 h (equilibrium binding), the cells were solubilized in 20 mM Tris.HCl pH 7.4 + 1% SDS (SDS solution). The radioactivity present in the solubilized cell extracts was counted in a liquid scintillation counter. The non-specific binding was determined in the presence of a 100-fold excess of unlabeled HA and was subtracted from the total binding. For competition binding experiments, ECs were incubated with 1 \(\mu\)g/ml of \[^{3}\text{H}\]-HA in the presence of 1-250 \(\mu\)g/ml of unlabeled HA, F1, F2 and F3 HA fragments, in the binding buffer. Following incubation at 4\(^0\) C for 4 h, the cell-associated radioactivity was measured as described above.

Preparation of HA fragments: Human umbilical cord HA (500 mg) was digested with 20,000 units of testicular hyaluronidase for different time intervals. The aliquots collected at different time intervals were precipitated with trichloroacetic acid to remove protein, dialyzed against distilled water, lyophilized and then dissolved in 0.1 M acetic acid. The mixture of HA fragments was separated on a Sephadex G-50 column (1.5 x 110 cm) and the column fractions were assayed for uronic acid content and the number of reducing ends. The chain length of each fragment was calculated from the number of reducing ends per mole of uronic acid. Three different uronate peaks were isolated from the column and these peaks were designated as F1 (10-15 disaccharide units), F2 (2-3 disaccharide units) and F3 (\(<2\) disaccharide units) (14).
**Cell adhesion assay:** EC cultures at ~ 80% confluence were incubated in L-methionine free DMEM (Gibco Life Technology, Gaithersburg, MD) at 37°C for 2 h, followed by pulse labeling with Tran ³⁵S-label (20 µCi/ml) for 1 h. Following labeling, the cells were washed in PBS and incubated with PBS containing 5 mM EDTA solution at 37°C to obtain a non-adherent single cell suspension. An aliquot of the labeled cells, solubilized in the SDS solution, was counted in the liquid scintillation counter. The cell density was determined by counting cells in a hemocytometer slide, which enabled us to determine cpm/50,000 cells. The HA-coated surfaces were prepared by incubating the wells of a 24-well plate with 1 mg/ml HA solution, prepared in 0.1 M sodium bicarbonate pH 9.6, at 37°C for 4 h. Following incubation, the wells were washed in PBS and incubated with PBS + 1% BSA at 37°C for 1 h, to block non-specific sites on the wells. Fifty thousand ³⁵S-labeled cells were incubated on HA-coated wells in binding buffer at 4°C for 30 min either alone or in the presence of 300 µg/ml of soluble HA (to determine non-specific cell adhesion). Following incubation, the wells were washed three times in binding buffer, solubilized in SDS solution and the radioactivity was counted in a liquid scintillation counter. The counts due to non-specific adhesion were subtracted from total cell adhesion counts.

**Mitogenic assay:** ECs were cultured on 48-well plates. At ~ 80% confluence, the cultures were incubated with “Endothelial basal medium” (EBM) which lacks all the additives (i.e., bovine pituitary extract, VEGF, h-EGF, ascorbic acid, hydrocortisone, and IGF-1) and serum present in EGM. ECs were incubated in EBM at 37°C for 12 h. The cultures were then incubated with various concentrations (0 – 5 µg/ml) of HA or HA fragments in EBM, at 37°C for 18 h. Following incubation, [³H]-thymidine (1 µCi/ml) was added to these cultures and the assay was terminated.
after a 2 h incubation, as described previously (26). The results are represented as mean ± SD from triplicate determinations.

**Immunoprecipitation, immunoblotting and gel analysis:**

**Immunoprecipitation with anti-CD44 antibody:** ECs grown on 6-cm dishes (~ 80% confluence) were incubated in L-methionine free DMEM at 37°C for 4 h. The cultures were incubated with Tran35S label (200 μCi/ml; ICN, Costa Mesa, CA) at 37°C for 1 h. The radioactively labeled cells were washed in PBS, and 2 x 10^5 cells were solubilized in RIPA buffer and immunoprecipitated using rat anti-CD44 monoclonal antibody as described previously (21). The immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis and fluorography.

**Immunoblotting with anti-RHAMM antibody:** EC cultures (~ 80% confluence) were treated with PBS + 5 mM EDTA to obtain a single cell suspension. ECs (2 x 10^5) were solubilized in SDS sample buffer, separated on a 10% SDS-polyacrylamide gel and blotted on to a PVDF membrane. The blot was incubated with rabbit anti-RHAMM antibody, R 3.6 (1:1500 dilution), at 4°C for 16 h (42). Following incubation, the blot was washed and incubated with alkaline phosphatase conjugated goat anti-rabbit antibody (1:7500 dilution) at room temperature for 2 h. The blot was then washed and developed using an alkaline phosphatase color detection system, involving nitroblue tetrazolium and 5-bromo-4-choloro-3-indoyl phosphate substrates (BioRad, Hercules, CA). To confirm that any differences observed in the intensity of bands, following immunoblot analysis are not due to the differences in protein loading, prior to incubation with anti-RHAMM antibody, the blots were stained with coomassie blue and processed as described below.

**Blotting with biotinylated HA:** Biotinylated HA was prepared according to a procedure by Hoare et al (43). ECs grown on 6-well culture plates (~ 80% confluence) were extracted in SDS sample buffer as described above. The cell extracts were separated on an 8.5% SDS-
polyacrylamide gel, blotted on to PVDF and probed with biotinylated HA (60 μg/ml) at 4°C for 16 h. After washing the blot was developed using an anti-biotin alkaline phosphatase-conjugated antibody (1:7500 dilution), as described above.

Alternatively, ~ 80% confluent cultures of ECs grown on 6-well cultures plates were solubilized in RIPA buffer and the cell lysates (prepared from 2 x 10^5 cells) were immunoprecipitated by sequential incubations with anti-RHAMM antibody (1:200 dilution) at 4°C for 16 h and goat anti-rabbit IgG agarose beads at 4°C for 90 min. The immunoprecipitates were washed 5 times in RIPA buffer and separated on a 10% SDS-polyacrylamide gel. The gel was blotted on to a PVDF membrane and the blots were processed with biotinylated HA as described above. To confirm that any differences observed in the intensity of bands, following immunoblot analysis are not due to the differences in protein loading, prior to incubation with biotinylated HA binding protein, the blots were stained with coomassie blue and processed as described below.

**Immunoblotting with anti-phosphotyrosine antibody:** ECs, grown on 6-well culture plates (~ 80% confluence) were incubated in EBM at 37°C for 16 h. The cells were then incubated with HA or the F1 HA fragment at different concentrations (0 – 10 μg/ml) for 5 min or at 2 μg/ml for different time intervals (0 – 10 min). At the end of the incubations, the cultures were washed with PBS and solubilized in 0.2 ml of SDS sample buffer. Equal amounts (i.e., 25 μl) of the cell extract from each sample were separated on a 10% SDS-polyacrylamide gel, and blotted onto a PVDF membrane. The blotted membranes were stained with 0.15% coomassie blue in 30% methanol for 1 min and then destained in 30% methanol for 5 min. This procedure allows the visualization of the total protein profile in each lane. The coomassie blue stained blots were photographed using a Kodak DC-20 camera that comes with gel analysis software (EDAS, Kodak, Rochester, NY). Using this system, the intensity of protein bands in each sample lane with molecular weights between 83 kDa and 50 kDa and 50 kDa and 36 kDa were compared for detecting differences in protein loading and possible artifacts during protein transfer. These differences were less than 5%. The coomassie stained blots were completely destained in 50%
methanol, rehydrated in distilled water for 10 min and then blocked with 3% bovine serum albumin in 20 mM Tris, 150 mM NaCl, pH 7.4 buffer containing 0.05% Tween-20. The blots were probed with anti-phosphotyrosine monoclonal antibody RC20 (1: 7000 dilution) and developed using a chemiluminescence system (NEN Dupont). The autoradiographs were photographed using the same Kodak system and intensity of bands among different samples were compared. To be sure that the differences observed in various samples are not due to the differences in protein loading, a ratio of the intensity of a tyrosine phosphorylated protein band to the intensity of total protein bands was calculated for each lane and then the ratios between different lanes were compared.

Alternatively, HA or F1 treated (2 μg/ml for 5 min) HMVEC-L (6-cm dishes, ~ 80% confluence) were solubilized in the RIPA buffer and immunoprecipitated using anti-FAK, anti-paxillin, anti-pan ERK antibodies at 5μg/ml concentration, according to the protocol provided by the manufacturer. The immunoprecipitates were separated on an 8.5% SDS-polyacrylamide gel, blotted on to a PVDF membrane and probed with anti-phosphotyrosine antibody as described above. In control experiments, HMVEC-L stimulated with HA or F1 were directly extracted in SDS sample buffer, separated on a 10% gel, and blotted on to a PVDF membrane. The blots were probed with anti-FAK (1:1000 dilution), anti-paxillin (1:5000 dilution) or anti-pan ERK (1:5000 dilution) antibodies at 4°C for 16 h. After washing, the blots were incubated with alkaline phosphatase conjugated goat anti-mouse IgG (1:7500 dilution) at room temperature for 2 h. The blots were then developed using an alkaline phosphatase system as described above.

In one set of experiments, HMVEC-L incubated in EBM at 37°C for 16 h were treated with either rat anti-CD44 monoclonal antibody (25 μg/ml) or rabbit anti-RHAMM antibody (25 μg/ml) or normal rabbit IgG (25 μg/ml) at 37°C for 15 min. The cells were then stimulated with HA or F1 HA fragment at 2 μg/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.

**RT-PCR and cloning:** Total RNA was extracted from HPAEC and HMVEC-L using the Quiagen RNA extraction kit. Approximately 5 \( \mu \)g of total RNA was reverse transcribed using Superscript II<sup>TM</sup> (Gibco Life Technology, Gaithersburg, MD). The first strand cDNA was amplified using Ampli Taq Gold<sup>TM</sup> (Perkin Elmer), Taq Gold buffer (with final MgCl2 concentration 1.5 mM), 5% dimethylsulfoxide and the following RHAMM primer pair: RHAMM L1 (This primer sequence is at nucleotide positions 1639-1659 in a RHAMM clone, GenBank accession number: AF032862): GAACCAACTCAAGCAACAGG; RHAMM L2 (This primer sequence is at nucleotide positions 2005-2025 in the RHAMM clone AF032862): AGCAAGCTGACAGCGGAGTT. The PCR conditions were as follows: 95<sup>0</sup> C for 10 min (initial melting), 10 cycles (with annealing temp decreasing by 1 degree in each cycle): 94<sup>0</sup> C for 30 sec; 70-60<sup>0</sup> C for 30 sec (touch down PCR) and 72<sup>0</sup> C for 1 min. Then 30 cycles of 94<sup>0</sup> C for 30 sec; 60<sup>0</sup> C for 30 sec, 72<sup>0</sup> C for 1 min. Final extension at 72<sup>0</sup> C for 7 min. The PCR product was analyzed on a 1.2% agarose gel and visualized by ethidium bromide staining. The PCR product was cloned into PCR TOPO vector, using the TOPO kit and a protocol supplied by the manufacturer (Invitrogen, Carlsbad, CA). Both strands of the cloned cDNA insert were sequenced in the University of Miami’s DNA core facility, using an automated DNA sequencer and T7 and SP6 universal primers.

**RESULTS**

**Binding of HA to human ECs:**

To determine whether different vascular ECs differ in their ability to bind HA, we examined [3<sup>H</sup>]-HA equilibrium binding in HMVEC-L (lung microvessel), HPAEC (pulmonary artery) and HUVEC (vein-derived). As shown in Fig. 1 A, [3<sup>H</sup>]-HA binds HMVEC-L and HPAEC in a dose-dependent manner that is saturable and specific. However, HUVEC do not display
specific HA binding at any concentration tested (even up to 5 \( \mu \text{g/ml} \)). Scatchard plot analyses indicate that both HPAEC and HMVEC-L contain a single class of high affinity HA binding sites with dissociation constants (\( K_d \)) of 1 nM and 2.3 nM, respectively. The analyses also showed that there are 16,690 and 17,580 binding sites on HMVEC-L and HPAEC, respectively. It has been previously shown that the angiogenic HA fragments (3-25 disaccharide units) are capable of binding to cell surface HA receptors (28). Therefore, we determined the ability of F1, F2 and F3 HA fragments to compete with \([^3\text{H}]-\text{HA} binding. Our results indicated that only the F1 fragment was able to effectively compete with \([^3\text{H}]-\text{HA} to bind to both HMVEC-L and HPAEC. The dissociation constants for the binding of F1 fragment to HPAEC and HMVEC-L, as determined by the competition binding curves, were 1.8 nM and 2.6 nM, respectively (data not shown).

**Adhesion of ECs to HA-coated surface:**

In this experiment we tested the ability of \( ^{35}\text{S} \)-labeled primary human ECs to adhere to HA-coated surface. As shown in Table 1, both HPAEC and HMVEC-L specifically adhere to the HA-coated surface. However, HUVEC, which do not show specific HA binding (as shown in Fig. 1 A), do not specifically adhere to a HA-coated surface. Some differences are also observed between the ability of HMVEC-L and HPAEC to adhere to the HA-coated surface. Out of the 50,000 cells of each type added onto the HA-coated surface, 24.7% of HMVEC-L (12,739 cells/well) and 16.9% of HPAEC (8468 cells/well) adhered specifically on the HA-coated surface (Table 1). Thus, HMVEC-L show 1.5-fold better adherence to the HA-coated surface than HPAEC. Statistical significance of differences observed in the adhesion of HMVEC-L, HPAEC and HUVEC to HA-coated surface were analyzed by Tukey Kramer Multiple Comparison Test. The differences in cell adhesion observed between HMVEC-L and HPAEC (\( p < 0.05 \)), HMVEC-L and HUVEC (\( p < 0.001 \)) and HPAEC and HUVEC (\( p < 0.001 \)) are statistically significant. Next we tested whether HA fragments are capable of blocking the adhesion of ECs to a HA-coated
surface. As shown in Table 1, the addition of the F1 fragment blocks the HA-mediated adhesion of both HMVEC-L and HPAEC by > 95%. The smaller HA fragments (i.e., F2 and F3) had no effect on the HA-mediated cell adhesion (data not shown). These results demonstrate that primary human ECs, HPAEC and HMVEC-L, which display high affinity HA binding also show HA-mediated cell adhesion. Furthermore, different ECs differ in their HA-binding and HA-mediated adhesion capabilities.

**Effect of HA and HA fragments on the proliferation of primary human ECs:**

To investigate the functional significance of the observed heterogeneity among primary human ECs with respect to HA-binding, we tested the effect of HA and HA fragments on the mitogenic response of HMVEC-L, HPAEC, and HUVEC. As shown in Fig. 2 A, HA and the F1 fragment induce a mitogenic response in HMVEC-L in a dose-dependent manner, and cause a maximum increase of 1.8- and 2.5-fold, respectively, at 2 μg/ml concentration (Fig. 2 A). The F2 and F3 fragments are not mitogenic to HMVEC-L. In HPAEC, although the mitogenic response was dose-dependent, HA and the F1 fragment are slightly less mitogenic. As shown in Fig. 2 B, HA and the F1 fragment cause a maximum mitogenic response of 1.4- and 1.8-fold, respectively, at 2 μg/ml concentration (Fig. 2 B). As expected from the HA binding and HA-mediated cell adhesion results, neither HA nor the F1 fragment is mitogenic to HUVEC (Fig. 2 C).

These results show that the F1 fragment is a better mitogen to primary human ECs than HA. Furthermore, heterogeneity exists among ECs of different origins with respect to HA/F1-induced mitogenic response.

**Identification of HA receptor in ECs:**
To identify the receptor that might be responsible for HA-induced functions as well as HA-related heterogeneity among different human ECs, we initially examined the expression of CD44 in HMVEC-L, HPAEC and HUVEC. We had previously shown that an 116 kDa isoform of CD44 (CD44 Ex14/v10) acts as the HA receptor in a chemically transformed bovine EC line (21). The expression of CD44 has also been reported in bovine aortic ECs and actively proliferating HUVEC (35). We immunoprecipitated CD44 from $^{35}$S-methionine labeled extracts of HMVEC-L, HPAEC and HUVEC, using a rat anti-CD44 monoclonal antibody that detects all CD44 isoforms. As shown in Fig. 3 A, all three EC lines express a ~ 110 kDa CD44 isoform. The minor differences observed in the mobility of CD44 among three EC lines, are most likely due to a gel artifact. This artifact was apparent in the coomassie blue stained dried gel that was exposed to the x-ray film. The expression of this CD44 isoform in the 3 EC lines appears to be directly opposite of their HA binding profile. HUVEC which do not bind HA or display any HA-mediated functions, express the highest level of CD44, followed by HPAEC and HMVEC-L, both of which bind HA and display HA-mediated adhesion and mitogenic response (Fig. 3 A).

Since CD44 expression could not explain the observed heterogeneity among EC lines with respect to HA binding and HA-mediated functions, we next examined the expression of RHAMM in these cells. Although RHAMM expression has not been previously demonstrated in ECs, RHAMM is expressed on many cell types and RHAMM-HA interaction has been shown to induce cellular signaling and cell motility (36,37). As shown in Fig. 3 B, immunoblot analysis using an anti-RHAMM antibody reveals the expression of an ~ 75-80 kDa RHAMM-related protein in all 3 ECs. Densitometric analysis of the RHAMM-related protein bands detected in three EC lines (calculated as the ratio of intensities of RHAMM-related band/total protein), showed that HUVEC express ~ 5- and 3-fold less RHAMM-related protein than HMVEC-L and HPAEC, respectively. Furthermore, RHAMM expression is the highest in HMVEC-L followed by HPAEC and the lowest expression of RHAMM is observed in HUVEC. These results show that
the expression of a RHAMM-related protein is consistent with the HA-binding profile of different ECs.

We next examined the profile of HA-binding proteins expressed in the three EC lines, by probing cellular proteins with biotinylated HA in a blotting experiment (43). As shown in Fig. 3 C, all three EC lines show the presence of a single ~ 75-80 kDa protein that binds biotinylated HA. The expression of this HA-binding protein in HMVEC-L, HPAEC, and HUVEC is consistent with their HA-binding profile and response to HA-induced cellular functions. As determined by the densitometric analysis ratio, HUVEC which do not bind HA, express 4.4- and 3.1-fold less expression of HA binding protein than HMVEC-L and HPAEC, respectively. (Fig. 3 C and densitometric data). The molecular mass of the ~ 75-80 kDa HA-binding protein is very similar to that of RHAMM and not CD44 (Fig. 3 A and C), suggesting that RHAMM may be the major HA-binding protein in primary human ECs. To further confirm this notion, we immunoprecipitated RHAMM from ECs using an anti-RHAMM antibody and probed these immunoprecipitates by biotinylated HA blotting. As shown in Fig. 3 D, the immunoprecipitated ~ 75-80 kDa RHAMM protein binds to biotinylated HA on the blot, suggesting that the ~ 75-80 kDa HA binding protein expressed in primary human ECs is mostly likely RHAMM.

Detection of RHAMM expression by RT-PCR and cloning analyses:

To confirm the expression of RHAMM in human ECs, we performed RT-PCR analysis on the RNA isolated from HMVEC-L, HPAEC and HUVEC using RHAMM specific primers. As shown in Fig. 4, an expected 386 bp PCR product is amplified from the RNA isolated from all three EC lines. PCR cloning and sequencing showed that the 386 bp product has 100% sequence identity to the published RHAMM sequence (GenBank accession number AF032862; data not shown). This result confirms the expression of RHAMM at the RNA and protein levels, in primary human ECs.
Effect of HA and F1 fragment on protein tyrosine phosphorylation:

A. Dose- and time-dependent effect: It has been previously shown that HA-RHAMM interaction induces tyrosine phosphorylation of several cellular proteins, including the focal adhesion kinase, p125<sub>FAK</sub> in fibroblasts (41). More recently, RHAMM has been shown to regulate platelet derived growth factor induced tyrosine phosphorylation of ERK (42). Since we found that RHAMM might be the functional HA receptor expressed on human ECs, we examined the effect of HA and the F1 fragment on protein tyrosine phosphorylation in HMEVC-L and HPAEC. In these experiments, following the treatment of intact cells with HA or the F1 fragment, extracts of the treated cells were probed by anti-phosphotyrosine antibody blotting.

As shown in Fig. 5 A, HA induces the tyrosine phosphorylation of 3 major proteins (mol. wt. approximately 125 kDa, 80 kDa and 42/44 kDa) in HMVEC-L. The increase in the tyrosine phosphorylation of these proteins is dose dependent and the maximum increase occurs at 2 μg/ml concentration (as determined by the densitometric ratio of the intensities of tyrosine phosphorylated band to total protein band(s): ~ 125 kDa: 1.4-fold; ~ 80 kDa: 1.6-fold; ~ 42/44 kDa: 3.3-fold). The tyrosine phosphorylation of these 3 proteins decreases at higher HA concentrations (e.g., 10 μg/ml). The F1 fragment also induces tyrosine phosphorylation of the same 3 proteins and the maximum increase occurs at 2 μg/ml concentration of F1 (Fig. 5 B; ~ 125 kDa: 4.2-fold, ~ 80 kDa: 2.5-fold; ~ 42/44 kDa: 3.6-fold).

The increase in HA- or F1 fragment-induced tyrosine phosphorylation of HMVEC-L proteins is also time-dependent. As shown in Fig. 5 C and D, the maximum increase in the tyrosine phosphorylation of all three proteins occurs, 1 min after HA/F1 treatment. No further increase or decrease in the phosphorylation of the ~ 125 kDa and ~ 80 kDa proteins is observed even after the cells are treated with HA or the F1 fragment for up to 10 min (Fig. 5 C and D). However, the tyrosine phosphorylation of the ~ 42/44 kDa protein is only ~ 50% after 10 min of treatment in comparison to that after a 1 min treatment (as determined by the densitometric...
It is possible that the 42/44 kDa protein is rapidly dephosphorylated after the maximum level of phosphorylation is achieved following HA/F1 treatment. It is also clear from the time course experiment that the F1 fragment is more efficient than HA in inducing the tyrosine phosphorylation of the ~ 125 kDa and ~ 42/44 kDa proteins (Fig. 5 C and D). In these experiments, the increase in the tyrosine phosphorylation of the ~ 125 kDa (HA: 1.5-fold, F1: 5.1-fold) and 42/44 kDa (HA: 1.8-fold; F1: 3.8-fold) proteins was ~ 3-fold higher than that observed following HA treatment. It is noteworthy that possibly due to hyperphosphorylation, the mobility of the 42/44 kDa protein is retarded following treatment with the F1 fragment for 1 min.

We next examined the dose and time-dependent effect of HA and F1 fragment on the protein tyrosine phosphorylation in HPAEC. As shown in Fig. 6 A, as is the case in HMVEC-L, HA induced the tyrosine phosphorylation of 3 proteins of mol. wt. ~ 125 kDa, ~ 80 kDa and ~ 42/44 kDa. The maximum increase in the tyrosine phosphorylation of these 3 proteins is observed at 2 and 5 μg/ml concentrations of HA (~ 125 kDa: 3.5-fold, ~ 80 kDa: 2.8-fold and ~ 42/44 kDa: 2.4-fold), and the increase in tyrosine phosphorylation is reduced at 10 μg/ml concentration. The effect of HA was more significant on the phosphorylation of ~ 125 kDa and ~ 80 kDa proteins, when compared to the phosphorylation of ~ 42/44 kDa protein (Fig. 6 A). The F1 fragment also induced the tyrosine phosphorylation of ~ 125 kDa, ~ 80 kDa and ~ 42/44 kDa proteins. The maximum increase in the tyrosine phosphorylation occurred at 2 μg/ml concentration of the F1 fragment (~ 125 kDa: 1.5-fold, ~ 80 kDa: 3.2-fold, and 42/44 kDa: 5.3-fold) and remained the same at 5 μg/ml concentration (Fig. 6 B). It is noteworthy that the F1 fragment was able to induce the tyrosine phosphorylation of the ~ 42/44 kDa and ~ 80 kDa proteins more significantly. The time course of HA and the F1 fragment-induced tyrosine phosphorylation of HPAEC proteins shows that the maximum increase in the phosphorylation of all the 3 proteins occurs between 0.5 to 1 min of treatment (Fig. 6 C and D). At longer treatment intervals (2-10 min), the increase in the tyrosine phosphorylation is less than that observed at 1 min of treatment (Fig. 6 C and D). The reduction in tyrosine phosphorylation is more significant.
for the ~ 42/44 kDa protein, suggesting that the ~ 42/44 kDa protein may be getting rapidly dephosphorylated, following tyrosine phosphorylation.

b. Identification of the proteins tyrosine phosphorylated following HA/F1 treatment:

Since both the HA and the F1 fragment induce tyrosine phosphorylation of three proteins of the same mol. wt., in both HMVEC-L and HPAEC, we next attempted to identify these proteins. The size of the ~ 125 kDa protein resembles that of the focal adhesion kinase or p125<sub>FAK</sub> (44). p125<sub>FAK</sub> has been shown to be tyrosine phosphorylated following the interaction of RHAMM with HA (41). Paxillin is an ~ 80 kDa protein that is present in the adhesion complex (45). It binds p125<sub>FAK</sub> and this interaction is important for the maximal tyrosine phosphorylation of paxillin by the p125<sub>FAK</sub>.src complex (46). The size of the ~ 80 kDa protein that is tyrosine phosphorylated following treatment of ECs with HA or the F1 fragment, is the same as that of paxillin. The size of the ~42/44 kDa protein doublet that is tyrosine phosphorylated following HA/F1 treatment is the same as that of p42/44 ERK (i.e., ERK1 and ERK2). To determine whether p125<sub>FAK</sub>, paxillin and p42/44 ERK proteins are tyrosine phosphorylated in HA/F1-treated ECs, we immunoprecipitated these proteins from HA/F1-treated HMVEC-L using respective specific antibodies and analyzed the immunoprecipitates by anti-phosphotyrosine antibody blotting. As shown in Fig. 7, both HA and F1 treatment induce the tyrosine phosphorylation of p125<sub>FAK</sub>, paxillin and p 42/44 ERK (panels ptyr-FAK, ptyr-paxillin and ptyr-ERK). It is noteworthy that the F1 fragment induces the tyrosine phosphorylation of ERK 3- to 4-fold more than that induced by HA and this hyperphosphorylation causes an upward shift in the electrophoretic mobility of tyrosine phosphorylated ERK in the F1 treated sample (Fig. 7, panel ptyr-ERK). The increase in tyrosine phosphorylation of FAK, paxillin and ERK is not due to differences in the levels of these proteins in the control untreated sample and the HA/F1-treated samples, because the immunoblot analyses using anti-FAK, anti-paxillin and anti-ERK antibodies show comparable levels of FAK, paxillin and ERK proteins in these samples (Fig. 7,
panels FAK, paxillin and ERK). At this point, however, we do not rule out the possibility that other proteins with molecular weights similar to those of FAK, paxillin and ERK are also tyrosine phosphorylated following HA and F1 treatments. Further studies are needed to test this possibility.

Effect of anti-RHAMM and anti-CD44 antibodies on HA/F1 fragment-induced tyrosine phosphorylation:

Since both CD44 and RHAMM proteins are expressed in HMVEC-L, but RHAMM appears to be involved in HA-binding, we examined which of these two proteins is responsible for HA/F1 fragment-induced protein tyrosine phosphorylation. As shown in Fig. 8, both HA and the F1 fragment-induced tyrosine phosphorylation of p125\(^{FAK}\), paxillin and ERK proteins is not inhibited by the treatment of HMVEC-L with anti-CD44 antibody. However, the anti-RHAMM antibody inhibits both HA- and the F1 fragment-induced tyrosine phosphorylation of all the three proteins over 90%. The control antibody has no effect on the HA/F1 fragment-induced tyrosine phosphorylation of HMVEC-L proteins. These results demonstrate that the interaction of RHAMM with HA or the F1 fragment is responsible for stimulation of protein tyrosine phosphorylation in HMVEC-L.

DISCUSSION

The regulation of EC functions (i.e., proliferation, migration and capillary formation) is important in pathophysiologic processes such as angiogenesis, wound healing, atherosclerosis and ischemia (1,2). While growth factors (i.e., VEGF, and b-FGF) stimulate EC functions, certain extracellular matrix components also directly regulate these functions. For example, small HA fragments stimulate angiogenesis (17). Although, some studies have examined the effect of HA and HA fragments on bovine endothelial cell functions, their effect on primary human ECs has not been examined (15,16,47). An important finding of our study is that the
human ECs derived from a large artery (i.e., HPAEC), a microvessel (i.e., HMVEC-L) and a vein (i.e., HUVEC) differ in their ability to bind HA/HA fragments and consequently, in their responsiveness to these ligands. While HPAEC and HMVEC-L both displayed high affinity HA binding sites, HUVEC did not bind HA (Fig. 1). Furthermore, HMVEC-L responded better to the F1 fragment-induced mitogenic response (2.5-fold) than HPAEC (1.8-fold) and also displayed better HA-mediated cell adhesion (Fig. 2 and Table 1). We have also observed that primary human coronary artery ECs and primary human dermal microvessel ECs display high affinity HA binding and some heterogeneity in HA-induced functions (Unpublished results). Our findings support the notion that ECs from different vascular origins show heterogeneity in their structures, cell surface expression of adhesion molecules, metabolic properties and response to growth factors (48,49).

The observed heterogeneity among various ECs with respect to their response to HA/F1 fragment, is related to the expression of functional HA receptors. It has been previously shown that the CD44 expression in cultured HUVEC depends upon their proliferation status (35). However, in our study HUVEC were found to express comparable, if not significantly more, amounts of CD44 when compared to CD44 expression in HMVEC-L and HPAEC (Fig. 3). The discrepancy between CD44 expression and no HA binding observed in HUVEC is unclear at the present time. However, it has been shown that although both CD44 and RHAMM are expressed in B cells from multiple myeloma patients, RHAMM and not CD44 is responsible for the motile behavior of these cells (50). Furthermore, clustering of CD44 molecules, as well as, the N-glycosylation of CD44 isoforms has been shown to be necessary for HA binding (51). There is also some evidence that CD44 may be expressed as a non-functional receptor in N-myc amplified cell lines (52). Thus the expression of CD44 alone may not confer HA binding properties.
The expression of RHAMM in ECs has not been reported previously. In this study we demonstrate the expression of RHAMM both at the RNA and protein levels. The observations, (a) HA-binding, HA-mediated cell adhesion, and HA-induced proliferation appear to correlate with RHAMM expression in primary human ECs, (b) ~ 80 kDa RHAMM protein binds HA in a HA-overlay transblot experiment, and (c) the anti-RHAMM antibody blocks HA/F1 fragment-induced tyrosine phosphorylation of EC proteins, suggest that RHAMM is the major functional HA receptor in primary human ECs.

The stimulation of p125\(^{\text{FAK}}\) and paxillin tyrosine phosphorylation, following the treatment of ECs with HA/F1 fragment, could explain the basis for certain HA-induced EC functions such as adhesion and migration. It has been shown that upon integrin-dependent cell adhesion p125\(^{\text{FAK}}\) is tyrosine phosphorylated and becomes catalytically active (53,54). Paxillin is another focal adhesion complex-associated protein, which contains two FAK-binding sites (55). The binding of paxillin to FAK is necessary for its maximum tyrosine phosphorylation, possibly by the FAK.src complex (46). Since paxillin is also tyrosine phosphorylated following the treatment of ECs by HA/F1 fragment, suggests that the RHAMM-HA/F1 interaction at the cell surface may activate FAK and cause recruitment of paxillin in the focal adhesion complex, followed by its tyrosine phosphorylation. Furthermore, HA/F1 fragment may regulate EC motility through focal adhesion complex.

The treatment of bovine ECs with HA oligosaccharides has been shown to induce the tyrosine phosphorylation of ERK1 (47). Our studies are consistent with these finding. It is interesting to note that the F1 fragment appears to be a better stimulator of tyrosine phosphorylation in ERK than HA. The 42/44 kDa doublet of ERK that gets phosphorylated following F1 treatment for 2 to 5 min most likely represents ERK1 and ERK2 proteins. Two reasons could explain why the ERK2 band appears to be strongly phosphorylated when
compared to the ERK1 protein. First is that F1 treatment preferentially induces tyrosine phosphorylation in ERK2. The other explanation is that, since the pan specific ERK antibody is generated against a peptide sequence in ERK2 it recognizes ERK2 better. The latter possibility may explain why we do not observe the ERK doublet consistently (Fig. 5, 6, and 7). We are trying to resolve the issue of ERK1 tyrosine phosphorylation by the F1 fragment, using antibodies specific to ERK1 and ERK2. The concentration at which the F1 fragment induces maximum cellular proliferation (2 \( \mu \text{g/ml} \)) is the same as that at which it induces tyrosine phosphorylation of ERK. This suggests that the F1 fragment may induce mitogenic response in primary human ECs through the MAP-kinase pathway.

Although, RHAMM is a well studied HA receptor, its expression on the cell surface has recently become a controversial issue. RHAMM lacks a transmembrane domain, and the mechanism by which it is expressed on the cell surface is not clear (56,57). Assmann et al failed to detect any cell surface expression of RHAMM in breast cancer cells and have suggested that RHAMM is an intracellular HA binding protein (58). One of the possibilities why Assmann et al were unable to detect the cell surface expression of RHAMM in breast cancer cells is that, most tumor cells produce HA \textit{in vitro} and this could possibly down regulate RHAMM from the cell surface due to HA binding (13). Nevertheless, in our study addition of an anti-RHAMM antibody blocked HA/F1 fragment-induced tyrosine phosphorylation of key signaling molecules, suggesting that at least in primary human ECs RHAMM is expressed on the cell surface. In addition to CD44 and RHAMM (as demonstrated in this study), ECs have been found to express other HA receptors (13). For example, microvessel ECs in human aorta express a novel EC specific HA receptor WF-HABP (59). These findings stress that many functionally active HA receptors may be expressed in human ECs in an organ and vessel type specific manner.
The vascular endothelium is of key importance in normal physiology and in the pathophysiology of several vascular diseases and tumor angiogenesis. Since HA levels are elevated in these diseases and angiogenic HA fragments are generated in certain tumors (11), the vascular endothelium is exposed to HA and possibly to angiogenic HA fragments under pathophysiologic conditions. The identification of at least one of the functional HA receptors in primary human ECs, demonstration of EC heterogeneity with respect to HA receptors and responsiveness to HA-mediated functions, and understanding some of the steps in HA-mediated signal transduction in primary human ECs, may help suggest new avenues to promote EC regeneration in vascular diseases while inhibiting critical steps in tumor angiogenesis.

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Table 1: Adhesion of human ECs to HA-coated surface. Tran$^{35}$S-labeled HMVEC-L (5.8 x $10^5$ cpm/50,000 cells), HPAEC (4.3 x $10^5$ cpm/50,000 cells) and HUVEC (7.6 x $10^5$ cpm/50,000 cells) were incubated on HA-coated surfaces at $4^0$ C for 60 min in a binding buffer. The non-specific binding was measured by pre-incubating cells with 300 $\mu$g/ml of soluble HA at $4^0$ C for 15 min prior to adding them on the HA-coated surfaces. The non-specific adhesion was 29% of the total HA adhesion and was subtracted. To examine the effect of HA fragments on HA-mediated cell adhesion the cells were incubated with 50 $\mu$g/ml of each fragment (i.e., F1, F2 and F3) at $4^0$ C for 15 min prior to adding them on the HA-coated surface. Following binding, the cells were solubilized and the cell-associated radioactivity was calculated as described in “Materials and Methods”. From the specific cell-associated radioactivity counts, number of cells adhered to the well was calculated. The results represent mean ± SD from triplicate determinations. Since F2 and F3 fragments did not inhibit HA-mediated adhesion of ECs, the inhibition data related to only the F1 fragment is shown.

| Cell type   | Specific adhesion (cells/well) | Effect of F1 (% inhibition of specific adhesion) |
|-------------|--------------------------------|-----------------------------------------------|
| HMVEC-L     | $12739 \pm 1239$               | $95.4 \pm 2.7$                                |
| HPAEC       | $8468 \pm 1651$                | $96.3 \pm 4.9$                                |
| HUVEC       | N.D.                           | N.D.                                          |
FIGURE LEGENDS:

Fig. 1: [3H]-HA binding to primary human ECs. HPAEC, HMVEC-L and HUVEC were incubated with various concentrations of [3H]-HA (0.15 to 3 μg/ml) in a binding buffer at 4°C for 4 h, under equilibrium binding conditions, as described in “Materials and Methods”. Non-specific binding was determined in the presence of a 100-fold excess of unlabeled HA and was subtracted from the total binding. The results represent an average of triplicate determinations from the same experiment. A: Equilibrium binding isotherm of [3H]-HA binding to ECs. B: Scatchard plot analysis of the equilibrium binding data on HMVEC-L and HPAEC that are presented in Fig. 1 A. To calculate the dissociation constant (K_d) of HA binding and receptor numbers per cell, the mol. wt. of [3H]-HA was considered as 10^6 Dalton.

Fig. 2: Effect of HA and HA fragments on the mitogenic response of primary human ECs. HMVEC-L (A), HPAEC (B), and HUVEC (C) were incubated in the presence of various concentrations of either HA or HA fragments (F1, F2 and F3) in serum-free EBM. The DNA synthesis was measured in terms of [3H]-methyl thymidine incorporation as described in “Materials and Methods”.

Fig. 3: Detection of HA receptor in primary human ECs. A: Detection of CD44. HMVEC-L, HPAEC and HUVEC were labeled with Tran^{35}S-label and were solubilized in RIPA buffer. The cell extracts were immunoprecipitated using a rat anti-CD44 monoclonal antibody that recognizes all CD44 isoforms. The immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis and fluorography. B: Detection of RHAMM. HMVEC-L, HPAEC and HUVEC were detergent solubilized and the cell extracts were separated on a SDS-polyacrylamide gel and then blotted. The membrane blot was probed with a rabbit anti-RHAMM antibody and a goat anti-rabbit alkaline phosphatase conjugated secondary antibody. The blots were developed using an alkaline phosphatase color detection system, as described in
“Experimental procedures”. **C: Detection of HA binding proteins.** The membrane blots containing cell extracts of HMVEC-L, HPAEC, and HUVEC were prepared as described in “B”. The blot was sequentially incubated with biotinylated HA, and an alkaline conjugated goat anti-biotin conjugated antibody. The blots were developed using an alkaline phosphatase detection system. **D. Binding of RHAMM to biotinylated HA.** HMVEC-L, HPAEC and HUVEC were detergent solubilized and immunoprecipitated using a rabbit anti-RHAMM antibody. The immunoprecipitates were analyzed by transblotting using biotinylated HA and then developed as described in “C”.

**Fig. 4: Examination of RHAMM expression by RT-PCR analysis.** Total RNA extracted from HPAEC (lane 1) and HMVEC-L (lane 2) was reverse transcribed and the cDNA was amplified using RHAMM-specific primers. The PCR product was analyzed by agarose gel electrophoresis and ethidium bromide staining.

**Fig. 5: Effect of HA and the F1 fragment on protein tyrosine phosphorylation in HMVEC-L.**

**A and B:** HMVEC-L were incubated with various concentrations of HA (A) or the F1 fragment (B) at 37°C for 5 min. Following incubation, the cells were solubilized in SDS sample buffer. An equal volume of cell extract from each sample was electrophoresed on SDS-polyacrylamide gels, blotted onto PVDF membrane and probed with anti-phosphotyrosine antibody, as described in “Materials and methods”. **C and D:** HMVEC-L were incubated with 2 μg/ml of HA or the F1 fragment at 37°C for various time periods. Following incubation, the cells were lysed in SDS-sample buffer and the extracts were analyzed as described in “A and B”.

**Fig. 6: Effect of HA and the F1 fragment on protein tyrosine phosphorylation in HPAEC.**

**A and B:** HPAEC were incubated with various concentrations of HA (A) or the F1 fragment (B) at 37°C for 5 min. Following incubation, the cells were solubilized in SDS sample buffer. An equal
volume of cell extract from each sample was electrophoresed on SDS-polyacrylamide gels, blotted onto PVDF membrane and probed with anti-phosphotyrosine antibody, as described in “Materials and methods”. C and D: HPAEC were incubated with 2 μg/ml of HA or the F1 fragment at 37°C for various time periods. Following incubation, the cells were lysed in SDS-sample buffer and the extracts were analyzed as described in “A and B”.

Fig. 7: Effect of HA and the F1 fragment on the tyrosine phosphorylation of p125^FAK, paxillin, and ERK. HMVEC-L were incubated with HA or the F1 fragment at 2 μg/ml for 5 min. Following treatment, the cells were lysed and equal amounts of cell lysates were directly analyzed by immunoblotting using anti-FAK, anti-paxillin or anti-ERK antibodies. Alternatively, the cell lysates were immunoprecipitated using anti-FAK, anti-paxillin or anti-ERK antibodies. The immunoprecipitates were then analyzed by anti-phosphotyrosine immunoblotting (panels ptyr-FAK, ptyr-paxillin and ptyr-ERK) as described in “Materials and Methods”.

Fig. 8: Effect of HA receptor antibodies on HA and the F1 fragment-induced tyrosine phosphorylation. HMVEC-L were treated with HA or F1 fragment (2 μg/ml for 5 min) in the presence or absence of anti-CD44, anti-RHAMM or the control normal rabbit IgG (NRIgG). Following incubation the cells were lysed and the cell lysates were analyzed by anti-phosphotyrosine antibody blotting as described in “Materials and Methods”.
Fig. 1 A

Fig. 1 B

- HMVEC-L
- HPAEC
- HUVEC

$^3$H-HA Bound (dpm/ml)

$^3$H-HA (µg/ml)

Bound/Free
FIGURE 4
FIGURE 5
FIGURE 6

HPAEC

HA

CONCENTRATION

F1

CONCENTRATION

A

0 0.5 1 2 5 10 (µg/ml)

B

0 0.5 1 2 5 10 (µg/ml)

C

0 0.5 1 2 5 10 (min)

D

0 0.5 1 2 5 10 (min)
FIGURE 7
FIGURE 8
Differences in hyaluronic acid-mediated functions and signaling in arterial, microvessel and vein-derived human endothelial cells
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