Human Mesenchymal Stem Cells Inhibit Vascular Permeability by Modulating Vascular Endothelial Cadherin/ β -Catenin Signaling

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The barrier formed by endothelial cells (ECs) plays an important role in tissue homeostasis by restricting passage of circulating molecules and inflammatory cells. Disruption of the endothelial barrier in pathologic conditions often leads to uncontrolled inflammation and tissue damage. An important component of this barrier is adherens junctions, which restrict paracellular permeability. The transmembrane protein vascular endothelial (VE)-cadherin and its cytoplasmic binding partner β -catenin are major components of functional adherens junctions. We show that mesenchymal stem cells (MSCs) significantly reduce endothelial permeability in cocultured human umbilical vascular endothelial cells (HUVECs) and following exposure to vascular endothelial growth factor, a potent barrier permeability-enhancing agent. When grown in cocultures with HUVECs, MSCs increased VE-cadherin levels and enhanced recruitment of both VE-cadherin and β -catenin to the plasma membrane. Enhanced membrane localization of β -catenin was associated with a decrease in β -catenin-driven gene transcription. Disruption of the VE-cadherin/ β -catenin interaction by overexpressing a truncated VE-cadherin lacking the β -catenin interacting domain blocked the permeability-stabilizing effect of MSCs. Interestingly, a conditioned medium from HUVEC-MSC cocultures, but not from HUVEC or MSC cells cultured alone, significantly reduced endothelial permeability. In addition, intravenous administration of MSCs to brain-injured rodents reduced injury-induced enhanced blood-brain barrier permeability. Similar to the effect on in vitro cultures, this stabilizing effect on blood-brain barrier function was associated with increased expression of VE-cadherin. Taken together, these results identify a putative mechanism by which MSCs can modulate vascular EC permeability. Further, our results suggest that the mediator(s) of these vascular protective effects is a secreted factor(s) released as a result of direct MSC-EC interaction.

Introduction

THE BARRIER FORMED BY vascular endothelial cells (ECs) restricts passage of circulating molecules and inflammatory cells into the underlying tissues. This barrier function is critical for the maintenance of tissue homeostasis and cellular function, especially for neural cells in the central nervous systems. Permeability across this barrier is regulated in a dynamic manner, in part, by adherens junctions (AJs) [1]. AJs are composed of membrane spanning vascular endothelial (VE)-cadherins, whose extracellular domains interact with VE-cadherins expressed on neighboring cells via a homo-

tropic mechanism to restrict paracellular permeability. The cytoplasmic tail of VE-cadherin also binds to catenin linker proteins (eg, β -catenin) to communicate with the cytoskeleton. Breakdown of the endothelial barrier following injury or disease can lead to the unrestricted passage of molecules and inflammatory cells, triggering uncontrolled inflammation and causing further tissue damage.

Stem cell transplantation has shown promise as an important tool for the treatment of several disease processes and injured tissues. Intravenous administration of mesenchymal stem cells (MSCs), the stromal progenitor cells found within the bone marrow [2], have been shown to be

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beneficial in a variety of disease models, including sepsis [3], acute renal failure [4], graft versus host disease [5], acute lung injury [6], and myocardial infarction [7]. MSCs are able to home to sites of active tissue injury and to specific types of tumors [8], where they are thought to modulate vascular function [9]. Consistent with this, we have previously reported [8] that intravenously administered MSCs exert potent antitumorigenic effects in a model of Kaposi's sarcoma, a highly angiogenic tumor thought to be of EC origin [10]. When administered intravenously for therapeutic purposes, MSCs first contact the vascular endothelium. Recently, it has been shown that the vast majority of infused cells home to, as well as take residence adjacent to, the vascular endothelium of the lung and the liver [8,11]. It has been proposed that signals from ECs, possibly following contact with MSCs, influence the differentiation of MSCs into EC-like cells [12]. Thus, it is possible that some of the therapeutic effects of MSCs observed in preclinical studies may relate to effects resulting from MSC-EC interactions at the site of injury and/or at the lung and liver endothelium. The cellular and molecular basis of this interaction and the influence of MSCs on endothelial function are currently not well understood.

We therefore examined the effects of MSCs on ECs and vascular function using both in vitro and in vivo experimental approaches. Our results show that MSCs, when cocultured with ECs, decrease paracellular permeability, and that this effect requires VE-cadherin/ β -catenin interaction. Further, the influence of MSCs on endothelial permeability is likely mediated by a secreted factor(s) that is released as a result of MSC-EC interaction. Similar to their effect in vitro, intravenous administration of MSCs postinjury significantly improved blood-brain barrier (BBB) function in brain-injured mice. These data have implications for the potential use of MSCs as a cellular therapy for disease conditions characterized by compromised vascular integrity.

Materials and Methods

Primary cells and cell lines

First-passage human MSCs and HUVECs were purchased from Lonza. For experiments with MSCs, cells were pooled from 5 separate donors. MSCs were routinely characterized by flow cytometry for a representative set of markers characteristic of MSCs using previously described methods [8]. As we previously reported [8], MSCs were found to have undetectable levels of major histocompatibility complex (MHC) class II and very low levels of MHC class I; intermediate levels of the cellular adhesion proteins VCAM and ICAM; and high levels of CD105, CD29, and CD44.

MSCs were cultured in an MSC growth medium (MSCGM; Lonza); HUVECs were maintained in the endothelial growth media (EGM)-2 medium. MSCs were used at passage 3–7 for all experiments. MSCs were labeled for tracking purposes with Quantum Dots (525 nm) as per the manufacturer's instructions (Invitrogen).

Transwell and coculture

HUVECs were cocultured in contact with MSCs or with MSCs in transwells (0.4- μ m pore size polyester (PET) membrane from BD Biosciences, San Jose, CA). About 5×10^5 cells were seeded on a 4.67 cm² surface area. The ratio of MSCs to

HUVECs was 0.2. In the dose-response experiments, the total number of cells per well (5×10^5) was held constant and the ratio of MSCs/HUVECs was adjusted to achieve seeding ratios of 0.2, 0.5, and 1.0. Cells were harvested by trypsinization 24 h after coculture and sorted by magnetic cell sorting (MACS) using beads conjugated with CD44 antibodies (Miltenyi Biotec). The CD44⁺ population (HUVECs) was used in subsequent assays of endothelial function. An aliquot of this population of cells was confirmed to be CD31⁺ by flow cytometry prior to use.

EC permeability

Collagen-coated 0.4- μ m pore size inserts were obtained from BD Biosciences. HUVECs were seeded at 40,000 cells per insert well in a total volume of 500 μ L of the medium and cultured for 8 h to allow for cell attachment and adhesion. HUVEC monolayer permeability was tested 8 h later by adding 10 μ L of 10 mg/mL 40-kDa fluorescein isothiocyanate (FITC)-Dextran (Sigma-Aldrich) to the upper chamber of each well. Monolayers were treated with 10 ng/mL vascular endothelial growth factor (VEGF)-A165 from R&D 1 h before addition of FITC-Dextran. Measurements were determined with a fluorimeter (Biotek-Synergy HT) using excitation and emission wavelengths of 485 and 530 nm, respectively. When making fluorescence determinations, the Synergy HT uses a tungsten halogen lamp with interference filters for wavelength specificity in conjunction with a photomultiplier tube (PMT) detector. The tungsten halogen lamp produces a large amount of light at a constant intensity, providing increased sensitivity and repeatability. Specially designed fluorescent interference filters prevent light leakage from excitation wavelength to the emission wavelength.

The fluorimeter reads the FITC component of the FITC-Dextran from samples obtained 30 min after addition of FITC-Dextran to the top well of the transwell insert. Specifically, 100 μ L of the medium was withdrawn from the lower well after 30 mins, added to a 96 well plate, and read on the Synergy. Data represent the mean $\pm$ standard deviation (SD) from 3 independent experiments. For experiments in which the effects of the conditioned medium from MSCs or MSC/HUVEC cocultures were studied, the medium from HUVEC monolayer cocultures that had been plated on collagen-coated inserts and allowed to adhere was replaced with an undiluted conditioned medium and maintained in culture for 4 h before addition of FITC-Dextran. Data represent the mean $\pm$ SD from 3 independent experiments.

Plasmids and transfections

About 1×10^6 HUVECs or MSCs were transfected with 1 μ g plasmid constructs using the Amaxa Nucleofection system (Lonza). Transfection efficiencies were estimated using a green fluorescent protein plasmid (Green Lantern; Stratagene). Lithium chloride (LiCl) was added to cultures at a final concentration of 10 mM 6 h before the cells had been harvested for luciferase reporter activity, which was performed 48 h after transfection of reporter constructs. The truncated VE-cadherin construct cloned into the pECE expression plasmid was a generous gift from Dr. Elisabetta Dejana (Istituto di Ricerche Farmacologiche Mario Negri, Milan) and was constructed as previously described [13].

Luciferase reporter gene assays

The TOPFLASH reporter construct expresses the firefly luciferase gene driven by a synthetic promoter containing 2 sets of 3 copies of T-cell/lymphoid enhancer factor (TCF/LEF) binding sites (Stratagene). HUVECs were cotransfected with the TOPFLASH vector and the Renilla-luciferase control pGL47.5 (Promega). At 24 h posttransfection, cells were seeded in 12-well plates. MSCs were added to HUVECs at a ratio of 0.2. Cells were lysed after 24 h after coculture and readings were obtained on a luminometer (Turner Designs). Firefly luciferase activity was normalized to Renilla-luciferase activity and normalized data are reported as relative luciferase units.

Animals and traumatic brain injury model

Male C57BL6 mice were purchased from Charles River Laboratories. The animals were housed on a 12-h light/dark cycle with *ad libitum* access to food and water. All experimental procedures were approved by the Institutional Animal Care and Use Committee and were conducted in accordance with the recommendations provided in the *NIH Guide for the Care and Use of Laboratory Animals*. A controlled cortical impact (CCI) injury model [3,4] was used to cause traumatic brain injury (TBI) in the animals. Mice were initially anesthetized with 5% isoflurane and 1:1 O₂:N₂O mixture. Animals were mounted on a stereotaxic frame and were secured by ear bars and an incisor bar. Anesthesia was maintained at 2.5% isoflurane with a 1:1 O₂:N₂O mixture. A 5-mm-diameter craniotomy was made midway between bregma and lambda on the right side, with the medial edge of the craniotomy 1 mm lateral to midline. Injury was produced using a magnetic impactor mounted at an angle of 10° from the vertical plane. A single impact at a velocity of 4 m/s with a deformation depth of 1.6 mm was delivered. After injury, the incision was closed. Sham animals received all aforementioned surgical procedures except for impact injury. Core body temperature was monitored with a rectal thermometer and maintained at 36.8°C–37.2°C with a heating pad during the surgery.

In vivo cell administration

At 2 and 24 h after injury, 1×10^6 MSCs, HUVECs, or vehicle control (phosphate-buffered saline, PBS) was administered via tail vein injections in a total final volume of 100 μ L. Cells were harvested via trypsinization and washed twice in PBS before in vivo administration.

Evans Blue dye extravasation

Three days after injury, Evans Blue dye (EB) (3% in saline, 4 mL/kg) was administered via jugular vein infusion and allowed to circulate for 1.5 h as previously described [5]. The animals were then transcardially perfused with 25 mL of PBS. The brains were removed, divided into ipsi- and contralateral hemispheres, and weighed. Tissue was homogenized in 1 mL cold 50% trichloroacetic acid and centrifuged at 20,000 g for 20 min. Supernatant solution was collected and neutralized with NaOH. The absorbance was measured at 610 nm and normalized per gram of tissue.

Immunohistochemistry

Mice were sacrificed 3 days after injury and brains quickly removed and snap frozen in -80°C isopentane. Coronal

sections (20 μ m) were cut on a cryostat, mounted on slides, postfixed for 20 min with -20°C methanol, and rehydrated in PBS. Sections were incubated in primary antibodies (1.0 μ g/mL in PBS containing 2.5% goat serum and 0.25% Triton X-100) for 48 h at 4°C , washed in PBS, and then incubated with Alexa Fluor-conjugated species-specific secondary antibodies for 3 h at RT. Sections were cover slipped with Fluormount-G (Fisher Scientific), and imaged using an upright Olympus BX-51 microscope (Bio-Rad) attached to a MagnaFire digital camera (Optronics).

Western blotting

The injured hemicortex was harvested and homogenized in 0.5% Triton X-100 in PBS (pH 7.4) with protease inhibitors. Equal amounts of protein homogenate were resolved on 4%–12% sodium dodecyl sulphate–polyacrylamide gel electrophoresis gels and transferred onto polyvinylidene fluoride (PVDF) membranes. Blots were blocked in PBS-T containing 5% milk and probed with primary antibodies directed toward β -catenin (Cell Signaling), activated β -catenin (Upstate Biotech-Millipore), VE-cadherin (Cell Signaling), phospho-VE-cadherin (Y731) (Abcam), phospho-GSK-3 β , or total GSK-3 β (Cell Signaling). Blots were then washed in PBS-T, incubated in alkaline phosphatase-conjugated secondary antibodies, and immunoreactivity detected using CDP Star (Pierce Biochemicals). Blots were exposed to a film, and densitometric readings of immunoreactive bands were obtained using Image-J (NIH).

Statistical analysis

Data in all graphs are shown as mean $\pm$ SD. For permeability and BBB permeability measures, data were analyzed using a Student's *t*-test for 2 group comparisons. Repeated measures 1-way analysis of variance was used for statistical analysis of dose–response permeability studies.

Results

MSCs reduce paracellular permeability of EC monolayers in vitro

Human MSCs were obtained from 4 young, healthy donors and were pooled for use in all experiments. Before using these cells for in vitro and in vivo experiments, fluorescence-activated cell sorting analysis was performed to examine expression of characteristic MSC cell surface markers. Cells were confirmed to display characteristic MSC cell surface markers [8], including high levels of CD44 and CD105, and an absence of CD31 expression (data not shown). Expression of CD44 on HUVECs is markedly lower than that on MSCs (Fig. 1A), while HUVECs uniformly express high levels of CD31. These differentially expressed markers allowed us to negatively or positively select MSCs from MSC-HUVEC mixed cultures using MACS. As determined by fluorescence-activated cell sorting analysis, MACS separation of MSC-HUVEC cocultures yields a very pure population of CD44⁺ HUVECs (Fig. 1B) that can be subsequently tested using in vitro permeability assays.

Figure 1C shows a schematic of culture conditions employed to study the effects of MSCs on the permeability characteristics of HUVECs. To determine if MSCs influenced barrier function of ECs, we cultured HUVECs alone or with

FIG. 1. Mesenchymal stem cells (MSCs) reduce paracellular permeability of endothelial cell (EC) monolayers in vitro. **(A)** Flow cytometry staining of isotype control (red), MSCs (blue), or human umbilical vascular endothelial cells (HUVECs) (green) for CD44, the marker used for negative selection to obtain a pure HUVEC population for in vitro study. **(B)** Representative flow cytometry data for CD44 staining of HUVEC-MSC cocultures before (*left panel*) and after (*right panel*) magnetic cell sorting (MACS) separation using negative selection for CD44⁺ cells. CD44-fluorescein isothiocyanate (FITC) signal is shown on the Y-axis, and forward scatter (FSC) is shown on the X-axis. **(C)** Schematic of experiments used to determine the effects of MSCs on EC monolayer permeability. **(D)** Permeability to 40-kDa dextran-FITC is reduced in HUVECs cocultured with MSCs. HUVECs were cultured with or without MSCs and separated as described in C. CD44⁻ HUVECs were subsequently seeded in transwell chambers (0.4- μ m pore size) and allowed to adhere for 8 h. Permeability of the HUVEC monolayer was assessed by diffusion of 40-kDa dextran-FITC into the bottom chamber. Stimulation with vascular endothelial growth factor (VEGF)-A (10 ng/mL; 60 min) increased permeability in control HUVEC cultures, but was significantly reduced in HUVECs cocultured with MSCs. Data shown are the average $\pm$ SEM of 3 independent experiments.

MSCs for 24 h. Cells from both culture conditions were then sorted to obtain a pure population of CD44⁻/CD31⁺ HUVECs, which were subsequently tested in permeability assays. An equal number of purified CD44⁻/CD31⁺ HUVECs that had been cultured alone or with MSCs were seeded on transwell inserts and allowed to attach for 8 h. Permeability to 40-kDa dextran-FITC was measured in unstimulated conditions and following VEGF stimulation, as described in the Materials and Methods section. HUVECs cocultured with MSCs were significantly less permeable to 40-kDa dextran-FITC (Fig. 1D) than HUVECs cultured alone. Further, treatment of HUVEC monolayers with VEGF-A increased permeability to 40-kDa dextran-FITC, which was significantly reduced in HUVECs that were cocultured with MSCs.

MSCs increase VE-cadherin/ β -catenin interaction at the cell surface of ECs

AJs regulate vascular integrity and permeability. VE-cadherin-VE-cadherin homophilic interactions on ECs are crucial for maintaining normal vascular integrity and per-

meability characteristics [14,15]. Cadherin junctions link the extracellular environment to the intracellular environment by recruiting and binding to β -catenin, which then binds to the actin cytoskeleton via other intermediate proteins within the cytoplasm [16]. To determine if β -catenin is involved in the decrease in barrier permeability, we observed the effects of the MSCs on β -catenin localization in cocultured HUVECs (Fig. 1D). Compared with HUVECs alone, we found that HUVECs that had been cocultured with MSCs demonstrated enhanced β -catenin immunoreactivity at the membrane, and an increased propensity to grow in clusters (Fig. 2A). Similarly, HUVECs cultured with MSCs demonstrated increased localization of VE-cadherin at the cell membrane compared with HUVECs cultured alone (Fig. 2B). Further, levels of activated, dephosphorylated β -catenin in the nucleus were markedly reduced in HUVECs cocultured with MSCs compared with HUVECs cultured alone (Fig. 2C). To determine if enhanced expression of VE-cadherin after coculture is associated with increased β -catenin binding to VE-cadherin, we immunoprecipitated homogenates from cocultured HUVECs with antibodies to VE-cadherin. Immunoprecipitation

FIG. 2. MSCs enhance vascular endothelial (VE)-cadherin/ β -catenin interaction at the cell membrane. After coculture with MSCs or culture alone, HUVECs were separated using CD44-based MACS and replated for experiments displayed in A–C. Representative 100 $\times$ fields from images captured under identical conditions are shown. Immunofluorescence staining of β -catenin (A) and VE-cadherin (B) in HUVECs after coculture (+MSCs) or alone (–MSCs). Coculture with MSCs resulted in clustering of HUVECs and increased staining of β -catenin and VE-cadherin at the membrane as compared to HUVECs alone. (C) Immunofluorescence staining of activated (de-phosphorylated) β -catenin in HUVECs after culture alone or together with MSCs. (D) Immunoprecipitation of VE-cadherin from lysates of HUVECs cultured alone (–) or together with MSCs (+) and then magnetically sorted, demonstrating that for a fixed amount of HUVEC lysate (25 μ g) increased expression of VE-cadherin is observed along with increased β -catenin binding.

of a fixed amount of homogenate from control or MSC + HUVEC cultures revealed increased β -catenin and VE-cadherin immunoreactivities in the coculture condition, suggesting that MSCs enhanced VE-cadherin levels and interaction with β -catenin (Fig. 2D).

Coculture with MSCs reduces β -catenin-dependent transcriptional activity in ECs

β -catenin regulates cell adhesion by interacting with VE-cadherin at the membrane, and also contributes to the regulation of TCF/LEF-mediated gene transcription in the nucleus [17]. Enhanced binding of β -catenin to VE-cadherin at the cell membrane is associated with decreased β -catenin-mediated TCF/LEF gene transcription [18]. To determine if coculture with MSCs results in decreased β -catenin-dependent TCF/LEF-mediated transcription in ECs, we co-transfected HUVECs with a β -catenin expression plasmid and a TOPFLASH reporter gene construct containing 6 TCF/LEF binding sites [19]. HUVECs cocultured with MSCs showed a marked reduction in T-cell factor/lymphoid enhancer factor (TCF/LEF) reporter transcription compared with HUVECs cultured alone (Fig. 3A). In contrast, no decrease in reporter activity was observed when a mutant construct lacking β -catenin binding sites (FOPFLASH [20]) was used (Fig. 3B).

We further explored the biochemical consequences of MSCs on the enhancement of VE-cadherin/ β -catenin cell surface interaction in HUVECs. Western blot results shown in Fig. 3C indicate that the levels of active, dephosphorylated β -catenin were decreased and VE-cadherin were increased in HUVECs after coculture with MSCs, corroborating the results shown in Fig. 2B and C. In addition, we found that

tyrosine phosphorylation of VE-cadherin (Y⁷³¹) in HUVECs cocultured with MSCs was decreased, consistent with an increase in intercellular homophilic interactions of VE-cadherin at the membrane [21]. Expression of cyclin D1, a TCF/LEF-driven gene [22], was decreased in coculture extracts, consistent with the decrease we find in TCF/LEF transcriptional activity. Notably, coculture of HUVECs with MSCs resulted in a decrease in phosphorylated (inactive) GSK-3 β , whereas total GSK-3 β immunoreactivity remained unchanged. Equal loading of the western blots was confirmed by equivalent glyceraldehyde 3-phosphate dehydrogenase immunoreactivity in each lane.

Previous studies have established that inhibition of GSK-3 β activity is required for β -catenin-mediated transcription [19]. Since phosphorylation by GSK-3 β targets β -catenin for proteosomal degradation, it is possible that GSK-3 β activation by MSCs may be required for the observed decrease in TCF/LEF-mediated transcriptional activity in HUVECs. To test this possibility, HUVECs alone or HUVECs cocultured with MSCs were assayed for TCF/LEF-mediated transcription in the presence of LiCl at a concentration known to potentially inhibit GSK-3 β activity. As shown in Fig. 3D, LiCl was unable to completely rescue the inhibitory effects of MSCs of TCF/LEF-mediated transcription, suggesting that these effects are independent of MSC effects on GSK-3 β activity in HUVECs.

MSCs' effect on endothelial paracellular permeability requires VE-cadherin/ β -catenin interaction

Intercellular homophilic VE-cadherin interactions and the intracellular association with β -catenin are critical for EC permeability [23]. Further, in a variety of contexts, β -catenin

FIG. 3. Coculture with MSCs inhibits β -catenin-dependent transcriptional activity in ECs. **(A)** Firefly luciferase activity normalized to Renilla-luciferase activity (relative luciferase units) from HUVECs cotransfected with a T-cell factor/lymphoid enhancer factor (TCF/LEF) reporter construct (TOPFLASH), a constitutively active Renilla-luciferase construct, and wild-type β -catenin, and then cultured alone or with MSCs overnight. Data from 3 independent experiments are shown as mean $\pm$ SEM. **(B)** Luciferase activity, normalized to Renilla, from HUVECs cotransfected with a mutant TCF/LEF reporter construct lacking β -catenin binding sites (FOPFLASH), a constitutively active Renilla-luciferase construct, and wild-type β -catenin and then cultured alone or with MSCs overnight. Group data from 3 independent experiments are shown as mean $\pm$ SEM. **(C)** Western blots of HUVECs that were cultured with (H + M) or without MSCs (H), MACS separated, lysed, and probed with antibodies directed against the indicated antigens. **(D)** Luciferase activity, normalized to Renilla, from HUVECs cotransfected with a TCF/LEF reporter construct (TOPFLASH), a constitutively active Renilla-luciferase construct, and wild-type β -catenin, and then cultured alone or with MSCs overnight followed by culture in the absence or presence of lithium chloride (LiCl) (10 mM) for 6 h.

plays a key role in the maintenance of endothelial cell-cell junctions through linkage of VE-cadherin complexes to the cytoskeleton [24,25]. Since MSCs reduced endothelial monolayer permeability (Fig. 1D) and increased VE-cadherin/ β -catenin association (Fig. 2D), we next examined if the MSC influence on HUVEC permeability required VE-cadherin/ β -catenin interaction. We transfected HUVECs with a truncated expression construct for VE-cadherin (truncated VE-cadherin [TVCad]) that lacks the C-terminal β -catenin binding domain and acts as a dominant negative inhibitor of β -catenin/VE-cadherin interaction [13]. HUVECs transfected with TVCad (TVCad-HUVECs) and cocultured with MSCs exhibited reduced surface VE-cadherin immunoreactivity displayed in a noncontiguous pattern (Fig. 4A, lower panel) compared with control-transfected HUVECs (pECE-HUVECs) cocultured with MSCs (Fig. 4A, upper panel). In addition, TVCad-transfected HUVECs did not mobilize β -catenin to the membrane after coculture with MSCs (Fig. 4B, lower panel), compared with control-transfected HUVECs cultured with MSCs (Fig. 4B, upper panel). Strik-

ingly, transfection of TVCad into HUVECs completely abrogated the inhibition of permeability induced by MSCs (Fig. 4C). These results indicate that VE-cadherin interaction with β -catenin is required for the effects of MSCs on EC permeability.

Conditioned medium from MSC-HUVEC coculture recapitulates the effects of MSCs on EC permeability

To gain further insight into the mechanism by which MSCs modulate EC permeability, we investigated whether a secreted factor(s) may be responsible for enhanced barrier integrity in MSC/EC cocultures. We tested the conditioned medium from cells grown in 3 different formats to reduce paracellular permeability: HUVECs alone, MSCs alone, and HUVECs + MSCs. In each format, the total number of cells seeded in the culture was held constant at 2×10^5 cells per well. For the cocultured conditions, MSCs were added to give an MSC-to-HUVEC ratio of 0.2, 0.5, or 1.0. The conditioned medium was harvested 24 h after seeding and

FIG. 4. Effects of MSCs on HUVEC paracellular permeability requires VE-cadherin/ β -catenin interaction. Transfection of HUVECs with a truncated expression construct for a mutant VE-cadherin (truncated VE-cadherin [TVCad]) results in reduced VE-cadherin membrane staining (**A**, lower panel) and reduced β -catenin membrane staining (**B**, lower panel). Control, vector-only transfected HUVECs (pECE-HUVECs) exhibit robust membrane expression of VE-Cadherin (**A**, upper panel) and β -catenin (**B**, upper panel) upon replating after coculture with MSCs and magnetic separation, similar to findings seen in Fig. 2 using untransfected HUVECs (Fig. 2A, B). Images are representative of at least 10 independent high-powered fields from 2 independent experiments. Images shown here were acquired under identical conditions as those shown in Fig. 2. (**C**)

Coculture with MSCs has no effect on permeability of HUVECs overexpressing TVCad compared with permeability of unmodified HUVECs or HUVECs transfected with TVCad and cultured alone.

tested for their effect on permeability. Permeability to 40-kDa dextran-FITC for each culture condition was assessed, and normalized to the permeability values obtained using the conditioned medium from HUVECs alone. The experimental design described above is depicted schematically in Fig. 5A. Figure 5B shows that addition of the conditioned medium from MSCs did not significantly decrease permeability as compared to HUVEC alone. In contrast, the conditioned medium from HUVEC + MSC cocultures significantly reduced paracellular permeability in a dose-dependent manner. Figure 5C shows that addition of the conditioned medium from coculture conditions, but not the conditioned medium obtained from either HUVECs or MSCs alone, resulted in reduced activated (nonphosphorylated) β -catenin immunoreactivity. Exposure to the conditioned medium from HUVECs + MSCs also increased VE-cadherin expression. These molecular changes are consistent with what was observed when MSCs and HUVECs were grown in culture (Fig. 3D), suggesting that a secreted factor(s) resulting from MSC-EC interaction may be responsible for improving the barrier integrity of ECs.

Intravenous administration of MSCs inhibits BBB permeability in mice with TBI

Since we found a decrease in EC permeability and an increase in the AJ protein VE-cadherin in our in vitro model, we asked if MSCs could affect vascular permeability in vivo. We used a rodent TBI model of BBB permeability as an in

vivo model of pathologic vascular permeability [26,27]. Figure 6A depicts the scheme of our in vivo experiments in which MSCs were labeled with Quantum dots for tracking purposes (Qdots), and 1×10^6 MSCs were injected at 2 and 24 h post-TBI via the tail vein. BBB permeability was determined by extravasation of EB 3 days after TBI, a time at which the BBB is maximally impaired [27]. Mice that received MSCs had significantly decreased amounts of extravasated EB in the brain, as shown visually in Fig. 6B, and quantitatively in Fig. 6C. Control cells (HUVECs) administered in the same quantity and fashion as MSCs did not affect BBB permeability after TBI. To determine if MSCs were present in the brains of treated mice, sections within and adjacent to the injured region were analyzed for the presence of Q-dot labeled MSCs (red). MSCs (~ 4 cells/5- μ m section) were found to be present in the brain sections of treated mice. These cells appeared to be located in the perivascular space (Fig. 6D) adjacent to vessel lumens (denoted as "L" in Fig. 6D).

Enhanced expression of junctional proteins in the penumbra after TBI in MSC treated mice

VE-cadherin, an AJ protein, exhibited reduced immunoreactivity in the penumbral region of the lesion site in mice with TBI compared to sham control (Fig. 7A). A similar decrease was also observed in the immunoreactivities of the tight junction proteins occludin and claudin-5 in the injury

FIG. 5. Conditioned medium from MSC + HUVEC coculture recapitulates the effects of MSCs on EC permeability. **(A)** Experimental schematic of transwell cocultures with MSCs $\pm$ HUVECs to determine if a secreted factor(s) from MSCs cultured alone or cultured with HUVECs is able to reproduce the effects on EC permeability seen when HUVECs were directly cocultured with MSCs. **(B)** Permeability of HUVEC monolayers to 40-kDa FITC-Dextran when cultured in the conditioned medium from HUVEC alone (2×10^5 cells) MSC alone (2×10^5 cells), or HUVECs cocultured with increasing numbers of MSCs (total number of cells in coculture was held constant at 2×10^5). Results represent data from 3 independent experiments. **(C)** Western blot probed with antibodies directed against de-phosphorylated β -catenin, VE-cadherin, or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (loading control) of EC lysates after overnight treatment with the conditioned medium from HUVECs (EC), MSCs (MSC), or HUVEC/MSC cocultures (MSC + EC, 1:1 cell ratio).

penumbra (Fig. 7B, C). Intravenous delivery of MSCs resulted in a significant sparing of both VE-cadherin and occludin immunoreactivity in mice following TBI (Fig. 7A, B). Further, there was a marked increase in the tight junction protein claudin-5 immunoreactivity (Fig. 7C) in the penumbra of MSC-treated mice. This is consistent with the demonstration that VE-cadherin interactions upregulate claudin-5 expression [28]. Western blot analyses of brain lysates revealed increased occludin and VE-Cadherin expression in the injured brains of MSC-treated mice compared with vehicle controls or sham injured mice (Fig. 7D, quantified in Fig. 7E, F).

Discussion

Our results show that MSCs exert a potent stabilizing effect on HUVECs that result in decreased permeability in vitro. This effect was found to be dependent on increased VE-cadherin expression and enhanced VE-cadherin/ β -catenin

interaction. Further, the effects of MSCs on HUVEC permeability seen in coculture experiments were recapitulated by the conditioned medium harvested from HUVEC-MSC cocultures, but not from the conditioned medium obtained from HUVECs or MSCs cultured alone. The in vitro effects of MSCs on endothelial permeability were manifest in vivo as a significant inhibition of TBI-induced BBB permeability in a rodent model.

Two pathways are known to regulate permeability across the endothelial barrier: transcellular and paracellular. Transcellular permeability is primarily regulated by intracellular signals that regulate endocytosis, exocytosis, and vesicular trafficking. In contrast, paracellular permeability is primarily governed by AJs and tight junctions (TJs). ECs express high levels of VE-cadherins that interact homotypically with VE-cadherins expressed on neighboring ECs to form AJs. The importance of VE-cadherins in vascular permeability has been demonstrated using VE-cadherin-specific

FIG. 6. MSCs inhibit blood–brain barrier (BBB) permeability in mice with traumatic brain injury (TBI). **(A)** Schematic depiction of in vivo experiments to determine the effects of MSCs on blood–brain barrier permeability after TBI **(B)**. Representative images of the site of cortical impact injury from sham injured mice, injured mice treated with vehicle, MSC-treated injured mice, or mice treated with control (HUVEC) cells. Blue represents Evans Blue dye extravasation at the site of injury. **(C)** Quantification of absorbance at 610 nm/g of tissue (proportional to the amount of Evans Blue extravasation) from ipsilateral cerebral hemisphere tissue of mice from the indicated treatment groups. At least 6 mice were studied in each group. **(D)** Brain sections from MSC-treated mice (3 days post-TBI) reveal MSCs in the brain. Immunofluorescence imaging demonstrates the presence of Q-dot-labeled MSCs (indicated by arrows) in the brain. The MSCs present in the brain were typically located in the perivascular niche adjacent to blood vessel lumens (red = MSC, blue = DAPI nuclear staining).

antibodies and mutant mouse models. Injection of anti-VE-cadherin antibodies into adult mice causes a dramatic increase in paracellular permeability and hemorrhage [29]. Loss of VE-cadherin is lethal in embryonic life due to altered vascular function [30]. We found that coculturing HUVECs with MSCs decreased both unstimulated and VEGF-stimulated paracellular permeability (Fig. 1D). We also observed that MSCs increased VE-cadherin membrane localization (Fig. 2) and the overall levels of VE-cadherin (Fig. 3), which may underlie the vascular protective effect of MSCs. Consistent with this, intravenous administration of MSCs increased VE-cadherin levels in brain homogenates, and decreased TBI-associated enhanced BBB permeability (Figs. 6 and 7). The MSCs' ability to decrease BBB paracellular permeability may not be solely due to enhanced VE-cadherin levels since the levels of 2 tight junction proteins, occludin and claudin-5 (Fig. 7), were also increased in the brain homogenates of animals injected with MSCs.

The function of AJs is regulated by catenin proteins (p120, plakoglobin, and β -catenin) that bind to the cytoplasmic tail of VE-cadherin and communicate with the cytoskeleton [1]. The interaction of β -catenin with VE-cadherin is critical for barrier function, as illustrated by the embryonic lethality observed in mutant mice expressing a C-terminal-truncated version of VE-cadherin that is unable to bind β -catenin [30]. Consistent with this, we observed that the permeability protective effect of MSCs is lost when a truncated VE-cadherin is expressed in HUVECs cells (Fig. 4C). The importance of the VE-cadherin/ β -catenin interaction in MSC-mediated reduction in endothelial permeability is further supported by coimmunoprecipitation experiments (Fig. 2D), demonstrating enhanced precipitation of the complex from protein extracts prepared from purified HUVECs grown for 24 h in a HUVEC-MSC coculture. Our results show an increase in VE-cadherin levels in HUVEC-MSC cocultures with an associated decrease in β -catenin phosphorylation at Ser³⁷ or Thr⁴¹

FIG. 7. Enhanced expression of junctional proteins in the penumbra after TBI in MSC-treated mice. Immunofluorescent staining of brain sections from the penumbra region of MSC-treated mice with antibodies reveals preservation of VE-cadherin immunoreactivity (**A**), preservation of occludin immunoreactivity (**B**), and increased claudin-5 immunoreactivity (**C**) as compared to vehicle-treated injured mice (*middle panels*) or shams (*left panels*); 100× images that are representative of multiple fields examined from each experimental group are shown. (**D**) Western Blot analysis of ipsilateral hemispheric brain homogenates from these mice revealed that MSCs modestly increased occludin and VE-cadherin immunoreactivity after injury. Densitometric (normalized to glyceraldehyde 3-phosphate dehydrogenase) quantification of the increased immunoreactivity of VE-cadherin (**E**) and occludin (**F**) in brain homogenates from injured, MSC-treated mice as compared with vehicle-control-treated injured mice or sham injured mice ($n = 3$ animals/group).

(Fig. 3). The molecular mechanism for the MSC-induction of VE-cadherin levels in HUVEC is not known at present, but is a critical goal for our future studies.

Studies have shown that phosphorylation of VE-cadherin on tyrosine residues can increase paracellular permeability in response to permeability-enhancing agents such as VEGF [31] or histamine [32]. Our observation that VE-cadherin Tyr⁷³¹ phosphorylation is decreased when HUVECs are co-cultured with MSCs (Fig. 3C) is consistent with these previous reports. Although the signaling mechanism for the observed decrease in Tyr⁷³¹ phosphorylation is not presently known, a possible candidate that may be involved is the VE-protein tyrosine phosphatase (VE-PTP). VE-PTP is an EC-specific phosphatase associated with VE-cadherin, and it is known to play an important role in regulating vascular permeability [33]. Knockout of VE-PTP is embryonically lethal [34], a phenotype similar to that observed for VE-cadherin null mice. Further highlighting the importance of tyrosine phosphorylation of VE-cadherin, a complex containing VE-cadherin, β -catenin, PI3-kinase, and VEGFR-2 is involved in the VEGF-A-mediated survival of ECs through the activation of Akt signaling. Mutant mice that express a truncated version of VE-cadherin that cannot bind β -catenin display increased apoptosis. Tyr⁷³¹ is located within the β -catenin interacting domain and its decreased phosphorylation could increase VE-cadherin/ β -catenin interaction. Consistent with this, we observe that interaction of HUVECs with MSCs decrease Tyr⁷³¹ phosphorylation and increase VE-cadherin in the plasma membrane. VE-cadherin is highly susceptible to degradation by a number of proteases including metalloproteases, elastase, cathepsin G, and trypsin [35–37]. Increased VE-cadherin/ β -catenin binding may induce conformational changes in the complex leading to greater stability and/or protease resistance.

Our in vitro experiments showed that the conditioned medium from HUVEC-MSC cocultures, but not from HUVEC or MSCs cultured alone, can decrease endothelial permeability. Moreover, similar to the effect of MSCs, the conditioned medium also decreases β -catenin phosphorylation and increases VE-cadherin levels. Taken together, these results suggest that MSC-HUVEC interaction led to the production of a secreted factor(s) that has barrier protective activity. Whether this factor(s) is derived from MSCs or HUVECs is a question that we are addressing in our ongoing studies. Our data showed that MSCs had a significant effect on BBB permeability when administered intravenously to brain injured animals. However, a low number of MSCs were detected in the brains of MSC-treated mice. We previously reported that intravenous administration of MSCs resulted in the majority of cells homing to perivascular spaces in the lung and liver, a finding observed by other groups as well [3,8,11]. Although a number of previous reports suggest that the effect of MSCs are mediated locally through expression of factors that act in a paracrine manner [38,39], our current findings suggest the possibility that MSCs could also exert a systemic effect, possibly through the secretion of a soluble factor(s) induced by MSCs in contact with vascular ECs. Since MSCs interact with the vascular endothelium of brain, lung, and liver following intravenous administration, we intend to further explore how MSCs interact with ECs derived from these and other organs of interest.

It is of interest to us to find out what the soluble factors are that regulate the effects we have described in this article. There are several known factors that are candidate factors for the effects that we have observed of MSCs on vascular permeability and endothelial AJ function. An example of such candidates are members of the angiopoietin family, which are known to be expressed by MSCs [40] and which have been previously shown to enhance vascular integrity in a variety of pathologic conditions [41,42]. In support of a role for angiopoietin as a mediator of the effects of MSCs on vascular permeability is work describing an association between beneficial effects of MSCs on vascular integrity and expression of Ang-1 in a preclinical model of stroke [43].

Additionally, it is possible that previously undescribed or uncharacterized secreted factors derived from MSC-EC interaction may account for some of the observed effects of MSCs in vitro and in vivo. Such factors may be identified using broad-based unbiased gene expression and/or proteomics approaches, such as that recently employed to demonstrate that expression of the antiinflammatory protein TSG-6 significantly contributes to the beneficial effects of MSCs in preclinical models of myocardial infarction [44]. Our future studies will employ both candidate and biased approaches to identify the secreted factor(s) derived from MSC-EC interactions that enhance vascular integrity, which may prove to be important for mechanistic and therapeutic purposes.

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Author Disclosure Statement

No conflicts of interest exist for any of the authors.

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