Acute lung injury is characterized by injury to the lung epithelium that leads to impaired resolution of pulmonary edema and also facilitates accumulation of protein-rich edema fluid and inflammatory cells in the distal airspaces of the lung. Recent in vivo and in vitro studies suggest that mesenchymal stem cells (MSC) may have therapeutic value for the treatment of acute lung injury. Here we tested the ability of human allogeneic mesenchymal stem cells to restore epithelial permeability to protein across primary cultures of polarized human alveolar epithelial type II cells after an inflammatory insult. Alveolar epithelial type II cells were grown on a Transwell plate with an air-liquid interface and injured by cytomix, a combination of IL-1 and TNFα, and IFNγ. Protein permeability measured by 131I-labeled albumin flux was increased by 5-fold over 24 h after cytokine-induced injury. Co-culture of human MSC restored type II cell epithelial permeability to protein to control levels. Using siRNA knockdown of potential paracrine soluble factors, we found that angiopoietin-1 secretion was responsible for this beneficial effect in part by preventing actin stress fiber formation and claudin 18 disorganization through suppression of NFkB activity. This study provides novel evidence for a beneficial effect of MSC on alveolar epithelial permeability to protein.

Bone marrow-derived mesenchymal stem cells (MSC) are adult stem cells that are capable of differentiating into chondroblasts, osteoblasts, adipocytes, fibroblasts, and myofibroblasts. They have been translated to effective clinical treatment options, and innovative therapies are needed.

Alveolar injury during ALI also impairs the capacity of ATII cells to actively remove pulmonary edema fluid (alveolar fluid clearance), which results in further extravascular lung water accumulation and is associated with higher mortality (5).

We discovered that intrapulmonary treatment with MSC improved survival and reduced pulmonary edema formation in Escherichia coli endotoxin-induced lung injury in mice (26). However, the mechanisms underlying the beneficial effect of MSC were not well understood. In an ex vivo perfused human lung preparation, we also found that the intrabronchial instillation of MSC after E. coli endotoxin-induced lung injury restored alveolar fluid clearance in part through secretion of keratinocyte growth factor, which increased sodium and vectorial fluid transport across the alveolar epithelium (27). In addition to growth factors, several paracrine factors are secreted by MSC that can directly or indirectly reduce barrier injury as well. More recently, we found that allogeneic human MSC secrete a significant quantity of angiopoietin-1 (Ang1). Ang1 plays an essential role in embryonic vascular development as a ligand for the receptor-tyrosine kinase Tie2. In the postnatal state, Ang1 is responsible for a quiescent vascular...
phenotype and is known as an endothelial survival (28) and vascular stabilization factor that reduces endothelial permeability and inhibits leukocyte-endothelium interactions by modifying endothelial cell adhesion molecules and cell junctions (29–32). Several studies have investigated its anti-inflammatory, anti-permeability, and endothelial protective characteristics. In mice that were injured by LPS, MSC or MSC (used as a vehicle for gene delivery) transplanted with the human Ang1 gene reduced pulmonary injury and the recruitment of inflammatory cells into the lung (19, 25, 33). To our knowledge no data are available on the effect of Ang1 on lung epithelial permeability to protein.

We hypothesized that MSC may exert beneficial effects on injured alveolar epithelial cells. Therefore, we exposed primary cultures of human alveolar type II (ATII) cells, grown on a semi-permeable membrane with an air-liquid interface, to cytomix (IL-1β, TNF-α, and IFNγ, a mixture of the most biologically active cytokines found in ALI pulmonary edema fluid (34)) to induce an increase in alveolar epithelial permeability. We investigated the mechanism of increased permeability at the level of the tight junction proteins. We then tested the potential therapeutic role of MSC by adding the cells simultaneously into the bottom chamber of the Transwell plate. Finally, to identify possible mechanisms, we tested the role of Ang1 as a secreted paracrine soluble factor by MSC using small interfering RNA (siRNA) knockdown methodology.

MATERIALS AND METHODS

Primary Cultures of Human Alveolar Epithelial Type II Cells—Human epithelial ATII cells were isolated from human lungs declined for transplantation by the Northern California Transplant Donor Network as previously described (34). After cold preservation at 4 °C, the right middle lobe was selected for cell isolation if no obvious signs of consolidation or hemorrhage by gross inspection were seen. The pulmonary vasculature was first flushed antegrade and retrograde with phosphate-buffered saline (PBS) to remove remaining blood from the microcirculation. The distal airspaces were then lavaged 10 times with Ca2+,-Mg2+–free PBS solution (37 °C) containing 0.5 mM EGTA and 0.5 mM EDTA. Elastase, 13 units/ml in Ca2+-,Mg2+-free Hanks’ balanced salt solution, was instilled into the distal airspaces by segmental bronchial intubation. The lobe was then digested at 37 °C for 45–60 min. After digestion, the lobe was further minced in the presence of fetal bovine serum (FBS) and DNase (500 μg/ml). The cell-rich fraction was filtered sequentially through multiple layers of sterile gauze and 100- and 20-μm nylon meshes (Spectra/Mesh®). The filtrate was further layered onto a discontinuous Percoll (Sigma) density gradient of 1.04 and 1.09 g/ml solution and centrifuged at 400 × g for 20 min. The interface containing both ATII cells and alveolar macrophages was collected and further centrifuged at 800 rpm for 10 min, and the cell pellet was washed and resuspended in Ca2+,-Mg2+-free PBS containing 5% FBS. The cells were incubated with magnetic beads coated with anti-CD 14 antibodies (Dynabeads® CD14, Invitrogen) at 4 °C for 40 min, and the majority of the macrophages were then selectively depleted with a Dynal magnet (Dynal Biotech, Oslo, Norway). The cell suspension was further incubated on Petri dishes coated with human IgG antibodies (Sigma) for 4 h at 37 °C to remove the remaining macrophages. The unattached remaining cells consisted of the ATII cells and were seeded at a density of 1 × 106 cells/well on collagen I-coated Transwell plates with a pore size of 0.4 μm and a surface area of 0.33 cm2 (Transwell®, Corning, NY). The cells were cultured in a 37 °C and 5% CO2 incubator in DMEM high glucose 50%, F-12 50% mix medium containing 10% FBS and antibiotics (penicillin, streptomycin, gentamicin, and amphotericin). Cells reached confluence after 48 h and then were checked by measuring transmembrane electrical resistance with an Ag-AgCl electrode. Cell preparations with resistance of >1000 ohms were used for the experiments. At 72 h, the fluid in the upper compartment was removed, and the cells were then grown in an air-liquid interface.

Human Mesenchymal Stem Cells—Allogeneic human MSC were obtained from the Tulane Center for Gene Therapy (New Orleans, LA). These MSC were recovered from bone marrow aspirates from healthy donors. Upon arrival, cells were thawed and expanded in tissue-cultured treated flasks (BD Falcon™) at a density of 500,000 cells/150 cm2. Cells were passaged every 3–4 days by trypsinization when they reached 70–80% confluence and used for the experimental protocols between passages 5 and 10. Between each passage, viability was measured with trypan blue exclusion. MSC were cultured in α-minimum essential medium (MEM) without ribonucleosides or deoxyribonucleosides containing 2 mM l-glutamine, 16.5% FBS, penicillin, and streptomycin. Cells were cultured in a humidified incubator at 5% CO2 and 37 °C under sterile conditions. Normal adult human lung fibroblasts (Lonza Inc.) were used as controls.

Human Alveolar Epithelial Type II Cells Exposed to Cytomix with and without Human Allogeneic Mesenchymal Stem Cells—To study the effects of MSC and its secretion of Ang1 on the ATII monolayers injured by an inflammatory insult, we developed a co-culture system (Fig. 1) with the Transwell inserts (0.4-μm pore size and collagen I-coated, Costar, Corning) in 24-well plates. In this system ATII cells were grown on the Transwell microporous membranes with their apical side directed to the upper compartment. For experiments using MSC, the cells were plated in the bottom compartment of the 24-well plates at a density of 250,000 cells/well with no direct cell contact between MSC and ATII. To injure the cells, we exposed the ATII cells to a mixture of three different cytokines referred to as cytomix (IL-1β, TNFα, and IFNγ (all from R&D Systems)) at a final concentration of 50 ng/ml each. We previously reported that these were the major proinflammatory cytokines in pulmonary edema fluid from patients with ARDS (34). After 96 h, when the ATII cells grew into a tight polarized monolayer, the cells were washed with PBS and serum-starved for 24 h before use in the following different conditions: 1) ATII cells alone, 2) ATII cells with cytomix, 3) ATII cells with cytomix and MSC (with or without siRNA to Ang1 or negative siRNA control), 4) ATII cells with cytomix and normal human lung fibroblast as a control group (Lonza), 5) ATII cells with cytomix and recombinant human angiopoietin-1 (rhAng1; 100 ng/ml, R&D Systems), 6) ATII cells with cytomix and a selective inhibitor of the Rho-associated protein kinase, Y-27632 (10 μM, Calbiochem), or a selective inhibitor of myosin light chain.
Mesenchymal Stem Cells and Alveolar Epithelial Permeability

FIGURE 1. Transwell co-culture system. A lateral view of the Transwell system is shown. Primary cultures of human alveolar epithelial type II cells were seeded at a density of $1 \times 10^5$ cells/well in collagen I-coated Transwell plates (0.4-micron pore size and a surface area of 0.33 cm$^2$). Human allogeneic mesenchymal stem cells or normal human lung fibroblasts were added simultaneously to the lower compartment. There was no direct cell-contact between cell types. Cytomix was added to both compartments according to the experimental protocol. Alveolar epithelial permeability was assessed by adding labeled $^{131}$I-albumin only to the upper compartment and measuring its unidirectional flux to the lower compartment. There was no hydrostatic pressure gradient between compartments.

Transfection of MSC with Ang1 siRNA—MSC were transfected with siRNAs using a lipid-based nucleofection for reverse transfection (siPORT™ NeoFX™, Ambion®) to knock down Ang1 secretion according to the manufacturer’s instructions. Cells were then plated in 24-well plates with the transfection medium for 24 h. In a different set of experiments, MSC were cultured alone with or without cytomix to measure the secretion of Ang1.

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Assessment of RhoA and Rac1/2/3 Activation—GTP-bound RhoA and Rac1/2/3 were measured in protein lysates of ATII cells with a commercially available activation kit (G-LISA™, kit #BK124 and #BK125, Cytoskeleton, Inc., Denver, CO) according to the manufacturer’s instructions. These assays use a Rho-GTP-binding protein or Rac-GTP-binding protein linked to a 96-well plate. Active, GTP-bound protein is bound to the wells whereas inactive, GDP-bound protein is removed during washing steps. Each well was loaded with 15 μg of protein, and activation signals are expressed as % of control values.

Measurement of NFκB Pathway Activation—For each experimental condition, nuclear proteins of cultured AT II cells were extracted with a Nuclear Extraction kit (catalog #40010, Active Motif). Activated transcription factor p65 in the nuclear extract was measured with an ELISA kit (TransAM™ NFκB p65, #40096, Active Motif) according to the manufacturer’s instructions. Experiments were done at 30 min and 1, 2, 3, and 24 h.

Total Cellular Protein Isolation and Western Blotting of Junctional Proteins—Total cellular protein was extracted from the ATII cells exposed to different experimental conditions using 0.1 ml of lysis buffer per well containing 1% Triton X-100, 20 mM Tris-Base (pH 8.0), 137 mM NaCl, 10% glycerol, 2 mM vanadate, 2 μg/ml aprotinin, 5 μg/ml leupeptin, and 1 mM Pefabloc, and homogenized. Protein levels were measured by the bicinchoninic acid method (Pierce). Each sample was first reduced and denatured with sample buffer and run on a 4–12% gradient Bis-Tris gel (Invitrogen), 10–20 μg of proteins per lane, using a MOPS-SDS buffer (Invitrogen) at 100 V for roughly 2 h. The proteins were then transferred onto a nitrocellulose membrane and blocked with 5% milk in Tris-buffered medium was aspirated as the initial sample. After 24 h, another 20 μl was aspirated from the upper compartment as the final sample, and all the fluid in the lower compartment was collected. Each sample was weighed, and radioactivity was counted in a gamma counter (Packard MINAXI 5000 series). The unidirectional flux of labeled $^{131}$I-albumin from the apical (upper compartment) to the basolateral side (lower compartment) was then calculated and expressed as % per 24 h as protein permeability.

Angiopoietin-1, Angiopoietin-2, and Tie2 Phosphorylation—Concentrations of Ang1, Ang2, and phospho-Tie2 were measured using commercially available ELISA kits (R&D Systems). Ang1 and Ang2 were measured in the medium of the cell culture system and expressed as pg/ml. Phospho-Tie2 was measured in the protein lysates of ATII cells from different groups using an ELISA and expressed as a percentage of control.
saline with Tween 20 for 1 h. The nitrocellulose membrane was then exposed to the primary antibody overnight at 4 °C. Primary antibodies used were: rabbit anti-ZO-1 (Zymed Laboratories Inc.), rabbit anti-occludin (Zymed Laboratories Inc.), mouse anti-claudin 3 (Zymed Laboratories Inc.), mouse anti-claudin 4 (Zymed Laboratories Inc.), mouse anti-claudin 5 (Zymed Laboratories Inc.), rabbit anti-claudin 18 (Zymed Laboratories Inc.), and rabbit anti-JAM-A (Zymed Laboratories Inc.). The protein bands were then visualized with a chemiluminescence agent, ECL + (Amersham Biosciences) and quantitated with NIH Image software.

Confocal Microscopy with Immunofluorescence Labeling of Cytoskeletal, Tight Junctional, and Adherens Junction Proteins—For detection of the cytoskeletal proteins actin and phosphorylated myosin light chain-2 (MLC-2), ATII cells were seeded on Lab-Tek II chamber slides (Nalge Nunc International) at a density of 1.6 × 10⁶ cells/cm². Cells were grown to confluence and serum-starved before the different experimental conditions were applied. As a surrogate of the co-culture system of ATII with MSC, the conditioned medium of MSC (250,000 cells for 24 h in a 1-ml volume) was added to the slides in place of the cells. After completion of the experimental conditions, the cell monolayer was washed twice with cold PBS and fixed in 4% paraformaldehyde for 30 min. The cells were then washed three times with PBS for 10 min in a gently shaking chamber at room temperature. The slides were then permeabilized by 0.2% Triton X-100 for 2 min. The slides were incubated with a primary antibody for the phosphorylated form of myosin light chain-2 (Cell Signaling) for 2 h at 37 °C. The slides were again washed three times with PBS for 10 min and then exposed to a mixture of the secondary antibody, rhodamine-labeled anti-rabbit-IgG (red) for phosphorylated MLC-2. Then the cells were exposed to FITC-phalloidin (green, Molecular Probes) for F-actin staining. The slides were then washed with PBS and mounted with Vectashield mounting medium. Images were obtained by Zeiss LSM510 laser scanning confocal microscopy.

For detection of immunofluorescent staining of tight and adherens junction proteins, antibodies to zonula occludens-1 (ZO-1) (Zymed Laboratories Inc.), claudins (Zymed Laboratories Inc.), occludin (Zymed Laboratories Inc.), FITC anti-β-catenin (BD Bioscience), and anti-E-cadherin (BD Bioscience) were used. For claudin staining, cultured ATII cells on collagen I-coated polyester membrane (#3801, Costar, Corning, Inc.) were washed with PBS, fixed with methanol at −20 °C for 10 min, and then rehydrated with PBS. The cells were blocked with 5% BSA plus 0.2% Triton X-100 for 30 min at room temperature. The cells were then incubated with primary anti-claudin 4 (1:100 dilution) and anti-claudin 18 (1:50 dilution) antibodies overnight. After washing with PBS 3 times, the cells were incubated with secondary FITC conjugated goat anti-rabbit and goat anti-mouse antibody (Invitrogen) for 1 h at room temperature. Note that these secondary antibodies are designed for multiple labeling experiments and are specially purified to avoid cross species reactivity with primary IgG. For ZO-1 and occludin staining, cultured ATII cells on collagen I-coated polyester membrane were washed with PBS and fixed with 4% paraformaldehyde for 10 min. Then the cells were permeabilized with 0.2% Triton X-100 for 5 min. The cells were then incubated with primary anti-ZO-1 (1:200 dilution) and anti-occludin (1:50 dilution) antibodies overnight. After washing with PBS 3 times, the cells were incubated with secondary Cy3-conjugated goat anti-rabbit antibody (Invitrogen) for 1 h at room temperature. For immunostaining of β-catenin, the cells were incubated directly with FITC anti-β-catenin (1:200 dilution). For immunostaining of E-cadherin, the cells were first incubated with anti-E-cadherin (1:50 dilution) for 4 h and then followed by incubation with secondary FITC goat anti-mouse antibody (BD Bioscience). All the membranes with cells were cut out and mounted with Vectashield mounting medium. Images were obtained by Zeiss LSM510 laser scanning confocal microscopy.
alone and in the presence of cytomix was almost undetectable (0 ± 0 and 1.8 ± 3.2 pg/ml, respectively). However, co-culture of ATII cells on the transwell membrane together with cytomix and MSC in the lower compartment resulted in a significant increase in Ang1 secretion at 24 h (Fig. 3A). MSC alone or after stimulation with cytomix also secreted Ang1 (Fig. 3A). These results indicate that MSC were the primary source of Ang1 secretion. In addition, levels of Ang2 in the same conditioned medium were not detectable by ELISA (R&D Systems).

Role of Ang1 Secretion by Human Mesenchymal Stem Cells on Epithelial Protein Permeability across Human ATII Cells Injured by Cytomix—To test the potential role of Ang1 secretion by MSC on the restoration of protein permeability, we exposed MSC to Ang1 siRNA to selectively knockdown the secretion of the protein. Two different siRNAs (#121280 and #121281, Ambion) significantly reduced Ang1 secretion at 24 and 48 h (Fig. 3B). Transfection of MSC with a negative control siRNA (Ambion) did not affect Ang1 production. All further experiments were performed using siRNA #121280 due to its significant effect on Ang1 secretion at 48 h.

To investigate the role of Ang1 secretion by MSC on protein permeability, we exposed primary cultures of human ATII cells to cytomix and added MSC in the lower compartment of the Transwell system pretreated with or without Ang1 siRNA. Epithelial protein permeability was restored when ATII cells were exposed to cytomix with MSC or MSC transfected with a negative control siRNA on the bottom chamber. However, when MSC were transfected with Ang1 siRNA, the therapeutic effect of MSC on protein permeability was lost (Fig. 4). The addition of rhAng1 (100 ng/ml) to ATII cells exposed to cytomix had a similar effect as MSC in restoring epithelial protein permeability (Fig. 4). These results indicate that MSC mediate a protec-
active effect on alveolar epithelial permeability at least in part through the secretion of Ang1.

Effect of Human Mesenchymal Stem Cells and rhAng1 on the Tie2 Receptor on ATII Cells—In endothelial cells, Ang1 mediates its effect through the phosphorylation of the Tie2 receptor at the cell membrane (35). To understand the therapeutic effect of Ang1 secretion by MSC on alveolar epithelial protein permeability, we tested the signaling pathway of Ang1 on ATII cells. The level of phosphorylation of Tie2, the activated form of the receptor, in the protein lysates of ATII cells under the different experimental conditions was measured. Human ATII cells expressed a significant level of phosphorylated Tie2 at base line, which decreased with exposure to cytomix (Fig. 5). Exposure of ATII cells to recombinant human Ang1 or MSC in the presence of cytomix restored the level of phosphorylated Tie2 receptor. However, the effect of MSC was completely abolished when it was transfected with Ang1 siRNA (Fig. 5).

Effect of rhAng1 on RhoA Activation in ATII Cells after Cytomix Exposure—Based on prior studies, activated RhoA regulates MLC phosphorylation through Rho kinase, inducing actin stress fiber formation and leading to increased endothelial permeability. Therefore, we measured the activation of RhoA after cytomix stimulation in the protein lysates of ATII cells (Fig. 6). Activation of RhoA peaked after 10 min of stimulation with cytomix. When recombinant Ang1 was added to the cell culture system simultaneously, activation at 10 min was significantly reduced, comparable with control levels.

Effect of Human Mesenchymal Stem Cells on Rac1/2/3 Activation in ATII Cells Exposed to Cytomix—Because the activation of RhoA and Rac1/2/3 have opposing effects (RhoA aggravates and Rac1/2/3 enhances barrier integrity (36)), we investigated whether or not MSC were able to increase levels of Rac1/2/3 activation. Analysis of Rac1/2/3 activation in protein lysates of ATII cells indicated that co-culture of ATII with MSC in the presence of cytomix increased the levels of Rac1/2/3 activation significantly at 24 h compared with control values (Fig. 7). No changes compared with control were seen when ATII cells were exposed to cytomix only.

Effect of Human Mesenchymal Stem Cells on Actin Cytoskeleton Organization in Primary Cultures of Human AT II Cells Exposed to Cytomix—Increased protein permeability is the result of changes at the level of intercellular junction proteins connected to the cytoskeleton, primarily actin and myosin fibers. Centripetal force exerted on the actin skeleton can increase paracellular permeability by contraction of the cells. The intracellular cytoskeletal distribution of both actin and myosin can distinguish different states of protein permeability. A central distribution of actin indicates the formation of “stress fibers” with increased leakage at the sites of tight junction proteins, whereas a peripheral distribution of actin fibers indicates junction preservation and preserved barrier integrity. Phosphorylation of myosin light chain cross-bridges the actin-myosin fibers, leading to an increase in the centripetal forces or stress fiber formation and protein permeability.
We investigated the distribution of actin (green) and phosphorylated MLC-2 (red) proteins by immunofluorescence and confocal microscopy among primary cultures of ATII cells exposed to cytomix with and without MSC conditioned medium (as a surrogate of MSC) or rhAng1. In control conditions (Fig. 8), staining (green) was mainly distributed at the peripheral rim within the cells. Stimulation with cytomix resulted in a central distribution of actin fibers (Fig. 8). The overlap of actin and phosphorylated MLC-2 indicated an interaction between both proteins potentially leading to an increase in centripetal forces on intercellular junction proteins that in turn resulted in loss of barrier integrity and an increase in protein permeability. When cytomix-stimulated ATII cells were co-incubated with conditioned medium of MSC or recombinant human Ang1 to human ATII cells exposed to cytomix partially restored the peripheral distribution of actin and reduced the activated form of phosphorylated MLC-2. Images are representative for each condition run in triplicates.

FIGURE 8. Immunofluorescence staining of human ATII cells exposed to cytomix treated with and without MSC or rhAng1. Alveolar type II cells grown on glass-slides were stained with phalloidin (green) for actin and rhodamine (red) for phosphorylated myosin light chain-2. Base-line staining for actin (green) in control human ATII showed a typical peripheral distribution. There was minimal staining for phosphorylated myosin light chain 2 (P-MLC-2) (red). Exposure to cytomix for 24 h increased the total amount of phosphorylated MLC-2 levels. More significantly, total cellular actin was re-distributed toward the center of the cells along with the phosphorylated MLC-2 to form actin stress fibers. The overlap of actin and phosphorylated MLC-2 is shown by the orange/yellow staining. The addition of MSC-conditioned medium or recombinant human Ang1 to human ATII cells exposed to cytomix partially restored the peripheral distribution of actin and reduced the activated form of phosphorylated MLC-2. Images are representative for each condition run in triplicates.

**Effect of MSC, BMS-345541, or rhAng1 on Tight and Adherens Junction Proteins Expression**—Tight junctions and adherens junctions are dynamic structures linked to the actin cytoskeleton, which control the paracellular permeability of the epithelium. Based on the difference between cytomix-treated and untreated cells on epithelial barrier function, we studied the expression of tight and adherens junction proteins in human ATII cells. By Western blot analyses, we found no differences in ZO-1, occludin, claudin 3, claudin 4, claudin 5, and JAM-A protein expression between control and cytomix exposed type II cells (Fig. 10). There were also no differences in immunostaining of the distribution of these tight junction proteins ZO-1, occludin, and claudin 4 (Fig. 11A) or of the adherens junction proteins, E-cadherin and β-catenin (Fig. 11B). However, the expression and distribution by immunostaining of claudin 18 was significantly decreased by cytomix. By immunostaining, cytomix induced a significant re-distribution of claudin 18 away from the periphery. More significantly, the addition of the NFκB pathway inhibitor BMS-345541, MSC, or rhAng-1 restored the expression and distribution of Claudin 18 (Fig. 12A). By Western blot analyses, BMS-345541 or MSC also partially restored total protein levels of claudin 18 as well. In contrast, rhAng1 did not restore total cellular claudin-18 levels, suggesting that the Ang1 main effect on permeability was on the distribution of the tight junction protein (Fig. 12B).

**Effect of rhAng1 on NFκB p65 Activation**—To determine whether MSC might modulate NFκB activation through Ang1 secretion, we measured NFκB p65 activation in human AT II cells. Cytomix induced a significant increase in p65 activation as early as 1 h and reached a peak level at 3 h, 50× control levels. Although not quite statistically significant (p = 0.07), the simultaneous addition of rhAng1 to the co-culture system restored the level of NFκB activation to 30× control levels at 3 h. We did not perform experiments to determine the effect of MSC (the cells themselves) on NFκB activation because MSC only began to secrete Ang1 at ~12 h after seeding.
DISCUSSION

The major findings of this study are the following. 1) Allogeneic human MSC restored epithelial protein permeability across primary cultures of human alveolar epithelial type II cell monolayer after cytomix-induced injury. 2) Secretion of the paracrine-soluble factor Ang1 by human MSC mediated a major fraction of this protective effect on human ATII permeability after the inflammatory insult. 3) The beneficial effect of Ang1 secretion on human ATII permeability was mediated by a direct effect on cytoskeletal re-organization of both actin and claudin 18, in part through suppression of NFκB activity. To our knowledge, this is the first study to demonstrate the potential therapeutic use of allogeneic human MSC and/or recombinant Ang1 on lung epithelial permeability to protein in ATII cells after an inflammatory insult.

Alveolar barrier integrity is critical for the prevention of permeability pulmonary edema and is maintained by both lung endothelium and epithelium. The alveolar epithelium, consisting of both ATI and ATII cells, forms the tighter membrane of the two. ATII cells exert other critical functions in the lung as well such as surfactant protein synthesis and alveolar fluid clearance through sodium and chloride transport. Disruption of this epithelial barrier occurs due to mechanical and inflammatory stress and leads to the influx of protein-rich fluid and inflammatory cells. This is manifested by hypoxemia, atelectasis, and pulmonary edema, which are the main characteristics of patients with ALI/ARDS. We have reported that human ALI pulmonary edema fluid itself increased protein permeability in our in vitro model of primary cultures of human alveolar epithelial type II cells (34). In line with this prior study, we demonstrated that ATII cells exposed to a mixture of the major biologically active cytokines found in ALI pulmonary edema fluid, IL-1β, TNFα, and IFNγ (referred to as cytomix), resulted in a significant increase in protein permeability by 500% over baseline (Fig. 2). The most significant finding of the current study was that the simultaneous addition of allogeneic human MSC to the bottom chamber of the Transwell plate restored epithelial protein permeability after cytomix exposure (Fig. 2).

Several studies have demonstrated that stem cell-based therapies may offer an effective intervention to lung diseases (16–26). Initially, most early studies postulated that the therapeutic effect was from the capacity of MSC to localize to injured lung tissues and differentiate into specific cell types as the principal mechanism of tissue repair. Different biological mechanisms were postulated to mediate this lung engraftment (6, 37): 1) trafficking of MSC to a local niche of progenitor cells in the lung, 2) trans-differentiation into specific lung cells, or 3) fusion of MSC with differentiated resident cells. Using bleomycin as a model of lung injury, Ortiz et al. (23, 24) found engraftment of MSC in the lung localized to areas of injury. Liebler et al. (38) also demonstrated a low level of retention of human bone marrow-derived cells in a murine xenograft transplantation model after bleomycin-induced lung injury. Stromal derived factor-1 levels in the injured tissues seemed responsible for this engraftment and was shown to be necessary for MSC to localize to the lung injury site.

FIGURE 9. Effect of ROCK inhibitor, myosin light chain kinase inhibitor, or NFκB pathway inhibitor on protein permeability induced by cytomix. A, treatment with Y-27632, a Rho kinase inhibitor, had no beneficial effect on protein permeability across human ATII cell monolayer. B, ML-7, a myosin light chain kinase inhibitor, significantly restored epithelial protein permeability induced by cytomix. NFκB pathway inhibitor BMS-345541 (C) or a NFκB p50 (NLS) inhibitory peptide (D) significantly restored the increase in protein permeability induced by cytomix as well. Epithelial protein permeability is expressed as the mean ± S.D. (% change/24 h, n = 3; *, p < 0.02 versus Control; √, p < 0.05 versus cytomix-injured).

FIGURE 10. Effect of cytomix on total protein levels of the major tight junction proteins. Shown is a representative Western blot analyses of tight junctional protein expressions in human ATII cells injured by cytomix. There are no significant differences in expression of ZO-1, occludin, claudin 3, claudin 4, claudin 5, and JAM-A between control and cytomix exposed groups. Blots were normalized to GAPDH protein expression. Blots are representative for each condition run in triplicates.
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for homing and engraftment of CXCR4+ cells (38, 39). Rojas et al. (21) found a high retention and differentiation rate (up to 29%) of intravenously administered MSC in bleomycin-injured murine lungs. In this study the authors could not rule out potential cell fusion between donor and resident cells and hypothesized that additional chemotaxis of endogenous bone marrow cells contributed to the therapeutic effect. However, in most subsequent studies of MSC used in lung injury (19, 25, 26), engraftment rates approached <5%, suggesting that the magnitude of MSC on repair appeared out of proportion to the number of donor-derived mesenchymal cells found in the lungs.

Therefore, the mechanistic focus underlying the therapeutic benefit of MSC shifted largely to the immunomodulatory properties of secreted paracrine factors such as IL-10 (26, 40) and IL-1ra (24). We reported that MSC administration could shift the balance from a pro-inflammatory to an anti-inflammatory cytokine profile in a mouse model of LPS-induced lung injury (26). In that study, intratracheally administration of MSC resulted in lower levels of TNFα and MIP-2 in bronchoalveolar lavage fluid and plasma, which was associated with an up-regulation of the anti-inflammatory cytokines IL-10, IL-13, and IL-1ra. In another study, Xu et al. (18) showed a reduction of the systemic inflammatory response when MSC were injected intravenously in a mouse model of LPS sepsis. The cells localized initially to the lung but did not engraft, and the effect was associated with the reduction in pro-inflammatory cytokines produced by the injured lung tissue, suggesting a paracrine effect. Moreover, it has been subsequently shown that MSC produced and secreted a broad variety of cytokines, chemokines, and growth factors (41). From these data, we hypothesized that the beneficial effects of MSC treatment might be related to the secretion of paracrine soluble factors.

In search of such a potential soluble factor, we found that human allogeneic MSC secreted a significant amount of Ang1 that increased by 270% with an inflammatory stimulus (Fig. 3). More importantly, using siRNA techniques, Ang1 secretion by MSC was responsible for the therapeutic effect of MSC on epithelial permeability to protein across primary cultures of polarized human alveolar type II cells injured by cytomix (Fig. 4). Interestingly, exposure of MSC to cytomix in the presence of human ATII cells increased the secretion of Ang1 further than with MSC alone, suggesting a potential cross-talk between human ATII cells and MSC (Fig. 3). The biological importance of Ang1 was further supported by using rhAng1 as a positive control, confirming its anti-permeability effect in alveolar epithelial type II cells (Fig. 4).

Angiopoietin 1–4 are a family of growth factors that function as ligands for the Tie2 receptor. Tie2 is a member of a distinct family of receptor tyrosine kinases and is involved in angiogenesis including destabilization of existing vessels, endothelial cell migration, tube formation, and the subsequent stabilization of newly formed tubes by mesenchymal cells (42). Tie2 or Ang1 deficiency results in embryonic lethality, the absence of vessel remodeling, and decreased endothelial cell survival (35, 43). Ang1 (44) and Ang2 (45) are the most studied ligands of Tie2. Binding of the agonist Ang1 to Tie2 mediates rapid receptor autophosphorylation. In contrast, binding of Ang2 to Tie2 has an antagonistic effect. Tie2 protein is also present in quiescent endothelial cells in a range of adult tissues and is constitutively activated to maintain a resting state and cell survival (46). It has been demonstrated that Ang1 is an important stabilizing, anti-inflammatory, and anti-permeability factor targeting the mature vascular endothelium in addition to its angiogenic properties (29–32, 47, 48). In our transwell model, in accordance with studies of endothelial cells, we found high levels of Tie2 phosphorylation in alveolar epithelial type II cells at baseline. In several models of systemic inflammation (49), Ang-2 is responsible for Tie2 dephosphorylation resulting in increased vascular permeability. Among ATII cells, we found that cytomix reduced the activated Tie2 protein levels directly, whereas secretion of Ang1 by MSC restored the phosphorylated form of the receptor (Fig. 5).

The potential role of Ang1 in acute lung injury has recently been addressed by other investigators as well. Karmpaliotis et al. (50) observed that endogenous Ang1 expression was decreased in response to endotoxin in a murine model of ALI. McCarter et al. (33) demonstrated that skin fibroblasts transfected with plasmid vectors overexpressing human Ang1 improved morphological, biochemical, and molecular indices.

FIGURE 11. Effect of cytomix on the distribution of tight junctional proteins. Immunostaining of tight and adherent junctional proteins in human ATII cells injured by cytomix is shown. A, immunostaining of ZO-1, occludin, and claudin 4 in human ATII cells is shown. There are no significant differences in the distribution pattern of ZO-1, occludin and claudin 4, B, immunostaining of adherent junctional protein E-cadherin and β-catenin is shown. There are no significant differences in the distribution pattern of E-cadherin and β-catenin. Images are representative for each condition run in triplicates.
of lung injury and inflammation in a rat model of ALI. In Tie2 heterozygous-deficient mice (Tie2+/−) or binary transgenic mice in which doxycycline-conditional Ang1 overexpression was targeted to endothelium using the Tie1 promoter, the same authors found that overexpression of Ang1 blunted endothelial adhesion molecule expression, increased HO-1 and endothelial nitric-oxide synthase expression, and decreased ET-1 expression, all of which likely contributed to reduced airspace inflammation, intra-alveolar septal thickening, and early mortality. In similar experiments, Mei et al. (25) and Xu et al. (19) transfected mouse MSC with human Ang1 to use as a cellular vector in the treatment of murine models of LPS induced lung injury. Mei et al. (25) showed that treatment with MSC alone or MSC transfected with Ang1, both, reduced airspace inflammation, but MSC transfected with Ang1 had a more profound effect. In their study, Xu et al. (19) reported an improvement in several lung inflammatory markers (alveolar permeability, neutrophil activation, inflammatory cytokines) with MSC transfected with Ang1 after lung injury. In contrast with our findings, these investigators could not detect Ang1 production by mouse MSC alone, possibly due to the species specificity of the ELISA used. Interestingly, the levels of Ang1 produced by transfected MSC were comparable with our levels of Ang1 secretion by human MSC in the Transwell model. In this project, our finding that human alveolar epithelial type II cells contain the Tie2 receptor may have implications in interpreting previous in vivo studies of lung protein permeability in mice. In the future, studies will need to be performed using transgenic mouse studies using Tie2-cre for endothelial-specific gene deletion experiments to differentiate the contribution of the epithelium and endothelium to lung permeability.

For the first time, in addition to its anti-permeability effect on endothelium, we have found that the effect of MSC and Ang1 on epithelial permeability to protein in cultured monolayers of ATII cells after cytomix-induced injury depended in part on cytoskeletal reorganization. Epithelial barrier properties are maintained by intercellular junctional complexes composed of tight junctions, adherens junction, and desmosomes. Tight junctions form a physical barrier and limit the diffusion of proteins and lipids (51). Interaction with several

FIGURE 12. Effect of NFκB pathway inhibitor BMS-345541, rhAng-1, or MSC on claudin 18 protein distribution among human AT II cells injured by cytomix. A, immunofluorescent staining of claudin 18 expression in human AT II cells injured by cytomix demonstrated a re-distribution of the tight junction protein away from the periphery of the cell. The simultaneous addition of BMS-345541, rhAng-1, or MSC restored the re-distribution of claudin 18 expression caused by cytomix. B, Western blot analysis of claudin 18 expression in human AT II cells injured by cytomix demonstrated a decrease in total protein levels. The addition of BMS-345541 or MSC partially restored the protein expression of claudin 18. RhAng-1 did not show a significant effect on protein expression of claudin 18. All experiments were run in triplicates. Claudin 18 expression was normalized to GAPDH expression. A representative Western blot is depicted above each graph. Data are expressed as the mean ± S.D. (% of control, n = 3; *, p < 0.01 versus control; √, p < 0.05 versus cytomix-injured). Control is arbitrarily set at 100%.
scaffolding proteins ultimately links the tight junction to the actin cytoskeleton, and its integrity is reflected in limited paracellular tracer flux of large molecules such as labeled albumin. Interaction of actin and myosin, the main components of the anchored cytoskeleton, regulates the tension throughout the cells. In the resting state, they form a dense ring encircling the cell. Cellular activation such as by inflammatory insult (cytomix) promotes actin disorganization or stress fiber formation and increases the centripetal tension. This augmented physical stress on the tight junctions might explain the increase in paracellular permeability often seen (52). This mechanism involves activation of myosin light chain kinase, resulting in phosphorylation of MLC-2 and their interaction with actin fibers. In our experiments, immunofluorescence staining showed this redistribution of actin fibers and its interaction with phosphorylated MLC-2 after cytomix exposure, which was restored by MSC or rhAng1 treatment (Fig. 8). Two members of the small GTPases, RhoA and Rac1/2/3, have important opposing effects in this process. Activation of RhoA induces stress fiber formation and was activated immediately by cytomix administration (Fig. 6). Activation of Rac1/2/3 counterbalances this and increased by MSC treatment (36) (Fig. 7), suggesting one mechanism of MSC for improvement in lung epithelial protein permeability. However, the simultaneous addition of a ROCK inhibitor, Y-27632, preventing RhoA activation, had no beneficial effect on overall epithelial protein permeability after cytomix exposure in our Transwell model (Fig. 9A). In contrast, the simultaneous addition of a myosin light chain kinase inhibitor, ML-7, fully restored epithelial protein permeability to a normal level after cytomix exposure, suggesting that the effect of MSC may be distal to RhoA activation and/or directly involve the formation of actin stress fibers (Fig. 9B).

Based on the lack of effect of a RhoA inhibitor on epithelial protein permeability, we studied the effect of cytomix on tight junction proteins. By immunofluorescence and Western blot analyses, we were unable to detect an effect of cytomix on total protein levels or the distribution of occludin, claudin 3, 4, and 5, and JAM-A or its associated proteins, ZO-1, E-cadherin, or β-catenin (Figs. 10 and 11). In contrast to the studies by Wray et al. (53) in a model of ventilator induced acute lung injury, we were unable to detect an increase in claudin 4 expression in primary cultures of human alveolar type II cells injured with cytomix. Consequently, we were unable to establish the role of claudin 4 in response to an increase in paracellular epithelial permeability. However, in our model cytomix decreased total protein levels of claudin 18 and caused a re-organization away from the periphery to a disorganized state (Fig. 12). The simultaneous addition of MSC or BMS-345541, a NFκB inhibitor, partially restored total protein levels and the distribution of claudin 18 after cytomix exposure (Fig. 12), suggesting an important role of this tight junction protein in barrier permeability. The addition of rhAng1 instead of MSC also had a similar effect on the redistribution of claudin-18 to the periphery or cell junction (Fig. 12), again demonstrating the important role of Ang1 secretion. This conclusion was supported by a recent manuscript by Koval et al. (54) who demonstrated that increased claudin-18 localization to the plasma membrane was associated with higher barrier resistance in primary cultures of rat alveolar epithelial type II cells. To determine how MSC restored claudin 18 levels, we measured the effect of simultaneous addition of recombinant Ang1 among primary cultures of human type II cells injured by cytomix on NFκB activation. The addition of rhAng1 partially reduced NFκB activation among human type II cells after cytomix exposure at 3 h, suggesting how MSC may restore lung epithelial permeability. More importantly, the addition of BMS-34551 or a NFκB p50 inhibitory peptide fully restored epithelial protein permeability to control levels among ATII cells after cytomix exposure (Fig. 9, C and D). In the future, the individual contributions of “actin stress formation” and/or claudin 18 re-organization on epithelial protein permeability will need to be clarified further.

There are some limitations to the current study. The underlying hypothesis of these studies rested on the contribution of MSC to alveolar epithelial barrier integrity paracrine mechanisms or cell contact independent effects. However, as reported by Németh et al. (40), the role of cell-contact may influence the immunomodulatory effect of MSC, which may influence lung epithelial permeability by changing the inflammatory milieu of an injured alveolus. In the future we will need to know the role, if any, of both the immunomodulatory effects and cell contact effect of MSC on epithelial permeability; the Transwell model cannot be used to answer this question due to the lack of other cells types such as macrophages and neutrophils. In addition, the therapeutic efficacy of MSC on epithelial permeability may in part result from the effects of secreted growth factors on alveolar type II cell apoptosis or growth. However, the role of cell death is probably not relevant in this model because we found that cytomix did not cause an increase in human alveolar type II cell apoptosis or necrosis. Finally, our study does not address the role of alveolar epithelial type I cells.

In conclusion, angiopoietin-1 secretion by human MSC can restore epithelial permeability to a normal level in cultured human type II cells. These effects are at least partially regulated at the level of cytoskeletal reorganization. Although it has been well known that Ang1 can restore the barrier properties of endothelial cells, the capacity of Ang1 to restore protein permeability in lung epithelial cells is novel and may provide insight into the mechanisms by which MSC have therapeutic effects in experimental studies of acute lung injury.

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