Corneal Mesenchymal Stromal Cells Are Directly Antiangiogenic via PEDF and sFLT-1

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PURPOSE. To evaluate the angiogenic properties of corneal derived mesenchymal stromal cells (Co-MSC).

METHODS. Co-MSCs were extracted from human cadaver, and wild-type (C57BL/6J) and SERPINF1−/− mice corneas. The MSC secretome was collected in a serum-free medium. Human umbilical vein endothelial cell (HUVEC) tube formation and fibrin gel bead assay (FIBA) sprout formation were used to assess the angiogenic properties of Co-MSC secretome. Complete corneal epithelial debridement was used to induce corneal neovascularization in wild-type mice. Co-MSCs embedded in fibrin gel was applied over the debrided cornea to evaluate the angiogenic effects of Co-MSCs in vivo. Immunoprecipitation was used to remove soluble fms-like tyrosine kinase-1 (sFLT-1) and pigment epithelium-derived factor (PEDF, SERPINF1 gene) from the Co-MSC secretome.

RESULTS. Co-MSC secretome significantly inhibited HUVECs tube and sprout formation. Co-MSCs from different donors consistently contained high levels of antiangiogenic factors including sFLT-1 and PEDF; and low levels of the angiogenic factor VEGF-A. In vivo, application of Co-MSCs to mouse corneas after injury prevented the development of corneal neovascularization. Removing PEDF or sFLT-1 from the secretome significantly diminished the antiangiogenic effects of Co-MSCs. Co-MSCs isolated from SERPINF1−/− mice had significantly reduced antiangiogenic effects compared to SERPINF1+/+ (wild-type) Co-MSCs.

CONCLUSIONS. These results illustrate the direct antiangiogenic properties of Co-MSCs, the importance of sFLT-1 and PEDF, and their potential clinical application for preventing pathologic corneal neovascularization.

Keywords: cornea, mesenchymal stromal cells, sFLT-1, angiogenesis, PEDF

Mesenchymal stromal cells (MSCs) play an important role in tissue repair and maintenance. They provide an attractive candidate for cell-based therapies. Bone marrow MSCs, and to a lesser extent adipose and umbilical cord derived MSCs, have been investigated for a wide range of human diseases based on their anti-inflammatory, antifibrotic and other tissue repair properties. We hypothesized that corneal tissue MSCs may be particularly suitable for therapeutic applications in the cornea.

Absence of blood vessels in the cornea is integral to its transparency and its immune-privileged status. Corneal avascularity is largely maintained through the local production of antiangiogenic factors, including VEGF receptor 3; soluble fms-like tyrosine kinase-1 (sFLT-1; also known as soluble VEGF receptor 1); and pigment epithelium derived factor (PEDF). Corneal neovascularization is often the final consequence of severe corneal infections or inflammation, which alter the balance in favor of angiogenic factors. Importantly, neovascularization makes the cornea less amenable to treatments via cadaveric corneal transplantation given the loss of the corneal immune privilege.

In this current study, we test the hypothesis that cornea-derived MSCs have intrinsic antiangiogenic properties and further investigate the mechanism for their direct antiangiogenic effects.

METHODS

Mice

All experiments in mice were conducted in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The protocols were approved by the Animal Care & Use Committee of the University of Illinois at Chicago (UIC). SERPINF1−/− on C57BL/6J background mice were provided by Paul J. Grippo, PhD, (UIC). C57BL/6J mice were used as wild-type.

MSC Culture

Human and mice Co-MSCs were isolated and expanded as described before. Briefly, the corneoscleral button from
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Table. Antibodies Used for Flow Cytometry

<table>
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healthy cadaver eyes (provided by Eversight Eye Bank, Ann Arbor, MI, USA) or freshly enucleated mouse eyes were washed five times with PBS containing 2X Antibiotic-Antimycotic and 2X penicillin-streptomycin (both from Thermo Fisher Scientific, Waltham, MA, USA). After removing the central button with a trephine, the limbus was cut into three segments that were placed in 2.4 IU of protease (Dispase II; Thermo Fisher Scientific) for 1 hour at 37°C. Intact epithelial sheets were removed from stroma. The limbal segments were cut into small pieces and incubated in collagenase Type I (0.5 mg/mL) (Sigma-Aldrich Corp., St. Louis, MO, USA) in DMEM/F12 media (Thermo Fisher Scientific) overnight at 37°C or used directly for explant culture. The digests were filtered through a 70-μm nylon strainer to obtain a single-cell suspension. They were seeded onto 1% gelatin (Sigma-Aldrich Corp.)–coated wells of a 6-well tissue culture plate in alpha MEM media supplemented with 10% fetal bovine serum, 1X L-Glutamine, and 1X NEAA (all from Corning, Manassas, VA, USA). Culture media were changed every other day, and cells were subcultured by brief digestion with reagent (TrypLE Express; Thermo Fisher Scientific) when 80% confluent.

Cell Transplantation

Total limbal stem cell deficiency was induced by total corneal epithelial debridement as described before. A 6-0 nylon suture was prepassed through the lids (in preparation for closing the lids afterward). Subsequently, 0.2 μL thrombin (Ethicon, Somerville, NJ, USA) was placed over the debrided cornea. A total of 5000 cells, resuspended in 3 mL of thrombin (Ethicon), were transferred over the cornea. After the gel formed, the suture was tightened. Erythromycin ophthalmic ointment was applied. The suture was removed after 3 days.

MSC Secretome

Upon reaching 100% confluency in a T175 flask, the MSCs were washed with 30 mL of prewarmed PBS 3 times. The media was then changed to phenol red free alpha MEM media supplemented with 1X L-Glutamine, and 1X NEAA. The conditioned media was collected after 48 hours. The cells were trypsinized and counted at the same time. The conditioned media was centrifuged with 500 G speed for 15 minutes to remove any cells or debris. The supernatant was transferred to a new tube and was used for experiments or kept at 4°C for up to a week.

Tube Formation Assay

Human umbilical vein endothelial cells (HUVEC; Thermo Fisher Scientific) were cultivated in specialty media (Medium 200; Thermo Fisher Scientific) fortified with low serum growth supplement (LSGS). At passage 4, the cells were detached and resuspended in medium (Thermo Fisher Scientific) + 10% LSGS, which was used as the base media. The 96-well plate was filled with 75 μL gelatinous protein mixture (Matrigel; Thermo Fisher Scientific) and allowed to solidify at 37°C, after which HUVECs (2 × 10⁴ cells/75 μL) were gently seeded on top of the gel. Subsequently, 75 μL of secretome, complete media, or specialty media (Thermo Fisher Scientific) was added as the testing condition, positive, and negative controls, respectively. After 6 hours, network structures were analyzed and photographed at ×4 magnification (Leica Microsystems, Wetzlar, Germany). Total tube length per image was calculated using angiogenesis analyzer plugin of ImageJ (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health [NIH], Bethesda, MD, USA).

Fibrin Induced Bead Assay

Fibrin induced bead assay (FIBA) was performed as described previously. Briefly, HUVECs (passage 3 to 5) were stained with red dye (Cat. No. C34565, CellTracker Deep Red Dye Thermo Fisher). A total of 2500 presterilized microcarrier beads (Cytodex 3; Sigma-Aldrich Corp., Cat. No. C3275) were mixed with 1 × 10⁶ HUVECs in specialty medium (Thermo Fisher Scientific) + LSGS (complete media) and incubated overnight. A total of 250 precoated beads in 500 μL of 2 mg/mL fibrinogen (Cat. No. F8630; Sigma-Aldrich Corp.) + 0.15 units/mL aprotinin (Cat. No. A6106; Sigma-Aldrich Corp.) was seeded per well of a 24-well plate. The bead-fibrinogen solution was coagulated by adding 0.625 unit/mL thrombin (Cat. No. T7513; Sigma-Aldrich Corp.). Subsequently, 500 μL of specialty medium (Thermo Fisher Scientific) + 10% LSGS was added to the surface of the clotted fibrin gel dropwise, followed by 500 μL of secretome, complete media, or specialty medium (Thermo Fisher Scientific) as testing condition, positive, and negative controls, respectively. After 3 days, images were captured and analyzed using ImageJ software.

Immunostaining

Corneal whole mount immunostaining was done as described before. Briefly, after enucleation of the eye, the cornea was dissected using a spring scissors. It was then fixed with 4% paraformaldehyde at 4°C overnight. After washing with PBS, it was incubated with 20 μg/mL serine protease (Proteinase-K; Sigma-Aldrich Corp.) for 5 minutes at room temperature followed by 100% methanol for another 30 minutes. It was then incubated with 10% serum and 2% bovine serum albumin (BSA) at 4°C overnight. Purified anti-mouse CD31 (Cat. No. 102401; BioLegend) was used as primary antibody and Rhodamine-conjugated donkey anti-rat IgG (Cat. No. 712-02-153; Jackson ImmunoResearch, West Grove, PA, USA) as
FIGURE 1. Human and mouse corneal stromal cells demonstrate mesenchymal stromal cells (MSC) features. (A) Bright-field image of passage-4 human corneal MSCs. (B) Flow cytometry analysis demonstrated a homogenous MSC population. More than 95% of the cells were positive for cell surface markers CD73, CD90, CD105, and negative for CD19, CD45, HLA-DR, CD11b, and CD34 (n = 10). (C) Differentiation into the three mesenchymal lineages: I: Osteogenesis: calcium deposition stained with Alizarin Red; II: Adipogenesis: lipid formation stained with LipidTOX; III: Chondrogenesis: Glycosaminoglycans stained with Alcian Blue. (D) Bright-field microscopy image of passage-4 mouse corneal MSCs. (E) Flow cytometry analysis demonstrated a homogenous MSC population. More than 95% of the cells are positive for cell surface markers CD29, Sca-1, CD105, CD44 and CD106 and negative for CD11b and CD45. (F) Differentiation into the three mesenchymal lineages: I: Calcium deposition stained with Alizarin Red; II: Lipid formation stained with LipidTOX; III: Glycosaminoglycans stained with Alcian Blue.

FIGURE 2. Corneal MSCs (Co-MSCs) are directly antiangiogenic in vitro. (A) Co-MSC secretome significantly inhibited mean sprout count per bead and mean sprout length in a fibrin-induced bead assay (n = 5). *P < 0.005. Scale bars: 200 µm. (B) Co-MSC secretome (6.8 ± 1.0 mm/field) significantly reduced tubule formation in a HUVEC assay (n = 5). *P < 0.005. The values shown are mean ± SD (error bars). S, secretome.
secondary antibody. The slides were visualized and photographed using a commercial microscope (Zeiss LSM 710; Carl Zeiss Meditec, Jena, Germany). To make the quantification more objective, we used VesselJ to quantify the ratio of vascularized area to total corneal area.

Human Proteome Array Analysis
The expression levels of proteins known for their roles in angiogenesis were analyzed using an antibody array (Proteome Profiler Human Angiogenesis Array Kit; R&D System, Minneapolis, MN, USA) according to the manufacturer’s instruction.

Enzyme-Linked Immunosorbent Assay
Human PEDF (Cat. No. DY1177-05); sFLT-1 (Cat. No. DVR100B); and VEGF-A (Cat. No. DVE00) proteins were detected with commercially available ELISA kits (all from R&D Systems) according to the manufacturer’s protocol. The obtained values were normalized to total cell numbers.

Immunoprecipitation (IP)
Co-MSC secretome was incubated with 25 μg/mL anti-PEDF (Cat. No. AF1177); anti-sFlt1 (Cat. No. AF321); or normal Goat IgG (Cat. No. AB 108-C) antibody (all from R&D) at 4°C overnight. After washing of the beads with IP buffer (Cat. No. I-5779; Sigma-Aldrich Corp.), samples were incubated with 30 μl of protein G beads (Sigma-Aldrich Corp. Cat. No. P-3296) at 4°C for 2 hours. The samples were centrifuged at 12000 g for 30 seconds. The supernatant was checked for the efficiency of IP. It was then used for the experiments.

siRNA Transfection
After reaching 70% to 80% of confluency, Co-MSCs were transfected with 50 nM SERPINF1 siRNA (Cat. No. sc-40947; Santa Cruz Biotechnology, Dallas, TX, USA) or scrambled siRNA (Cat. No. D-001205; Dharmacon, Lafayette, CO, USA) in TransFECTKO siRNA transfection reagent (Cat. No. MIR2150; Mirus Bio LLC, Madison, WI, USA). The transfection efficiency was tested with ELISA on the secretome.

Flow Cytometry
The cells were detached as described above. They were incubated with Fc block (Cat. No. 564220 for human; BD Pharmingen, San Jose, CA, USA, and TruStain fcX [anti-mouse CD16/32], Cat. No. 101319 for mouse; BioLegend) and stained at 4°C for 20 minutes in antibody diluent (PBS with 2% FBS) with cell surface antibodies (Table). For intracellular staining, fixation buffer (Cat. No. 420801; BioLegend) and intracellular
staining perm wash buffer (Cat. No. 421002; BioLegend) were used according to manufacturer’s protocol. Flow cytometry data was acquired on the BD LSR Fortessa (BD Pharmingen). Data were analyzed using analytical software (FlowJo; FlowJo LLC, Ashland, OR, USA).

Western Blot Analysis
Cells cultured on 100-mm dishes were rinsed twice with PBS and harvested in SDS RIPA buffer (Sigma-Aldrich Corp.) supplemented with protease/phosphatase inhibitors (Sigma-Aldrich Corp.). After protein concentration measurement, equal amounts of each sample were mixed with 2X Laemmli buffer (Bio-Rad Laboratories, Hercules, CA, USA) and 5% beta-mercaptoethanol (Sigma-Aldrich Corp.), denatured by heating at 95°C for 10 minutes, and subjected to electrophoresis on 4% to 20% Tris-Glycine gels (Invitrogen, Grand Island, NY, USA). The protein bands were transferred to polyvinylidene difluoride membranes. The membranes were incubated in 5% BSA in tris-buffered saline (TBS) for 1 hour followed by an overnight incubation (4°C) with primary antibodies at the optimal concentration. The membranes were washed with TBS with 0.03% Tween 20 and incubated with the horseradish peroxidase–conjugated secondary antibody for 1 hour at room temperature. Detection was performed with commercial detection system (ECL Plus Western Blotting Detection System; Amersham, Buckinghamshire, UK). Mouse Serpin F1/PEDF antibody (AF1149) was purchased from R&D Systems.

Data Analysis and Statistical Comparisons
To obviate observer bias and to increase reproducibility, all the animal surgeries were done by one of the authors who was blinded to the treatment arms. Data collection and analyses were done in a masked fashion to minimize inter- and intraobserver bias. Eyes with infection were excluded from the study. Corneal infection was defined as corneal edema, hypopyon, and exudate within the first 7 days after the procedure. Results are presented as the mean ± SD of three independent experiments. Normality of the data was tested using D’Agostino & Pearson normality test. Based on normality in independent experiments. Normality of the data was tested with 2-sided student’s t-test or 2-sided student’s t-test was performed to determine significance, which was set at P < 0.05. For more than two arms comparison, 1-way ANOVA with Tukey’s post hoc correction was used. All statistics were performed using statistical and spreadsheet software (GraphPad Prism 7.0; GraphPad Software, Inc., La Jolla, CA, USA, and Excel; Microsoft Corp., Redmond, WA, USA).

RESULTS

Co-MSC Secretome Is Antiangiogenic
Co-MSCs were successfully isolated from human and mouse corneas and characterized to meet the minimal International Society of Cell Therapy criteria for defining MSCs (Fig. 1). Previous studies have demonstrated that the therapeutic effects of MSCs are largely mediated through their secreted factors.

We proceeded to test Co-MSC secretome using in vitro assays of angiogenesis. The results indicated that Co-MSC secretome significantly inhibits vascular sprouting and endothelial tube formation compared to control media (Figs. 2A, 2B).

Co-MSCs Have an Antiangiogenic Profile by Protein Expression
Human angiogenesis array was used to profile pro- and antiangiogenesis mediators in the Co-MSC secretome. It revealed it has high levels of antiangiogenic factors, whereas low levels of proangiogenic factors (Fig. 3A). Based on the results of proteome array and previous studies, we focused specifically on sFLT-1 and PEDF (product of gene serine proteases inhibitor F1; SERPINF1), two important soluble mediators of corneal angiogenic privilege.21 Co-MSCs from multiple donors consistently expressed and secreted high levels of sFLT-1 and PEDF, whereas Co-MSCs secretome from multiple donors contained low levels of VEGF-A. Co-MSCs also secreted high levels of TNF-stimulated gene 6 (TSG 6), a well-known anti-inflammatory mediator of MSCs (Fig. 3B).

Co-MSCs Have Antiangiogenic Effects In Vivo
We tested Co-MSCs in an in vivo model of pathologic corneal neovascularization. In this model, the entire corneal epithelium is mechanically debrided that results in severe corneal neovascularization. MSCs were embedded in a fibrin gel and applied to the cornea immediately after the injury (Fig. 4A). In vivo microscopy confirmed the presence of fluorescent-labeled MSCs in the cornea 3 days after the procedure (Fig. 4B). The results demonstrated that Co-MSCs could effectively prevent corneal neovascularization (Fig. 4C).
additive; removing both factors resulted in a more significant reduction in the antiangiogenic effects of Co-MSC secretome (Fig. 5A). The efficiency of immunoprecipitation for PEDF and sFLT1 was more than 95% as determined by ELISA (Fig. 5B).

Knocking down PEDF in Co-MSCs, using siRNA, likewise decreased the antiangiogenic activity of its secretome in vitro (Fig. 6). To examine the effect of PEDF in vivo, we isolated Co-MMSCs from SERPINF1−/− mice and compared them to wild-
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**FIGURE 7.** PEDF plays a key role on the antiangiogenic properties of Co-MSCs in vivo. (A) Western blot shows Co-MSCs isolated from SERPINF1<sup>+/+</sup> mice do not express PEDF (B) Co-MSCs isolated from SERPINF1<sup>/−</sup> mice had lower antiangiogenic effects, compared to wild-type (SERPINF1<sup>+/+</sup>) Co-MSCs, when placed in fibrin gels after corneal epithelial injury in wild-type mice (same model as in Fig. 4A; n = 5, 2-sided t-test: *P = 0.0001). Scale bars: 500 µm. The values shown are mean ± SD (error bars). Co-mMSC<sup>SF1+/+</sup>, corneal mouse MSCSERPINF1; NV, neovascularization.

Discussion

It is well accepted that the therapeutic effects of MSCs in many disease processes are mediated largely through their immunomodulatory and anti-inflammatory properties. They have been shown to modulate both innate and adaptive immune mechanisms. Local tissue derived MSCs, which share many features with other MSCs, can have additional special attributes that may be potentially useful for certain clinical applications. For instance, Co-MSCs were shown to have antibirotic effects and prevent the development of corneal scarring after injury.

While majority of studies have shown BM and adipose derived MSCs are proangiogenic; there has been reports that BM-MSCs can block corneal neovascularization. In particular, MSCs from different sources have been shown to secrete several antiangiogenic factors, including thrombospondin 1; tissue metalloproteinase inhibitor 1; pentraxin-3 (TSG-14); and PEDF. In general, the mechanisms that have been proposed for the antiangiogenic effects of MSCs are indirectly based on their anti-inflammatory properties rather than direct antiangiogenic effects. The current study provides the first direct evidence that Co-MSCs have a more distinctive feature. Given the feasibility of generating clinical grade Co-MSCs from human cadaveric corneas, they provide an attractive cell source for developing regenerative
FIGURE 8. Schematic illustration summarizes the mechanisms by which Co-MSCs confer their antiangiogenic properties. (A) After corneal epithelial injury, DAMPs, chemokines, and cytokines released by the injured and resident cells directly induce angiogenesis while also recruiting inflammatory cells that further promote angiogenesis. The net effect is shifting the antiangiogenic balance of the cornea toward angiogenesis. (B) Co-MSCs applied to the cornea after injury modulate neovascularization directly through the secretion of antiangiogenic factors including PEDF and sFLT-1 and indirectly by suppressing inflammation via TSG6 and other anti-inflammatory factors. DAMP, damage-associated molecular pattern.
antiangiogenic and immunomodulatory therapies for the cornea and potentially other tissues.

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References


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