The role for hyaluronan (HA) and CD44 in vascular barrier regulation is unknown. We examined high and low molecular weight HA (HMW-HA, ~1,000 kDa; LMW-HA, ~2.5 kDa) effects on human transendothelial monolayer electrical resistance (TER). HMW-HA increased TER, whereas LMW-HA induced biphasic TER changes ultimately resulting in EC barrier disruption. HMW-HA induced the association of the CD44s isoform with, and AKT-mediated phosphorylation of, the barrier-promoting sphingosine 1-phosphate receptor (SIP1) within caveolin-enriched lipid raft microdomains, whereas LMW-HA induced brief CD44s association with SIP1 followed by sustained association of the CD44v10 isoform with, and Src and ROCK 1/2-mediated phosphorylation of, the barrier-disrupting SIP3 receptor. HA-induced EC cytoskeletal reorganization and TER alterations were abolished by either disruption of lipid raft formation, CD44 blocking antibody or siRNA-mediated reductions in expression of CD44 isoforms. Silencing SIP1, AKT1, or Rac1 blocked the barrier enhancing effects of HA whereas silencing SIP3, Src, ROCK1/2, or RhoA blocked the barrier disruption induced by LMW-HA. In summary, HA regulates EC barrier function through novel differential CD44 isoform interaction with SIP receptors, SIP receptor transactivation, and RhoA/Rac1 signaling to the EC cytoskeleton.

Endothelial cells (EC) constitute an inner lining of blood vessels to regulate the interface between the blood and the vessel wall including vascular barrier regulation, passive diffusion, and active transport of substances from the blood, regulation of vascular smooth muscle tone, and blood clotting (1, 2). Disruption of this semi-selective cellular barrier is a critical feature of inflammation as well as an important contributing factor to atherosclerosis and tumor angiogenesis (3, 4). A number of bioactive agonists contribute to EC barrier regulation via direct effects on the integrity of EC tight junctions, cell-cell, and cell-matrix adhesions. One important extracellular matrix component, hyaluronan (HA), and its cell surface receptor, CD44, has been implicated in normal EC function and angiogenesis (5, 6).

Hyaluronan (HA) is a major glycosaminoglycan (GAG) component of the extracellular matrix of many tissues. Structurally, high molecular weight (HMW) HA (>500,000 daltons) is composed of repeating disaccharide units of D-glucuronic acid and N-acetylgalcosamine, which exists as a random coil structure that can expand in aqueous solutions (6, 7). Aqueous HA is highly viscous and elastic, properties which contribute to its space filling and filtering functions (7) and is synthesized by at least three hyaluronan synthases (HAS1, HAS2, and HAS3) (8). Studies utilizing HAS genes knock-out mice reveal that only HAS2 is required for viability with HAS2 deletion resulting in lethal abnormalities in cardiac development, which is rescued by addition of exogenous HA (9, 10). Proinflammatory cytokines (TNFα, IL-1β and LPS induce HA production in EC in vitro (11) and increased HA levels are observed in bronchoalveolar lavage fluid (BALF) from patients with inflammatory lung disorders such as pulmonary fibrosis, acute lung injury, and chronic obstructive pulmonary disease (12–15). Further, intratracheal administration of nebulized high MW HA has been used to prevent injury in experimental emphysema (16).

HA is degraded by hyaluronidases, under certain pathological inflammatory conditions, to produce lower molecular weight fragments found in tissue injury and serum of patients with certain malignancies (17, 18). Further, low MW fragments of HA (LMW, 1,350–4,500 Da) are potent inducers of angiogenesis in vitro and in vivo (19, 20). Six hyaluronidase genes encode Hyal-1,2,3,4, PHYLAL1 (a pseudogene) and PH-20 with high MW HA and its fragments binding hyaladherin proteins including CD44, a major HA receptor (5, 8).

CD44 belongs to a family of transmembrane glycoproteins, which are expressed in a variety of cells including EC (21, 22). Multiple CD44 isoforms result from extensive, alternative exon splicing events (23, 24) with the alternative splicing often occurring between exons 5 and 15 leading to a tandem insertion of one or more variant exons (v1-v10, or exon 6 through exon 14) within the membrane proximal region of the extracellular domain (25, 26). The variable primary amino acid sequence of different CD44 isoforms is further modified by extensive N- and O-glycosylations and glycosaminoglycan (GAG) additions (5, 26). The extracellular domain of CD44, containing clusters of conserved basic residues, plays an important role in HA binding, whereas the cytoplasmic domain is

The abbreviations used are: EC, endothelial cell; HMW, high molecular weight; LMW, low molecular weight; IL, interleukin; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; TER, transendothelial monolayer electrical resistance; HA, hyaluronan; HGF, hepatocyte growth factor; PDGF, platelet-derived growth factor; SIP, sphingosine 1-phosphate; VEGF, vascular endothelial growth factor; CEM, caveolin-enriched microdomain; MβCD, methyl-β-cyclodextrin.
HA/CD44/S1P Receptor Signaling in Human EC

A

| Immunoblot: | Triton X-100 | % Optiprep™ |
|------------|--------------|-------------|
|            | Lysate Sol. | Insol.      |
| a. Anti-Caveolin-1 |             |             |
| b. Anti-Flotillin-1 |             |             |
| c. Anti-Lamin A/C |             |             |
| d. Anti-GRP75    |             |             |
| e. Anti-GRP78    |             |             |
| f. Anti-GRASP65  |             |             |
| g. Anti-VEGF R.  |             |             |
| h. Anti-Vimentin |             |             |

Marker:
- Lipid Raft
- Lipid Raft
- Nucleus
- Mitochondria
- ER
- Golgi
- Plasma Membrane
- Intermediate Filament Network

B

Cholesterol Quantitation

C

Immunoblot:
- M.W. (kDa)
- IM-7
- CD44v
- CD44v3
- CD44v6
- CD44v10

D

RT-PCR

Markers CD44 CD44v10

EC Lysate
both structurally and functionally linked to cytoskeletal elements and signaling molecules (5, 26). In particular, HA binding to CD44 isoforms can activate several downstream events including the PI 3-kinase/AKT pathway, the tyrosine kinase Src and the serine/threonine kinase, ROCK (22, 27, 28). The signaling properties of CD44 are required for a variety of cellular activities including EC adhesion, proliferation, migration, and angiogenesis (5, 20–22, 26). The effects of HA and CD44 on human vascular barrier regulation, however, are unknown but are explored in the present study.

The S1P family of receptors regulate a number of functions common to CD44 including migration and angiogenesis and like CD44, localize to lipid rafts in activated EC (21, 29–31). S1P binds to the plasma membrane heptahelical S1P receptors 1 (Edg1), 2 (Edg5), 3 (Edg3), 4 (Edg6), and 5 (Edg8) expressed in a variety of cell types including endothelium (4, 31, 32). Human EC exhibit high expression of S1P1 and S1P3 with S1P1 signaling coupled to the G_i pathway and Rac1 activation whereas S1P3 signaling couples to the G_p, G_{q/11}, and G_{12/13} pathways and activates RhoA to a much greater extent than Rac1 (4, 33).

In this study, we examined the effects of low MW HA (~2,500 Da) and high MW HA (~1 million Da) on selective CD44 isoform-specific S1P receptor transactivation leading to EC barrier regulation. We further examined the role of caveolin-enriched microdomains (CEM) or lipid rafts and cytoskeletal regulatory GTPases (i.e., RhoA and Rac1) on HA-induced regulation of EC barrier integrity.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Reagents**—Human pulmonary artery EC were obtained from Cambrex (Walkersville, MD) and cultured as previously described in EBM-2 complete medium (Cambrex) at 37 °C in a humidified atmosphere of 5% CO_2, 95% air, with passages 6–10 used for experimentation (4). Unless otherwise specified, reagents were obtained from Sigma. Reagents for SDS-PAGE electrophoresis were purchased from Bio-Rad, Immobilon-P transfer membrane from Millipore (Millipore Corp., Bedford, MA), and gold microelectrodes from Applied Biophysics (Troy, NY). Rat anti-CD44 (IM-7, common domain) antibody was purchased from BD Biosciences (San Diego, CA). Goat anti-CD44v4 (v3–v10) antibody and mouse anti-KDR (VEGF receptor 2) antibody were purchased from Chemicon, International (Temecula, CA). Rabbit anti-CD44v3, anti-CD44v6 and anti-CD44v10 antibody were purchased from Calbiochem. Rabbit anti-caveolin-1, anti-flotillin-1, anti-lamin A/C, anti-GRP75, anti-GRP78, anti-GRASP65, anti-vimentin, anti-AKT1, anti-phosphothreonine (308) AKT, anti-phosphoserine (473) AKT, anti-ROCK1, anti-ROCK2, anti-p115 rho-GEF, and anti-Tiam1 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-S1P_{1} receptor was purchased from Affinity Bioreagents (Golden, CO). Rabbit anti-phosphoserine and anti-phosphothreonine antibodies were purchased from Zymed Laboratories, Inc. (South San Francisco, CA). Mouse antibodies were purchased for S1P_{3} receptor (Exalpha Biologicals, Watertown, MA), RhoA, Rac1, pp60Src, and phosphotyrosine antibody (Upstate Biotechnology, Lake Placid, NY). Mouse anti-β-actin antibody and rabbit anti-phosphotyrosine (418) Src antibody were purchased from Sigma. Recombinant active Src, ROCK1, and ROCK2 were purchased from Upstate Biotechnology. Secondary horseradish peroxidase-labeled antibodies were purchased from Amersham Biosciences (Piscataway, NJ). Texas Red-conjugated phalloidin was purchased from Molecular Probes (Eugene, OR).

**Preparation and Quantitation of Low and High MW HA**—
The method of preparation is similar to that described previously (19). For HMW-HA, 500 mg of rooster comb HA (~1 million Da polymers) (34) was dissolved in distilled water and centrifuged in an Ultrafree-MC™ Millipore 100,000 Da MW cutoff filter and the flow-through (less than 100,000 Da) was discarded. For LMW-HA, 500 mg of rooster comb HA was digested with 20,000 units of bovine testicular hyaluronidase in digestion buffer (0.1 M sodium acetate, pH 5.4, 0.15 M NaCl) for 24 h, and the reaction stopped with 10% trichloroacetic acid. The resulting solution was centrifuged in an Ultrafree-MC™ Millipore 5,000 Da MW cutoff filter and the flow-through (less than 5,000 Da) was dialyzed against distilled water for 24 h at 4 °C in 500-Da cutoff Spectra-Por tubing (Pierce-Warriner, Chester, UK). Low and High MW HA were quantitated using an ELISA-like competitive binding assay with a known amount of fixed HA and biotinylated HA-binding peptide (HABP) as the indicator (35). In some cases, both Low and High MW HA were subject to boiling, proteinase K (50 µg/ml) digestion, hyaluronidase SD digestion (100 milliunits/ml) or addition of boiled (inactivated) hyaluronidase SD to test for possible protein/lipid contaminants (36). LMW and HMW-HA with DNA standards were run on 4–20% SDS-PAGE gels and stained with combined Alcian blue and silver staining to further determine HA purity and size (37).

**Lipid Raft Isolation**—Caveolin-enriched microdomain known as lipid rafts were isolated from human lung EC as we previously described (29). Triton X-100-insoluble materials were mixed with 0.6 ml of cold 60% Optiprep™ and overlaid with 0.6 ml of 40–20% Optiprep™ and the gradients centrifuged (35,000 rpm) in SW60 rotor for 12 h at 4 °C and different fractions were collected and analyzed. In some cases, different fractions were analyzed for total cholesterol content using

![FIGURE 1. Characterization of caveolin-enriched microdomains and CD44 expression in human pulmonary EC.](image-url)
HA/CD44/S1P Receptor Signaling in Human EC

A

B

C

D

E

Cholesterol Quantitation from Human EC Lipid Rafts

High MW HA (30 min.)

Low MW HA (30 min.)

% Inhibition of HA-Mediated Permeability

Control IgG  Anti-CD44 Antibody (PBS)  Vehicle  Cholesterol Depletion

Control IgG  Anti-CD44 Antibody (PBS)  Vehicle  Cholesterol Depletion
the Amplex Red™ cholesterol assay kit (Invitrogen (Molecular Probes)).

**Immunoprecipitation and Immunoblotting**—Cellular materials associated within the 20% Optiprep™ fractions (lipid raft fraction) were incubated with IP buffer A (50 mM HEPES (pH 7.5), 150 mM NaCl, 20 mM MgCl₂, 1% Nonidet P-40 (Nonidet P-40), 0.4 mM Na₃VO₄, 40 mM NaF, 50 μM okadaic acid, 0.2 μM phenylmethylsulfonyl fluoride, 1:250 dilution of Calbiochem protease inhibitor mixture 3) and immunoprecipitated with either anti-S1P₁ receptor (Im-7, common domain) (B, panel a and c), anti-S1P₃ receptor (B, panel b), or anti-S1P₃ receptor (B, panel d) antibody. Experiments were performed in triplicate with highly reproducible findings (representative data shown).

**FIGURE 3.** Analysis of HA-induced CD44 isoform-specific interaction with and activation of S1P receptors in caveolin-enriched microdomains. A, EC were plated on gold microelectrodes, serum-starved for 1 h and either untreated (Control) or treated with 100 nM of Low or High MW HA for 5, 15 or 30 min and CEM (Lipid Raft) fractions (20% Optiprep™ layer) were then prepared as described in the “Experimental Procedures.” The CEM fractions were run on SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-Caveolin-1 (A, panel d), anti-CD44 (IM-7, common domain) (A, panel b), anti-S1P₁ receptor (A, panel d), or anti-S1P₃ receptor (A, panel e) antibody. Experiments were performed in triplicate with highly reproducible findings (representative data shown). B, EC were grown to confluency, serum-starved for 1 h, and either untreated (Control) or treated with 100 nM of Low or High MW HA for 5, 15, or 30 min and CEM fractions (20% Optiprep™ layer) were then prepared as described under “Experimental Procedures.” The CEM fractions were solubilized in IP buffer A (50 mM HEPES (pH 7.5), 150 mM NaCl, 20 mM MgCl₂, 1% Nonidet P-40 (Nonidet P-40), 0.4 mM Na₃VO₄, 40 mM NaF, 50 μM okadaic acid, 0.2 μM phenylmethylsulfonyl fluoride, 1:250 dilution of Calbiochem protease inhibitor mixture 3) and immunoprecipitated with either anti-S1P₁, or anti-S1P₃ receptor antibody. The resulting immunobeads were run on SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-CD44 (IM-7, common domain) (B, panel a and c), anti-S1P₁ receptor (B, panel b), or anti-S1P₃ receptor (B, panel d) antibody. Experiments were performed in triplicate with highly reproducible findings (representative data shown).

**FIGURE 2.** Characterization of low and high MW HA-induced CD44-mediated regulation of human EC permeability. A, EC were plated on gold microelectrodes, serum-starved for 1 h, and either untreated (Control) or treated with 1 nM, 10 nM, or 100 nM High MW HA. The TER tracing represents pooled data ± S.E. from three independent experiments as described under “Experimental Procedures.” The arrow indicates the time of High MW HA addition. A, inset, bar graph inset demonstrates that pretreatment of High MW HA by boiling (column b) or proteinase K digestion (column c) have little effect on High MW HA-induced EC TER (column a). Treatment with hyaluronidase SD (column d) blocked the effects of High MW HA, which were reversed by treating the HA with boiled (inactive) hyaluronidase SD (column e). B, EC were plated on gold microelectrodes, serum-starved for 1 h and either untreated (Control) or treated with 1, 10, or 100 nM Low MW HA. The arrow indicates the time of Low MW HA addition. The TER tracing represents pooled data ± S.E. from three independent experiments as described under “Experimental Procedures.” B, inset, bar graph inset demonstrates that pretreatment of Low MW HA by boiling (column b) or proteinase K digestion (column c) have little effect on Low MW HA-induced EC TER (column a). Treatment with hyaluronidase SD (column d) blocked the effects of Low MW HA, which were reversed by treating the HA with boiled (inactive) hyaluronidase SD (column e). C, graphical representation of percent inhibition of HA-induced change in EC permeability. EC were plated on gold microelectrodes, serum-starved for 1 h and either treated with 100 nM High MW HA + control (rat preimmune) IgG (10 μg/ml), 100 nM High MW HA + anti-CD44 (IM-7) antibody (10 μg/ml), 100 nM High MW HA + vehicle, (phosphate-buffered saline, pH 7.4), or 5 mM MβCD (a cholesterol depletion agent that abolishes CEM formation) + 100 nM Low MW HA + control (rat preimmune) IgG (10 μg/ml), 100 nM Low MW HA + anti-CD44 (IM-7) antibody (10 μg/ml), 100 nM Low MW HA + vehicle, (phosphate-buffered saline, pH 7.4), or 5 mM MβCD (a cholesterol depletion agent that abolishes CEM formation) + 100 nM Low MW HA. The bar graphs represent pooled TER data ± S.E. at 30 min after addition of agonist from three independent experiments as described under “Experimental Procedures.”
and pv10 Forward Primer: GGTGGAAGAAGAGACCCAAA-3’, GenBankTM L05419). Amplicons were analyzed by 1.25% agarose gel electrophoresis in 1X Tris borate EDTA.

### Determination of Complexity Formation between S1P1 Receptor/CD44s and S1P3 Receptor/CD44v10—EC monolayers were serum-starved for 1 h, treated with High or Low MW HA (100 nM) (5–30 min) and subsequently solubilized in IP buffer A (see above). The samples were then immunoprecipitated with either rabbit anti-S1P1 receptor or mouse anti-S1P3 receptor antibody followed by SDS-PAGE in 4–15% polyacrylamide gels and transfer onto Immobilon™ membranes (Millipore Corp.). After blocking nonspecific sites with 5% bovine serum albumin, the blots were incubated with either rabbit anti-S1P1 antibody, or mouse anti-S1P3 antibody followed by incubation with horseradish peroxidase-labeled goat anti-rabbit, goat anti-mouse, or goat anti-rat IgG. Visualization of immunoreactive bands was achieved using enhanced chemiluminescence (Amersham Biosciences).

### Determination of Tyrosine/Serine/Threonine Phosphorylation of the S1P1 and S1P3 Receptor—Solubilized proteins in IP buffer B (see above) were immunoprecipitated with either rabbit anti-S1P1 receptor or mouse anti-S1P3 receptor antibody followed by SDS-PAGE in 4–15% polyacrylamide gels and transfer onto Immobilon™ membranes (Millipore Corp.). After blocking nonspecific sites with 5% bovine serum albumin, the blots were incubated with either rabbit anti-S1P1 antibody, mouse anti-S1P3 antibody, mouse anti-phospho-tyrosine, rabbit anti-phosphoserine antibody, or rabbit anti-phosphothreonine antibody followed by incubation with horseradish peroxidase-labeled goat anti-rabbit, goat anti-mouse, or goat anti-rat IgG. Visualization of immunoreactive bands was achieved using enhanced chemiluminescence (Amersham Biosciences).

### Construction and Transfection of siRNA against S1P1, S1P3, CD44, AKT1, Src, ROCK1, ROCK2, Rac1, and RhoA—The siRNA sequence(s) targeting human against S1P1, S1P3, CD44, Rac1, and RhoA were generated using mRNA sequences from GenBank™ (gi:13027635, gi:38788192, gi:30353932, gi:62241010, gi:77415509, gi:4885582, gi:41872582, gi:29792301, gi:33876092, respectively). For each mRNA (or transcript), two targets were identified. Specifically, S1P1 target sequence 1 (5’-AAAGCTACA-CAAAAGCCTGGA-3’), S1P3 target sequence 2 (5’-AAAAAGGCTGATCCTACATC-3’), S1P1 target sequence 1 (5’-AAC-AGGGACTCGGAGACAGCA-3’), S1P3 target sequence 2 (5’-ATAATGATTGTTCCTTGGGCGC-3’), CD44 target sequence 1 (5’-AAATATACTGCCGTTTGC-3’), CD44 target sequence 2 (5’-AAAAATGTGCCTAGCATC-3’), AKT1 target sequence 1 (5’-AACTTGCCTACTGATCAGTTA-3’), AKT1 target sequence 2 (5’-AAAAACCTGACACCTGGC-3’), Src target sequence 1 (5’-AAAATGGAACCTCTAGCTGCG-3’), Src target sequence 2 (5’-AAAATCGGAACTTGGC-3’), ROCK1 target sequence 1 (5’-AAAAATGGGACCAACCTGG-3’), ROCK1 target sequence 2 (5’-AAAATGGGATGGCAGAGT-3’), ROCK2 target sequence 1 (5’-AACTGTAGCGAGAGGCGG-3’), ROCK2 target sequence 2 (5’-AACTGCTAGAGGACAGCG-3’), Rac1 target sequence 1 (5’-AAAAATCTGCCATCCTAGCTG-3’), Rac1 target sequence 2 (5’-AAAAATCTGCCATCCTAGCTG-3’), scrambled sequence 1 (5’-AAAGAAATCGGAGAATTCT-3’), and scrambled sequence 2 (5’-AAAGAAATCGGAGAATTCT-3’). For construction of the siRNA, a transcription-based kit from Ambion was used (SilencerTM siRNA construction kit). Human lung EC were then transfected with siRNA using siPORTamine™ as the transfection reagent (Ambion, TX) according to the protocol provided by Ambion. Cells (~40% confluent) were serum-starved for 1 h followed by incubated with 3 μM (1.5 μM of each siRNA) of target siRNA (or scramble siRNA or no siRNA) for 6 h in serum-free media. The serum–containing media was then added (1% serum final concentration) for 48 h before biochemical experiments and/or functional assays were conducted.

### Rho Family Activation Assay—RhoA and Rac activities in human lung EC were performed as described previously (38).

### TABLE 1

| Cortical actin quantitation | 5 Min treatment | 30 Min treatment | 5 Min treatment | 30 Min treatment |
|-----------------------------|----------------|----------------|----------------|----------------|
| 1. Control (Scramble siRNA)  | 8 ± 0.4        | 8 ± 0.4        | 8 ± 0.4        | 8 ± 0.4        |
| 2. High MW HA + Scramble siRNA | 73 ± 3.5      | 85 ± 4.2      | 73 ± 3.5      | 85 ± 4.2      |
| 3. High MW HA + S1P1 Receptor siRNA | 15 ± 0.8    | 18 ± 1.2      | 15 ± 0.8    | 18 ± 1.2      |
| 4. High MW HA + S1P3 Receptor siRNA | 75 ± 3.8      | 88 ± 4.5      | 75 ± 3.8      | 88 ± 4.5      |
| 5. High MW HA + Src siRNA | 74 ± 3.5      | 84 ± 4.3      | 74 ± 3.5      | 84 ± 4.3      |
| 6. High MW HA + AKT1 siRNA | 22 ± 1.2      | 25 ± 1.0      | 22 ± 1.2      | 25 ± 1.0      |
| 7. High MW HA + ROCK 1/2 siRNA | 71 ± 3.2      | 83 ± 3.9      | 71 ± 3.2      | 83 ± 3.9      |
| 8. High MW HA + RhoA siRNA | 78 ± 3.7      | 84 ± 3.8      | 78 ± 3.7      | 84 ± 3.8      |
| 9. High MW HA + Rac1 siRNA | 22 ± 0.9      | 25 ± 1.3      | 22 ± 0.9      | 25 ± 1.3      |
| 10. Low MW HA + Scramble siRNA | 64 ± 2.8      | 5 ± 0.2       | 64 ± 2.8      | 5 ± 0.2       |
| 11. Low MW HA + S1P1 Receptor siRNA | 14 ± 0.7      | 3 ± 0.1       | 14 ± 0.7      | 3 ± 0.1       |
| 12. Low MW HA + S1P3 Receptor siRNA | 68 ± 2.7      | 56 ± 2.5      | 68 ± 2.7      | 56 ± 2.5      |
| 13. Low MW HA + Src siRNA | 21 ± 1.3      | 15 ± 0.6      | 21 ± 1.3      | 15 ± 0.6      |
| 14. Low MW HA + AKT1 siRNA | 65 ± 2.4      | 52 ± 2.1      | 65 ± 2.4      | 52 ± 2.1      |
| 15. Low MW HA + ROCK 1/2 siRNA | 12 ± 0.5      | 10 ± 0.4      | 12 ± 0.5      | 10 ± 0.4      |
| 16. Low MW HA + RhoA siRNA | 59 ± 2.5      | 53 ± 2.3      | 59 ± 2.5      | 53 ± 2.3      |
| 17. Low MW HA + Rac1 siRNA | 18 ± 0.8      | 4 ± 0.1       | 18 ± 0.8      | 4 ± 0.1       |

### FIGURE 4.

**CD44, S1P1, and S1P3 silencing inhibits HA-induced endothelial cell barrier function.** A, immunoblot analysis of siRNA-treated or untreated human EC. Cellular lysates from untransfected (control, no siRNA), scramble siRNA (siRNA that does not target any known human mRNA), S1P1, S1P3, siRNA, or CD44 siRNA transfection were analyzed using immunoblotting with anti-S1P1 antibody (A, panel a), anti-S1P3 antibody (A, panel b), anti-CD44 (IM-7) antibody (A, panel c), anti-Caveolin-1 antibody (A, panel d), or anti-actin antibody (A, panel e) as described under “Experimental Procedures.” Experiments were performed in triplicate each with similar results. Representative data are shown. B, EC were plated on gold microelectrodes and treated with scramble siRNA (Control), S1P1 receptor siRNA, S1P3 receptor siRNA, or CD44 siRNA for 48 h. EC were then serum-starved for 1 h followed by addition of 100 nM Low MW HA. The arrow indicates the time of Low MW HA addition. The TER tracing represents pooled data ± S.E. from three independent experiments as described under “Experimental Procedures.” C, EC were plated on gold microelectrodes and treated with scramble siRNA (Control), S1P1 receptor siRNA, S1P3 receptor siRNA, or CD44 siRNA for 48 h. EC were then serum-starved for 1 h followed by addition of 100 nM Low MW HA. The arrow indicates the time of Low MW HA addition. The TER tracing represents pooled data ± S.E. from three independent experiments as described under “Experimental Procedures.” D, bar graph demonstrates the inhibitory effects of S1P1 receptor siRNA transfection of EC on S1P (the natural ligand for S1P1 receptor), HGF, PDGF, VEGF, ATP, and thrombin-induced maximal change in TER (at least n = 3 for each condition).
**In Vitro S1P Receptor Phosphorylation**—The S1P receptor phosphorylation reaction was carried out in 50 μl of the reaction mixture containing 40 mM Tris-HCl (pH 7.5), 2 mM EDTA, 1 mM dithiothreitol, 7 mM MgCl₂, 0.1% CHAPS, 0.1 μM calyculin A, 100 μM ATP, purified enzymes (i.e. 100 ng of recombinant active Src, ROCK1 or ROCK2) with or without immunoprecipitated S1P₁ or S1P₃ receptor obtained from human pulmonary EC that were serum-starved for 1 h. After incubation for 30 min at 30 °C, the reaction mixtures were boiled in SDS sample buffer and subjected to SDS-PAGE. Immunoblots were performed using mouse anti-phosphotyrosine, rabbit anti-phosphothreonine, rabbit anti-S1P₁, or mouse anti-S1P₃ antibody followed by incubation with horseradish peroxidase-labeled goat anti-rabbit or goat anti-mouse IgG. Visualization of immunoreactive bands was achieved using enhanced chemiluminescence.

**Immunofluorescence Microscopy and Cortical Actin Quantitation**—Polymerized actin rearrangement was assessed with Texas Red-conjugated phalloidin and analyzed using a Nikon Eclipse TE 300 microscope as we have described (4). Computer-recorded tiff images were analyzed with ImageQuant™ software from Amersham Biosciences. A standardized average gray value (SAGV) was generated for total phalloidin staining versus cortical phalloidin staining for each cell (29). To calculate percent cortical actin staining, the following equation was used: ((cortical actin SAGV × area) divided by (total actin SAGV × area)) × 100. At least ten cells per sample were analyzed. Experiments were performed in triplicate.

**Statistical Analysis**—Student’s t test was used to compare the means of data from two or more different experimental groups. Results are expressed as means ± S.E.

**Measurement of TransEC Electrical Resistance (TER)**—EC were grown to confluence in polycarbonate wells containing evaporated gold microelectrodes, and TER measurements performed using an electrical cell substrate impedance sensing system (Applied Biophysics) as previously described (4). TER values from each microelectrode were pooled at discrete time points and plotted versus time as the mean ± S.E.
HA/CD44/S1P Receptor Signaling in Human EC

**RESULTS**

Divergent Effects of Low and High Molecular Weight Hyaluronan on Human Lung Endothelial Cell Barrier Function: Role of Caveolin-enriched Microdomains (Lipid Rafts)—Initial experiments examined the effects of low and high MW-HA on human lung EC barrier function and the role of CD44 and lipid rafts in this process. Lipid rafts isolated from human lung EC contain specific markers (caveolin-1 and flotillin-1), are enriched in cholesterol and exclude other subcellular organelle markers (Fig. 1, A and B). These results demonstrate the purity and specificity of our lipid raft isolation procedure. Next, RT-PCR and isomorph-specific immunoblot analysis were performed to explore whether CD44 isoforms, a major cell surface HA receptor family, were present in human lung EC. Fig. 1, C and D demonstrate that human pulmonary EC express at least two major CD44 isoforms, CD44s (standard form, ~85 kDa) and CD44v10 (~116 kDa).

HMW-HA (~1 million Da) consistently produced a gradual and sustained rise in transmonolayer electrical resistance (TER) in dose-dependent fashion whereas LMW-HA (~2,500 Da) induced biphasic changes in TER with an initial rapid increase in barrier enhancement followed by significant and prolonged barrier disruption (Fig. 2, A and B). The dose response was significant when comparing equal nanomolar concentrations (but not equal concentrations in the range of 1.0–100 μg/ml (Fig. 2C)) of Low and High MW HA. Depleting cholesterol with methyl-β-cyclodextrin (MβCD) treatment (Fig. 2D) or using a pan-CD44 blocking antibody, which blocks HA binding to all CD44 isoforms, abolished both HMW- and LMW-HA-induced changes in TER (Fig. 2F). These results demonstrate that cholesterol-enriched microdomains regulate HA-mediated EC barrier function. Further, CD44 is the major HA receptor responsible for HA-mediated EC barrier alterations.

We previously demonstrated that CD44 localizes in activated EC to specialized cholesterol- and caveolin-enriched lipid rafts, plasma membrane microdomains implicated in a variety of cellular functions including potocytosis, cholesterol, and calcium regulation as well as signal transduction (21, 22, 39). Lipid rafts are biochemically defined by insolubility in 4 °C Triton X-100 and light buoyant density after discontinuous gradient centrifugation (40). Both HMW-HA and LMW-HA rapidly (5 min) recruit CD44s to the lipid raft fraction whereas LMW-HA promotes robust but delayed recruitment of CD44v10 (after 15 min) (Fig. 3A).
HA/CD44/S1P Receptor Signaling in Human EC

A

Immunoblot:

a. Anti-Tiam1

b. Anti-p115 RhoGEF

c. Anti-Caveolin-1

20% Optiprep™ Lipid Raft Fractions

B

Activated Rac1

|           | Scramble siRNA | S1P₁ siRNA | S1P₃ siRNA |
|-----------|---------------|------------|------------|
| 5”        | 15”          | 30”        |            |
| C         | 5”           | 15”        | 30”        |

Immunoblot: Anti-Rac1

C

Activated RhoA

|           | Scramble siRNA | S1P₁ siRNA | S1P₃ siRNA |
|-----------|---------------|------------|------------|
| 5”        | 15”          | 30”        |            |
| C         | 5”           | 15”        | 30”        |

Immunoblot: Anti-RhoA

D

Immunoblot:

a. Anti-RhoA

b. Anti-Rac1

c. Anti-Caveolin-1

d. Anti-Actin

E

High MW HA (1 hour)

Low MW HA (1 hour)

Normalized Resistance

| Control (No HA) | Scramble siRNA | RhoA siRNA | Rac1 siRNA | Control (No HA) | Scramble siRNA | RhoA siRNA | Rac1 siRNA |
|----------------|---------------|------------|------------|----------------|---------------|------------|------------|
|                |               |            |            |                |               |            |            |

Graph showing normalized resistance for high and low MW HA with different treatments.
Transactivation of S1P Receptors Are Involved in HA-mediated Lung Vascular Barrier Regulation in a CD44 Isoform-specific Manner—We next explored whether HA induces physical and/or functional associations between CD44 and S1P receptors, which may be involved in HA-mediated vascular barrier responses. HMW-HA (100 nM) induced CD44s association in lipid rafts with S1P1, the known barrier-promoting S1P receptor (Fig. 3B). In contrast, LMW-HA initially recruited the S1P1 receptor followed by recruitment of S1P3 receptors. Immunoprecipitation followed by immunoblotting from lipid raft fractions revealed that HMW-HA promotes S1P1 receptor association with CD44s. In contrast, LMW-HA (100 nM) induced an initial CD44s association with S1P1 followed by CD44v10 association with S1P3 receptor in lipid raft fractions. Both the spatially specific actin cytoskeletal reorganization and TER alterations evoked by HMW-HA and LMW-HA were abolished by either MβCD (to inhibit lipid raft formation), by anti-CD44 blocking antibody or by siRNAs specific for CD44 (Figs. 2 and 4, Table 1). Silencing S1P1 receptor blocked the EC barrier enhancing effects of High MW HA while silencing S1P3 receptor blocked the EC barrier disruptive effects of Low MW HA (Fig. 4). Consistent with HA-mediated S1P transactivation, HMW-HA promoted AKT1-mediated threonine phosphorylation of S1P1 receptor whereas LMW-HA induced sequential AKT1-mediated S1P1, and Src/ROCK1/2-mediated S1P3 receptor phosphorylation/activation (Figs. 5 and 6). These results were confirmed by using in vitro phosphorylation of S1P receptors with recombinant AKT1, Src, ROCK1 and ROCK2 (Fig. 5C). Further, silencing AKT1 expression blocks HWM-HA-mediated EC barrier enhancement while silencing Src or both ROCK 1 and 2 expression blocks LMW-HA-mediated EC barrier disruption (Fig. 6C). Thus, low and high MW HA promote differential CD44 isoform-specific association with and activation of S1P receptors in lipid rafts. Activation of S1P1 receptor is required for HA-induced EC barrier enhancement while S1P3 receptor activation promotes barrier disruption.

Role of RhoA and Rac1 Signaling on HA-induced EC Barrier Function—We have previously demonstrated that the Rho family GTPase, Rac1, regulates S1P-mediated EC barrier enhancement (4). We examined whether Rho family GTPases could play a role in the HA-specific regulatory responses and identified that either LMW-HA (5 min.) or HMW-HA (5, 15, 30 min) induced Rac1 activation in concert with recruitment of the Rac1-specific exchange factor, Tiam1, to EC lipid rafts (Fig. 7). Rac1 activation was inhibited by siRNA for S1P1 (but not S1P3) to reduce receptor expression. The HA-induced EC barrier enhancement was inhibited by silencing Rac1 (but not RhoA) expression. In contrast, LMW-HA (but not HMW-HA) recruited the RhoA exchange factor, p115 RhoGEP, to EC lipid rafts at 15–30 min. and promoted RhoA activation. LMW-HA-induced RhoA activation was inhibited by siRNA for S1P3 (but not S1P1) and LMW-HA-induced EC barrier disruption was inhibited by silencing RhoA (but not Rac1) expression.

Finally, silencing either S1P1 or Rac1 expression attenuated EC barrier-enhancing effects of HMW-HA and LMW-HA whereas silencing S1P3 or RhoA expression diminished the EC barrier-disruptive response to LMW-HA (Figs. 4 and 7). These results suggest that HA promotes cytoskeletal reorganization and EC barrier regulation through differential CD44 isoform interaction with S1P receptors via RhoA/Rac1 signaling in lipid rafts. Transactivation of S1P1 receptor may represent a common mechanism for receptor-mediated vascular barrier regulation.

HA-induced, CD44, and S1P Receptor-dependent, Cytoskeletal Reorganization in EC—TER measurements of EC barrier function in vitro revealed that reduction in expression of either S1P1 or Rac1 attenuated the barrier-enhancing effects of low and high MW HA; whereas reduction in S1P3 or RhoA expression attenuated the delayed barrier-disruptive response to low MW HA on EC (Figs. 4 and 7). As cytoskeletal reorganization is a fundamental element of virtually all EC barrier-regulatory responses, we compared phalloidin staining of HA- and S1P-challenged EC to visualize cellular F-actin localization (Fig. 8 and Table 1). At early time points (5 min) both low and high MW HA induced prominent cortical actin ring formation, which was attenuated by reduction of S1P1 (but not S1P3), AKT, or Rac1 (but not RhoA) expression, findings similar to that reported for S1P (4, 29). Low MW HA challenge for 30 min., however, resulted in a loss of cortical actin staining with increased F-actin stress fiber formation, which was significantly attenuated by silencing S1P3 (but not S1P1) or RhoA (but not Rac1) expression.
Role of S1P1 Receptor as a Central Regulator of EC Permeability—We recently demonstrated that the S1P1 receptor regulates activated protein C (APC)/endothelial cell protein C receptor (EPCR)-mediated EC barrier protection against edemagenic agents such as thrombin (41). As silencing the S1P1 receptor reduces the barrier enhancement induced by HA, and both LMW-HA and HMW-HA promote transactivation of S1P1 receptor during the EC barrier-enhancing stages of these agonists, we next explored whether S1P1 receptor serves as a central regulator of EC barrier function (Fig. 4D). Reductions in S1P1 receptor expression significantly modulated the barrier-regulatory effects of human lung EC challenged with HGF, PDGF, VEGF, or ATP (4, 42). In contrast, thrombin, a known EC barrier-disruptive agent, was unaffected by S1P1 receptor silencing, suggesting that the S1P1 receptor serves as a critical and central regulator of EC barrier function.

DISCUSSION

Agents that exhibit the capacity to restore barrier integrity after periods of increased vascular permeability have obvious therapeutic applications in diverse inflammatory syndromes as well as in conditions such as tumor angiogenesis and atherosclerosis (4, 42). We explored the effects of HA, a major glycosaminoglycan, which exists in multiple MW forms (6, 43), on EC permeability. We found that the high MW form (~1,000,000 Da) promotes increased EC barrier integrity in vitro and propose that high MW HA plays an important role in providing a protective barrier between endothelial cells and underlying vasculature in vivo. In contrast, hyaluronan fragments of ~2,500 Da (low MW HA), previously shown to be angiogenic (19), induces a biphasic effect on EC permeability with a brief, barrier-enhancing phase followed by a prolonged barrier-disruptive phase (Fig. 9).

CD44 is highly likely to be important in lung disease as CD44−/− mice develop lung fibrosis, inflammatory cell recruitment, and hyaluronan fragment accumulation at sites of lung injury (44). Both high MW HA and its fragments bind to CD44, however, high and low MW HA evoke highly specific cellular functions. The high MW HA induces CD44s-mediated transactivation (AKT-dependent threonine phosphorylation) of S1P1 receptor and consequent Rac1 signaling leading to cortical actin thickening and barrier enhancement in human pulmonary EC. Silencing S1P1 receptor, AKT1 or Rac1 reverses the barrier-protective effects of high MW HA. In contrast, low MW HA promotes CD44v10-mediated transactivation (Src-dependent tyrosine phosphorylation and ROCK1/2-dependent transactivation (threonine phosphorylation) of the S1P1 receptor in CEM (B-3), RhoGEF recruitment to lipid rafts (B-4), and RhoA activation (B-5) leading to cytoskeletal reorganization/decreased cortical actin (B-6), and decreased EC barrier function (B-7).
migration. In the present study, low MW HA was found to be a potent inducer of CD44v10 signaling, S1P3 receptor activation and RhoA-mediated EC barrier disruption. The effects of Ca2+ signaling on HA-induced EC barrier regulation are currently under investigation.

Caveolin-enriched microdomains or lipid rafts, are important plasma membrane microdomains that regulate numerous EC functions (45, 46). CD44 localization in lipid rafts is important for HA-mediated signaling (21, 30) as cholesterol depletion blocks both low and high MW HA-induced EC barrier responses. Further, CD44 isoform-specific activation of S1P receptors occurs in lipid rafts indicating that these microdomains play an important role in HA-induced EC functions. We have previously demonstrated that PI3 kinase and Rac1 signaling in lipid rafts are important for S1P1 receptor-mediated EC barrier enhancement (29). The ability to potentially target these microdomains as a means of drug delivery for edemagenic states has significant promise.

HA/CD44-mediated stimulation of Src (pp60Src, c-Src tyrosine kinase) activity has been shown to regulate cytoskeletal function (28). In agreement with our results, researchers have reported that activation of Src promotes cytoskeletal-mediated EC barrier disruption (47–49). In particular, Src regulates EC contraction and vascular permeability (48). Inhibition of Src reduces edema and stabilizes a VEGF receptor 2/cadherin complex after myocardial infarction (50). The role of Src activation on receptor tyrosine kinases and adhesion proteins in EC barrier function are currently being investigated in our laboratory.

Actin cytoskeletal reorganization plays a key role in EC barrier regulatory responses to a variety of agents (3, 4). We observed that HA promotes cytoskeletal reorganization and EC barrier regulation via differential CD44 isoform interaction with S1P receptors and RhoA/Rac1 signaling in lipid rafts. In particular, high MW HA induces cortical actin ring formation while low MW HA treatment of EC for 15 or 30 min promotes actin stress fiber formation. These results demonstrate the requirement for S1P1 receptor transactivation in agonist-induced EC barrier enhancement, and the potential for S1P1 activation to represent a common mechanism for receptor-mediated vascular barrier regulation.

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