Suppression of Corneal Neovascularization by PEDF Release from Human Amniotic Membranes

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PURPOSE. Human amniotic membrane (HAM) transplantation is commonly used in corneal surface reconstruction and is known to inhibit neovascularization of this tissue. The purpose of the present study is to reveal the molecular basis underlying antiangiogenic activity of HAM.

METHODS. The effects of HAM protein on proliferation of vascular endothelial cells and corneal epithelial cells were determined by quantifying viable cells using the MTT assay. The presence of pigment epithelium-derived factor (PEDF) in HAM was demonstrated at the protein level by Western blot analysis and immunohistochemistry using a monoclonal antibody specific to human PEDF. The PEDF concentration was measured by a specific ELISA. The expression of PEDF in HAM was confirmed at the RNA level by RT-PCR and DNA sequencing.

RESULTS. Soluble proteins from HAM inhibited proliferation of human umbilical vein endothelial cells and bovine retinal capillary endothelial cells (BRCECs) while promoting proliferation of bovine cornea epithelial cells. Moreover, the HAM-induced inhibition of BRCECs was neutralized by a specific anti-PEDF antibody. PEDF protein was identified with an abundance of 103.84 ± 33.21 ng/mg of soluble proteins, which is comparable to that in the retina, a PEDF-rich tissue. PEDF expression was predominantly localized in the basement membrane of HAM. RT-PCR using specific PEDF primers amplified a single product from HAM RNA. The PCR product has a sequence identical with that of human PEDF.

CONCLUSION. HAM specifically inhibits endothelial cell growth and thus suppresses neovascularization in the cornea. PEDF in HAM has a major role in eliciting this antiangiogenic activity. (Invest Ophthalmol Vis Sci. 2004;45:1758–1762) DOI: 10.1167/iovs.03-0882

Human amniotic membrane (HAM) is the innermost layer of the placenta and consists of a basement membrane and an avascular stromal matrix. The use of HAM as a graft in ocular surface reconstruction has become widespread because of its availability and convenience.1–3 This procedure has a high rate of success, and the results are highly reproducible.1–5 Currently, HAM is used increasingly to improve the outcome of surgeries treating various conjunctival6–10 and corneal11–13 diseases. HAM is applied as a patch in cases of chemical and thermal burns9,10 and of refractory and recalcitrant keratitis.11 It also provides an excellent substrate for expanding epithelial stem cells ex vivo.12,13 Clinical observation has shown that HAM transplantation decreases vascularization on the ocular surface.14 The antiangiogenic effect of HAM is one of the foremost reasons for its therapeutic application.14 However, the molecular basis underlying the antiangiogenic activity of HAM has not been revealed.

Angiogenesis in the eye is tightly controlled by two counterbalancing systems: angiogenic stimulators, such as vascular endothelial growth factor (VEGF), which promote the proliferation of endothelial cells,15 and angiogenic inhibitors, such as pigment epithelium-derived factor (PEDF), which instead inhibit proliferation of endothelial cells.16 In certain conditions such as pterygia and inflammation, the cornea and/or conjunctiva increase the production of angiogenic stimulators whereas levels of its angiogenic inhibitors decrease.17 As a result, the capillary endothelial cells overproliferate and form new blood vessels that infiltrate the cornea. The abnormal growth of new blood vessels in the eye is a major cause of blindness in people of all ages.

Endogenous angiogenic inhibitors are essential for keeping the cornea and vitreous avascular and inhibiting the neovascularization of ocular tissues.18–20 Several angiogenic inhibitors, such as PEDF and angiostatin, have been isolated from the eye.18–20 PEDF belongs to the serine protease inhibitor family and is present at high levels in ocular tissues, including the cornea, vitreous, and retinal pigment epithelium.18 PEDF was originally identified as a neurotrophic factor.22,23 Later, it was found to have potent antiangiogenic activity.24 It has been shown that PEDF is essential for maintaining the avascularity of the cornea and vitreous.25 Decreased PEDF levels have been shown to play a role in retinal neovascularization and formation of pterygia.16,17

The present study tests the hypothesis that HAM-induced inhibition of corneal neovascularization in the cornea surface reconstruction may be ascribed to the release of PEDF.

MATERIALS AND METHODS

Human tissue used in these experiments was obtained and managed in accordance with the provisions of the Declaration of Helsinki.

Cell Culture

Human retina capillary pericytes were isolated from donor eyes obtained through the South Carolina Lion’s Eye Bank Association, and bovine retina capillary endothelial cells (BRCECs) were isolated from bovine eyes according to a protocol described previously.26 Purity of the human pericytes was determined by immunostaining using an FITC-conjugated antibody specific to α-smooth muscle actin (Sigma-Aldrich, St. Louis, MO), as described previously.26 The identity of
BRCECs was confirmed by its characteristic cobblestone morphology and the incorporation of acetylated low-density lipoprotein labeled with a fluorescent probe, Dil (1,1′-diododecyl-3,3′,3′-tetramethyldiocarbocyanine iodide; Molecular Probes, Eugene, OR). Human umbilical vein endothelial cells (HUVECs) were purchased from Clonetics (San Diego, CA) and cultured in Molecular Cellular Developmental Biology 151 (MCDB151; Invitrogen-Gibco, Grand Island, NY) containing 10% fetal bovine serum (FBS), 200 μM 1-glutamine (Invitrogen-Gibco), 1% antibiotic-antimycotic (Invitrogen-Gibco), 1.4% heparin, and 1% endothelial cell growth supplement (EGS; Sigma-Aldrich). Primary bovine cornea epithelial cells (BCECs) were isolated from bovine cornea and cultured in DMEM/F12 (1:1) containing 10% FBS, 5 ng/mL epidermal growth factor (EGF; Sigma-Aldrich), 5 μg/mL insulin (Sigma-Aldrich), and 40 μg/mL gentamicin, as described previously. 25

HAM Protein Preparation

HAM was collected and immediately frozen in liquid nitrogen until it was used. HAM tissues were homogenized manually and then, sonicated four times on ice, each time for 15 seconds. The tissue homogenates were centrifuged twice for 30 minutes each at 15,000 rpm at 4°C. The soluble proteins in the supernatant were filtered through a 0.22-μm filter for sterilization. The protein concentration was measured using a protein assay (Bio-Rad, Hercules, CA).

MTT Cell Proliferation Assay

Cells were seeded in 12-well plates in triplicate and cultured in the growth medium until they reached 60% to 70% confluence. The culture medium was replaced with a medium containing 1% FBS. The HAM proteins were added to the culture medium at various concentrations and incubated with the cells for 72 hours. The viable cells were quantified by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide; Roche, Mannheim, Germany) assay according to a protocol recommended by the manufacturer. The effect of HAM protein on the number of viable cells was analyzed with Student’s t-test.

Western Blot Analysis

The same amounts (100 μg) of soluble proteins from human cornea, conjunctiva, and HAM were used for Western blot analysis performed with a monoclonal antibody to human PEDF (Chemicon, Inc., Temecula, CA). The signal was visualized with a chemiluminescence detection kit (ECL; Amersham International, Piscataway, NJ), as described previously. 26 The same blot was stripped and reblotted with an antibody specific to β-actin to normalize the PEDF level.

PEDF ELISA

Concentrations of PEDF in HAM, retina homogenates, serum, and vitreous were determined by using a commercial PEDF ELISA kit (Chemicon, Inc.), according to the manufacturer’s instructions. Briefly, HAM protein was prepared as described earlier. Human retina and HAM were homogenized and sonicated on ice for 2 × 3 minutes. The homogenates were centrifuged at 15,000 rpm for 10 minutes. The supernatants, serum, and vitreous were treated with 8 M urea on ice for 1 hour. The urea-treated samples were diluted 1:100 in assay diluent, added to the antibody-coated wells immediately, and incubated at 37°C for 1 hour. After four washes, 100 μL of the diluted biotinylated mouse anti-human PEDF monoclonal antibody was added to each well and incubated in the wells at 37°C for 1 hour. Then, 100 μL of diluted streptavidin-peroxidase conjugate was added and incubated at 37°C for 1 hour. After the addition of TMB/E for 5 to 10 minutes, 100 μL of stop solution was added and the absorbance was measured immediately at 450 nm in a microplate reader. For standardization, the PEDF concentration was normalized by the protein concentration in the samples.

Immunohistochemistry

The avidin-biotin complex (ABC) method (ABC kit; Vector Laboratories, Inc., Burlingame, CA) was used for immunostaining of the frozen sections of HAM. Briefly, after they were air dried, the sections were blocked with nonspecific goat serum for 30 minutes at room temperature. Excess blocking solution was removed, and the monoclonal antibody specific to PEDF was added to the slides and incubated overnight at 4°C. The slides were rinsed three times in PBS for 5 minutes each. The goat anti-mouse secondary antibody was added and incubated with sections for 60 minutes at 37°C. The slides were then rinsed three times in PBS. ABC was incubated with the slides for 30 minutes at room temperature, followed by three 5-minute rinse cycles in PBS. 3,3′-Diaminobenzidine (DAB; Vector Laboratories, Inc.) was added as the substrate and allowed to develop for 5 to 10 minutes. The slides were then rinsed in deionized water, counterstained with hematoxylin for 3 minutes, and mounted (Permount; Fisher Scientific, Pittsburgh, PA).

RT-PCR and DNA Sequence Analysis

Total RNA was isolated from HAM (Trizol reagent; Invitrogen Life Technologies, Carlsbad, CA), according to the protocol recommended by the manufacturer. RT-PCR was performed as described previously. 27 Total RNA was reverse transcribed using the Superscript II kit (Gibco BRL, Gaithersburg, MD). Specifi c PCR products were examined by electrophoresis in 1% agarose gel and isolated for DNA sequencing using an automated DNA sequencer. The sequence of the PCR product was compared with the PEDF cDNA using the GCG program.

RESULTS

Effects of HAM Proteins on Endothelial Cell and Cornea Epithelial Cell Proliferation

BCECs, human retina capillary pericytes, HUVECs, and BRCECs were treated with soluble HAM proteins at concentrations of 12.5, 25, 50, 100, and 200 μg/mL for 72 hours. The viable cells were quantified using the MTT assay. Treatment with HAM proteins caused a concentration-dependent inhibition in both types of endothelial cells analyzed: HUVECs and BRCECs (Fig. 1). In BRCECs, HAM proteins at all concentrations significantly decreased viable cells, compared with the control (P < 0.05, n = 3). HUVECs were relatively less sensitive to HAM proteins, compared with BRCECs, as significant decreases of cell viability in HUVECs were observed from 25 to 200 μg/mL but not at 12.5 μg/mL of HAM proteins (P < 0.05, n = 3). In the same concentration range, HAM proteins did not affect cell viability in primary pericytes (Fig. 1). In contrast, HAM protein treatment resulted in a concentration-dependent increase in the number of viable cells in BCECs. At concentrations of 50, 100, and 200 μg/mL, HAM proteins significantly increased viable BCECs compared with the control (P < 0.05; Fig. 1).

Partial Neutralization of the Antiangiogenic Activity of HAM Proteins by Anti-PEDF Antibody

To determine whether the inhibition of endothelial cells by HAM proteins can be ascribed to PEDF, HAM proteins were applied to BRCECs together with a purified anti-PEDF IgG. 14 In the presence of nonspecific rabbit IgG and the absence of the specific anti-PEDF antibody, 50 μg/mL HAM protein significantly decreased the number of viable BRCECs to 77% of the control (P < 0.01, n = 3; Fig. 2). When the anti-PEDF antibody was added to concentrations of 2, 20, or 200 ng/mL, together
with 50 μg/mL HAM proteins, the antibody reversed the HAM-protein-induced inhibition of BRCECs as a function of the antibody concentration. At concentrations of 20 and 200 ng/mL, the antibody increased viable cells from 77% to 89% \( (P < 0.05, n = 3) \) and 93% \( (P < 0.01, n = 3) \) of the control, respectively, suggesting that the antiangiogenic activity of HAM proteins can be partially neutralized by the anti-PEDF antibody (Fig. 2).

**Detection of PEDF Protein in HAM**

To detect PEDF at the protein level, six independent HAM samples were blotted with the monoclonal anti-PEDF antibody.

**FIGURE 3.** PEDF protein expression in the cornea, conjunctiva, and HAM. The same amount (100 μg) of soluble proteins from normal human cornea (lane 1) and conjunctiva (lane 2) and six independent HAMs (lanes 3–8) was analyzed by Western blot analysis using a monoclonal antibody against PEDF (top). The filter was stripped and reblotted with an anti-β-actin antibody (bottom).

Normal human cornea and conjunctiva were used as controls in the same Western blot analysis. The anti-PEDF antibody recognized a single band of approximately 50 kDa, identical with the molecular weight of human PEDF, in all six HAM samples analyzed. The antibody recognized an intense band in the cornea and a weaker one in the conjunctiva with the same molecular weight as that of HAM (Fig. 5).

**Quantification of PEDF in HAM**

PEDF concentrations in HAM, retina homogenates, serum, and vitreous were measured with an ELISA specific for PEDF and normalized by total protein concentration to obtain the level of PEDF in these tissues. The results showed that the PEDF level in HAM was 103.84 ± 33.21 ng/mg of soluble proteins \( \text{(mean} \pm \text{SD, } n = 6) \), which is comparable to that in human retina \( (113.64 \pm 16.44, n = 6) \), a known PEDF-rich tissue. The PEDF levels in human serum and vitreous were 57.09 ± 30.92 ng/mg \( (n = 6) \) and 427.32 ± 38.07 ng/mg, respectively.

**Cellular Localization of PEDF in HAM**

To determine cellular localization of PEDF in HAM, we stained sections of HAM with the monoclonal anti-PEDF antibody by immunohistochemistry. An intense PEDF signal was detected in the basement membrane and a relatively weaker signal in the stromal matrix (loose connective tissue) of HAM (Fig. 4). No signal was detected in the negative control in which the anti-PEDF antibody or the secondary antibody was omitted, demonstrating specificity of the labeling.

**PEDF mRNA Expression in HAM**

To determine whether PEDF is expressed in HAM, we probed for PEDF mRNA expression using RT-PCR for specific amplification of human PEDF cDNA. RT-PCR yielded a single 310-bp product in all six different HAM RNA samples (Fig. 5). The size of the product matched the anticipated length of the human PEDF mRNA. As the two PCR primers span two introns of 0.7 and 3 kb of the human PEDF gene, this product cannot be due to genomic DNA contamination in the RNA samples. To further confirm the identity of the PCR product, we isolated the 310-bp bands from the gel and determined their nucleotide sequences. The sequence showed 100% identity to the human PEDF cDNA registered in GenBank (Accession number AF400442).

**DISCUSSION**

Neovascularization in the cornea and conjunctiva induced by wound and other pathologic conditions often causes opacifi-
cation, scarring, and a loss of visual acuity. It has been reported that HAM transplantation is beneficial for reconstruction of the ocular surface by inducing epithelialization20,25 and reducing inflammation, vascularization, and scarring.1,14,30,31 The present study demonstrates that PEDF is expressed in HAM and is a major contributor to the antiangiogenic activity in HAM.

Proliferation of endothelial cells is an essential step in neo-vascularization.32 Although HAM transplantation has been shown to prevent corneal vascularization in patients, the direct effect of HAM proteins on proliferation of vascular endothelial cell has not been studied. Our results demonstrate that HAM proteins dose dependently inhibited primary endothelial cell growth, but they had no effect on pericytes, indicating that HAM-induced inhibition of cell growth is specific to endothelial cells. This specific inhibition of endothelial cells is consistent with the vascular activities of peptide angiogenic inhibitors such as PEDF, which has been shown to inhibit cell proliferation and induce apoptosis specifically in endothelial cells.16,18,20

The specialized anatomy of the eye is such that highly vascularized and avascular tissues exist side by side. PEDF has been shown to be a major contributor to the maintenance of the avascularity of some ocular tissues, such as vitreous and cornea.16,18,20 In the present study, PEDF was expressed in HAM at both the protein and mRNA levels. The abundance of PEDF protein in HAM was comparable to that in the retina higher than that in the serum. As retina is known to contain high levels of PEDF, our results suggest that HAM is also a PEDF-rich tissue. Moreover, inhibition of endothelial cells growth by HAM proteins can be partially neutralized by an anti-PEDF antibody. These results suggest that PEDF is an important factor contributing to the antiangiogenic activity of HAM. As PEDF has also been shown to reduce vascular leakage (Salti HI, et al. JOVS 2002;45-ARVO E-Abstract 1415), high levels of PEDF in HAM may also contribute to the anti-inflammatory activity of HAM transplantation in reconstruction of the cornea. However, high concentrations of anti-PEDF antibody cannot completely block the antiangiogenic activity of HAM, suggesting that there may be other, yet to be identified angiogenic inhibitors in HAM.

Recently, HAM has been widely used as an important support layer in ocular surface reconstruction. Many researchers have attempted to grow human limbal progenitor cells,33 limbal epithelium,34 conjunctival epithelial progenitor cells,35 and oral mucosal epithelial cells13 on HAM and to accelerate the repair of corneal and conjunctival epithelial damage using HAM transplantation. Our results demonstrate that HAM proteins inhibit vascular endothelial cell proliferation while promoting cornea epithelial cell growth. The latter activity may explain why HAM transplantation promotes epithelialization and wound healing in the cornea. It is not clear which growth factor in HAM is responsible for the effect promoting corneal epithelial cell growth. Nevertheless, promoting proliferation of cornea epithelial cells and inhibiting that of vascular endothelial cells are desired effects in the treatment of corneal diseases, as they enhance cornea epithelial wound healing while preventing vascularization in the cornea. In fact, these two different functions of HAM transplantation may be synergistic in the treatment of corneal injury (i.e., inhibition of neovascularization can lead to increased acceptance of the corneal grafts), and the enhanced repair of cornea epithelium can also further inhibit vascularization, because the cornea epithelium expresses high levels of PEDF.38

![Figure 4](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932926/ on 04/01/2017)

**FIGURE 4.** Cellular localization of PEDF in HAM. Sections of HAM were stained with the anti-PEDF antibody, using the ABC method (PEDF signal brown). (A) Negative control in which the primary antibody was omitted; (B) section stained with the anti-PEDF antibody showing PEDF staining; (C) intense PEDF staining in the basement membrane of HAM; and (D) relatively weaker PEDF signal in the stroma of HAM. Magnification: (A, B) ×100; (C) ×200; (D) ×400.

**FIGURE 5.** Detection of PEDF mRNA in HAM. The same amount of total RNA obtained from six different HAMs was used as the template for RT-PCR using a pair of primers specific for human PEDF. RT-PCR amplified a 310-bp product with a sequence identical with the human PEDF cDNA. Lane 1: molecular weight marker; lanes 2–7: HAM from six different subjects.

References