In Vitro Antiangiogenic Activity in Ex Vivo Expanded Human Limbocorneal Epithelial Cells Cultivated on Human Amniotic Membrane

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Purpose. To compare the in vitro antiangiogenic activities of ex vivo expanded human limbocorneal epithelial (HLE) cells cultivated on preserved human amniotic membrane (AM) and to identify factors responsible for the activities.

Methods. The antiangiogenic effects were compared of culture media conditioned by AM, HLE cells, or HLE cells cultivated on intact AM (HLE/IAM), on denuded AM (HLE/DAM), or on DAM cocultured with 3T3 fibroblasts (HLE/DAM/3T3). A monolayer culture of human umbilical vein endothelial cells (ECs) was used in a proliferation and migration assay. ECs suspended in type I collagen gel were used to assess capillary tube formation. Quantitative analyses of tissue inhibitor of metalloproteinase (TIMP)-1, thrombospondin (TSP)-1, pigment epithelium-derived factor (PEDF), and endostatin (proteolytic fragment of collagen XVIII) were performed by ELISA. Immunoconfocal microscopy was performed to localize the site of endostatin expression in HLE cells and AM.

Results. HLE cell– but not AM-conditioned medium (CM) inhibited the proliferation and migration of ECs, and coculture of HLE cells, but not of AM, with ECs inhibited capillary tube formation. Although some data from HLE cells alone are not significantly different from the control, increased inhibitory activity was expressed by HLE/IAM and HLE/DAM and was most significantly expressed by HLE/DAM/3T3. Quantitation of TIMP-1, TSP-1, PEDF, and endostatin revealed that only the level of endostatin showed an increased expression by HLE cells cultivated on AM. Neutralizing antibody to endostatin substantially abrogated the inhibitory effect on EC proliferation and migration, but was less effective on EC differentiation. Endostatin signal was more prominent in the basement membrane zone of HLE cells cultivated on denuded AM than in those cultivated on intact AM.

Conclusions. The antiangiogenic effect of HLE cells was enhanced when they were cultivated on AM and cocultured with 3T3 fibroblasts, and endostatin-related antiangiogenic factor may play a major role. This highlights the significance of cell–matrix and cell–cell interaction in the regulation of antiangiogenic factor secretion by HLE cells. (*Invest Ophthalmol Vis Sci.* 2004;45:2586–2595) DOI:10.1167/iovs.03-1338

The limbus is enriched with the stem cells of corneal epithelium.[1] Insults such as severe chemical burn may deplete the corneal epithelial cells, leading to the invasion of the conjunctival epithelium, which is often associated with persistent inflammation, neovascularization, and decreased vision.[2] Auto-[3] and allograft limbal transplantations[4] are effective in restoring the corneal epithelium and inhibiting inflammation and neovascularization. Preserved human amniotic membrane (AM) is now widely used as a substrate for ocular surface reconstruction.[5,6] The combination of limbal and AM transplantation has been shown to improve the surgical outcome in patients with total limbal deficiency.[7,8] Most recently, transplantation of human limbocorneal epithelial (HLE) cells cultivated on AM has been shown to reduce corneal inflammation and neovascularization effectively, and to preserve the progenitor cell population selectively.[9–14] To date, HLE cells have been cultivated on intact AM,[9,14,15] denuded AM,[11–13] or denuded AM with 3T3 feeder cells.[10] There have been reports showing that intact but not denuded AM preferentially preserves the progenitor cells.[14,16–20] We were therefore interested to see whether the antiangiogenic activity of the HLE cells could also be modulated by AM.

The anti-inflammatory activities of AM have been widely studied[21–25]; however, little is known about whether AM possesses direct antiangiogenic activity. There has been evidence to suggest that that AM modulates the repertoire of proteins produced by HLE cells, such as tissue inhibitor of metalloproteinase (TIMP)-1, an endogenous antiangiogenic factor.[26] This led us to postulate that AM may modulate the antiangiogenic nature of the HLE cells.

We have reported that limbal but not conjunctival epithelial cells inhibit the tube formation of vascular endothelial cells (ECs), indicating the possession of antiangiogenic activity by HLE cells.[27] Nevertheless, the underlying mechanism(s) remains to be elucidated. Because the in vivo antiangiogenic effect of AM transplantation may be secondary to the anti-inflammatory activities, as mentioned, to study exclusively the antiangiogenic activity of HLE cells, we examined the effect of differentially cultured HLE cells on the major components of the angiogenic process: the proliferation, migration, and differentiation of ECs.[28,29] Potentially involved antiangiogenic factors were examined.

Methods

Preparation of Human AM

Human corneas were handled according to the tenets of the Declaration of Helsinki, and the research was approved by the Committee of

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Medical Ethics and Human Experiment at the Chang Gung Memorial Hospital. AM was obtained with informed consent after cesarean section and was preserved and prepared according to published methods.[8] Different preparations of AM were used in the study. Live AM denotes AM with living epithelial cells, which was cleaned and cultured in a 37°C incubator immediately after retrieval from the delivery room. Cryopreserved, frozen AM denotes that the AM was kept at −70°C, and for the experiments, the epithelium was kept intact (FIAM) or denuded (FDAM), as previously described.[10,30] Cryopreserved, old AM implies that the AM was left intact or denuded (OIAM and ODAM, respectively) immediately after thawing, and was kept in a 37°C incubator for at least 3 weeks before use in experiments. The purpose of this control group was to evaluate whether the AM component of the HLE/AM composite graft would have any effect on the ECs.

The viability of AM cells was evaluated by trypan blue staining (0.25%), which was performed on the flatmounted AM preparations.

Primary Cultures of HLE Cells on AM

HLE cells were grown from explant culture of 2 × 2-mm pieces of corneoscleral rims on cell culture inserts (Costar; Corning Inc., Corning, NY), which were either precoated with type I collagen solution only or overlaid with intact or denuded AM. The cultures were maintained with supplemental hormonal epithelial medium (SHEM) with 5% fetal bovine serum (FBS). When the epithelial sheet was approximately 50% confluent, half of the inserts with HLE cells on denuded AM were transferred to a new six-well plate, with confluent culture of a mitomycin-C–treated 3T3 fibroblast feeder layer.[10,30] These explants remained submerged without air-lifting for at least another week. Cultures reaching 80% to 90% confluence were used for the experiments.

Coculture HLE Cells and Vascular ECs Isolated from Human Umbilical Vein

ECs were isolated as previously described.[27] The cells were grown with endothelial growth medium (EGM; Clonetics, San Diego, CA) containing 2% FBS and bovine brain extract, and were used for experiments below the fifth passage. The outer well contained 5×10^5 ECs in 1 mL type I collagen gel in EGM. The culture insert contained nothing; HLE cells alone; or HLE cells on intact AM (HLE/IAM), denuded AM (HLE/DAM), or denuded AM cocultured with 3T3 fibroblasts (HLE/DAM/3T3). Cultures were maintained with 1.5 mL SHEM-5% FBS in the insert, and the same volume of EGM with 5% FBS in the outer well. Basic fibroblast growth factor (bFGF 10 ng/mL; R&D Systems, Minneapolis, MN) was added as the source of angiogenic factor. As additional controls, live AM, FIAM, FDAM, OIAM, and ODAM were placed in inserts and cocultured with ECs similarly. Cultures were maintained for 3 days and then fixed with 4% formaldehyde in PBS. EC tube formation was quantified by image analysis (NIH/Scion Image; Scion, Frederick, MD) from five random fields at ×100. Each experiment was performed with at least five replicates. The HLE cells on AM were fixed and embedded in paraffin and processed for hematoxylin-eosin (HE) staining.

Preparation of Conditioned Medium from HLE Cells Cultivated on AM

Eighty percent to 90% confluent cultures of HLE cells on the inserts were washed twice with PBS and transferred to a new six-well plate, and 1.5 mL serum- and growth factor–free medium was added to the upper and lower chambers. The cultures were maintained for 48 hours, and the medium was collected and stored at −70 °C. Medium conditioned by AM of various preparations was processed similarly. To assay for effect on EC proliferation and migration, the conditioned medium (CM) was thawed and concentrated 10-fold with a centrifugal filter (Centricon; Millipore, Bedford, MA).

Inhibition of EC Proliferation

ECs were plated in a 24-well culture plate at 5×10^3 cells per well. On the following day, the medium was changed to 0.5 mL EGM with 5% FBS. Concentrated HLE cell CM collected under various conditions was adjusted to have similar protein concentrations and then was added at the equivalent of 20% or 40%. Medium was changed on day 3. On day 6, the cells were removed by trypsinization and counted (ZM counter; Coulter Electronics, Luton, UK). Medium conditioned by AM of various preparations was tested similarly.

Inhibition of EC Migration

A confluent EC culture was first wounded with a razor,[31,32] and the floating cells were removed and washed with PBS. Cultures were then incubated in EGM containing 5% FBS, and the CM was added to an equivalent of 40%. Cultures were fixed after 10 hours, and the number of cells crossing the original line was counted using a ×40 phase-contrast microscope.

ELISA Assay

ELISAs for TIMP-1, thrombospondin (TSP)-1, pigment epithelium-derived factor (PEDF), and endostatin were performed to identify the possible endogenous angiogenic inhibitors responsible for HLE cell–induced antiangiogenic activities. Serum- and growth factor–free CM of HLE cells or AM was prepared, and cell debris was removed by low-speed centrifugation. ELISAs for TIMP-1 (R&D Systems), TSP-1, PEDF, and endostatin (Chemicon, Temecula, CA) were performed according to the instructions provided by the vendors, and the plates were read with a microtiter plate reader (VERSAmax; Molecular Devices, Sunnyvale, CA). At least five samples were examined for each condition, and the values were further adjusted to a per protein basis.

Effect of Endostatin-Neutralizing Antibody on the Proliferation, Migration, and Differentiation of ECs

To see whether endostatin in the HLE cell CM plays a role in inhibiting EC proliferation and migration, concentrated CM was aliquoted and incubated overnight at 4°C, with or without 1.0 or 10.0 μg/mL polyclonal antibody to endostatin (Chemicon) before adding it to the cultures, as indicated. In the tube-formation assay, the same concentrations of antibody were used.

Immunohistochemistry and Confocal Laser Scanning Microscopy

Confocal microscopy was performed to localize endostatin protein in the HLE-AM composite graft. The AM, including cultured HLE cells was removed from the culture dish and embedded in optimal cutting temperature (OCT) compound for cryopreservation. Cryosections (8 μm) of the specimen were mounted on glass slides (Superfrost Plus; Fisher Scientific, Pittsburgh, PA) and fixed with cold acetone (−20°C) for 10 minutes. The slides were pretreated with 0.1% Triton X-100 in PBS for 10 minutes, and after they were washed three times with PBS, they were incubated with polyclonal antibody against the endostatin portion of collagen XVIII (10.0 μg/mL; Chemicon) for 1 hour at room temperature. Goat serum (5%) was added to the antibody diluent (PBS with 0.1% Tween-20 and 0.05% sodium azide) to block nonspecific binding sites. After another three rounds of washing, the slides were incubated with FITC-conjugated secondary antibody (made from goat; 1:200 dilution with PBS containing 2% goat serum and 0.05% sodium azide; Chemicon). After three additional PBS washes (15 minutes each), sections were mounted with an antifade solution (Vectashield mounting medium with propidium iodide; Vector Laboratories, Burlingame, CA) that simultaneously counterstained the DNA in the nuclei of AM cells and HLE cells. Negative controls were performed with PBS used in place of the primary antibody. Laser confocal microscopy (TCS SP2-MP system; Leica, Heidelberg, Germany) was performed using

filters for FITC (excitation 488 nm, emission 500–535 nm) and for propidium iodide (excitation 514 nm, emission 595–633 nm). The image was averaged from 14 scans within a thickness of 5 to 7 μm.

Statistical Analysis

All experiments were repeated at least three times and with at least triplicate incubations. Documentation and calculation of number of cells and capillary length were performed by one of the authors (L-KY), who was blind to each experimental group.

Number of cells, total capillary tube length, and the results of ELISAs among groups were compared by analysis of variance (ANOVA). If a significant ANOVA result was obtained, the Dunnett multiple comparison with the control group or Scheffé's multiple comparison among groups was made. All probabilities are two-sided and are considered significant when <0.05.

Results

Histology

In live AM, more than 90% of the HLE cells (Fig. 1A) and stromal cells (Fig. 1B) were viable and did not stain with trypan blue. In FIAM, the epithelial cells (Fig. 1C) and stromal cells (Fig. 1D) were universally positive for trypan blue staining. In the EDTA-treated and rubber policeman–scraped AM (DAM), very few trypan blue–positive HLE cells were left (Fig. 1E); however, the stromal cellularity remained similar (Fig. 1F). In the OIAM, most of the epithelial (Fig. 1G) and stromal cells (Fig. 1H) had disintegrated and could no longer be stained by trypan blue as conspicuously as those shown in Figure 1C. Similarly, in OFAM only a few epithelial cells were visible (Fig. 1I), and the remnants of the stromal cells showed marked disintegration (Fig. 1J).

HLE cell outgrowth was detected in approximately 4 to 7 days after explantation. In 2 to 3 weeks, the epithelial sheet of the HLE-only group was predominantly one to two cell layers; occasionally, three layers formed (Fig. 2A). The cells were flat, with elongated cytoplasm and nuclei. The HLE cell outgrowth on intact or denuded AM was predominantly two to three cell layers, and the cells were more cuboid (Figs. 2B, 2C). Occasionally, the epithelial remnant of intact AM was visible (Fig. 2B, arrows). In the HLE/DAM/3T3 group under submerged conditions, the cell sheet was predominantly two to three cells thick with occasional four-cell-thick patches (Fig. 2D). In previous observations, coculture with 3T3 fibroblasts and air-lifting promoted epithelial stratification,[10,30,33] suggesting that stratification is influenced more by air–liquid intersurface exposure than by substrate provision.

Inhibition of EC Proliferation by HLE Cell CM

After 6 days of incubation, the number of cells in the control group was $(34.0 \pm 10.7) \times 10^3$ cells per well. When cultured in the presence of 20% or 40% CM from live AM, the number of cells per well was increased, respectively, to $(54.2 \pm 6.6) \times 10^3$ and $(58.6 \pm 14.3) \times 10^3$ ($P < 0.01$, Fig. 3). Adding CM prepared from FIAM, FDAM, OIAM, or ODAM all resulted in an increase in the number of cells, but the difference did not reach statistical significance (Fig. 3).

In contrast, the addition of 20% or 40% HLE cell CM suppressed EC growth. Of note, CM from HLE cells on denuded AM cocultured with 3T3 fibroblasts exhibited the strongest inhibitory effect. A greater than 50% suppression was observed at a CM concentration of 40% ($P < 0.001$; Fig. 3).

Inhibition of EC Migration by HLE Cell CM

CM prepared from live or cryopreserved AM had a promoting rather than an inhibiting effect on EC migration. Among these, CM prepared from FIAM had the highest activity. It exerted a 45% increase in cell migration at a CM concentration equivalent of 40% ($P < 0.001$; Fig. 4Ac). However, the results from CM prepared from live AM, FDAM, OIAM, or ODAM were not significantly different from the control.

In contrast, CM prepared from HLE cells cultured on various AM substrata all had an inhibitory effect on EC migration. CM prepared from HLE cells on denuded AM (Fig. 4Ai) and on denuded AM cocultured with 3T3 fibroblasts (Fig. 4Aj) exerted the strongest inhibition ($P < 0.001$). A 75% reduction in cell migration was observed at a CM concentration equivalent of

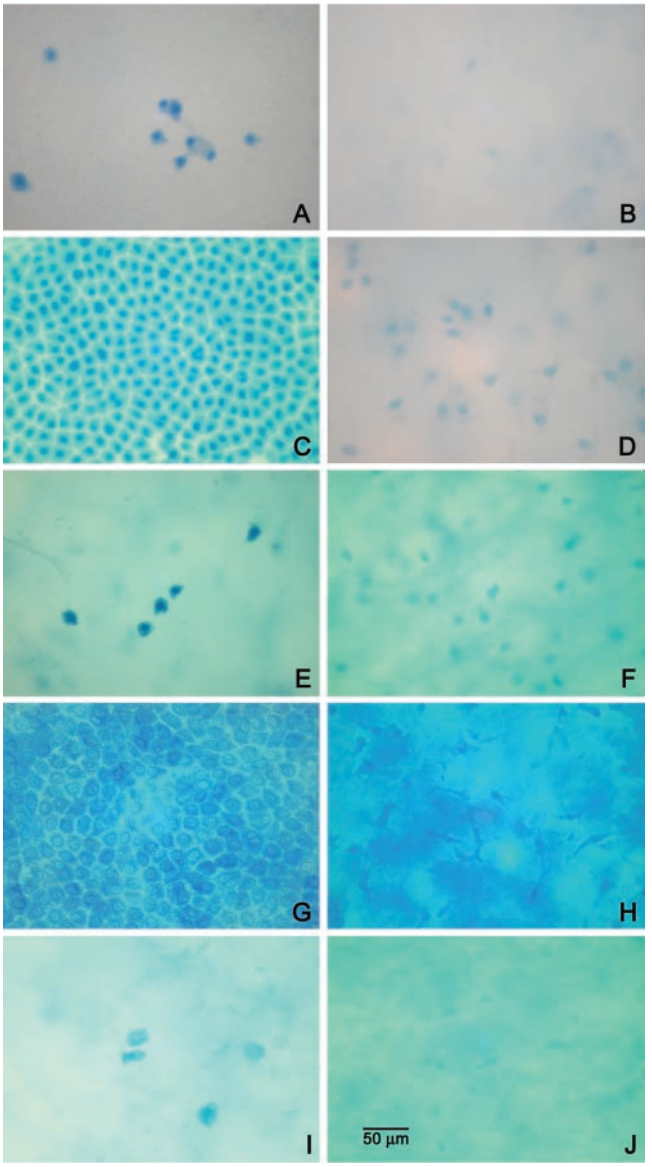

Figure 1. Vital staining for flatmounted AM in different preparations. Most epithelial cells (>90%; **A**) and stromal cells (**B**) in live AM were viable and trypan blue negative. In FIAM, the nuclei of AM epithelial cells (**C**) and stromal cells (**D**) were universally stained by trypan blue. After EDTA treatment and scraping to remove the AM epithelial cells (denuded AM), only a few trypan blue positive epithelial cells were left (**E**). However, the stromal cellularity remained similar (**F**). Unlike frozen AM, the nuclei of epithelial (**G**) and stromal cells (**H**) in the OIAM were disintegrated and were no longer stained. Similarly, in ODAM, only a few epithelial cells were visible (**I**), and the remnants of the stromal cells showed marked disintegration (**J**).

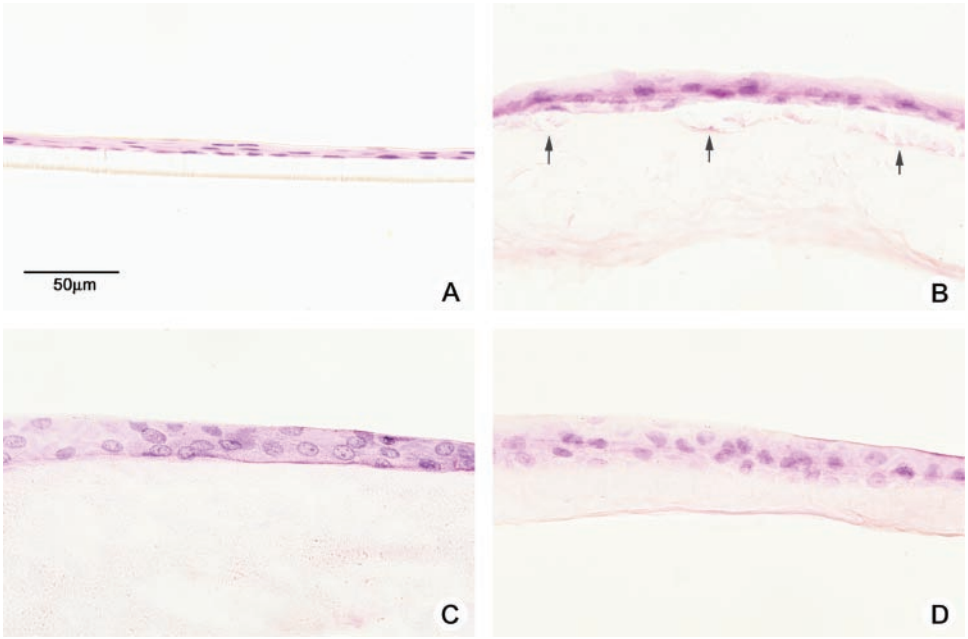

FIGURE 2. HE staining of HLE cells grown on a culture insert (**A**), on intact AM (**B**), on denuded AM (**C**), and on denuded AM cocultured with 3T3 fibroblasts (**D**). Two to 3 weeks after explantation, HLE cells cultured on an insert were predominantly one to two cell layers thick; occasionally three layers formed (**A**). The cells were flat with elongated cytoplasm and nuclei. The HLE cell outgrowth on intact (**B**) or denuded AM (**C**) was predominantly two to three cell layers, and the cells became more cuboid. Occasionally, the epithelial remnant of intact AM can be seen (**B**, *arrows*). When HLE cells on denuded AM were cocultured with 3T3 fibroblasts in submerged conditions, the cell sheet was also predominantly two to three cells thick, with occasional four-cell-thick patches (**D**).

40% in the latter. CM prepared from HLE cells only or HLE/IAM did not significantly inhibit the migration rate of the ECs compared with that of the control.

Inhibition of EC Tube Formation by Coculture with HLE

ECs in collagen gel organized into a capillary-like network in the presence of exogenous bFGF (Fig. 5Aa). Coculture of ECs with live AM made no obvious difference in tube formation (Fig. 5Ab). Coculture with FIAM and FDAM caused an increase in tube length (Fig. 5Ac, Ad), and coculture with OIAM or ODAM did not significantly reduce EC tube formation (Figs. 5Ae, Af, respectively). In contrast, coculture of ECs with HLE cells cultured on various AM preparations significantly reduced tube formation. The inhibition was most prominent when ECs were cocultured with HLE cells on denuded AM and previously cocultured with 3T3 fibroblasts ($P < 0.001$; Fig. 5Aj).

Production of TIMP-1, TSP-1, PEDF, and Endostatin by HLE Cells Cultivated on Intact or Denuded AM

The amounts of TIMP-1, TSP-1, PEDF, and endostatin in HLE cell CM collected under various culture conditions were compared on a per protein basis with ELISAs. HLE cells cultivated on intact or denuded AM produced lesser amounts of TIMP-1 (Fig. 6A), TSP-1 (Fig. 6B), and PEDF (Fig. 6C) than did HLE cells cultured on a plastic surface. In contrast, the production of endostatin was increased, especially when HLE cells on denuded AM were cocultured with 3T3 fibroblasts ($P = 0.007$ compared with the HLE-only group; Fig. 6D). In addition,

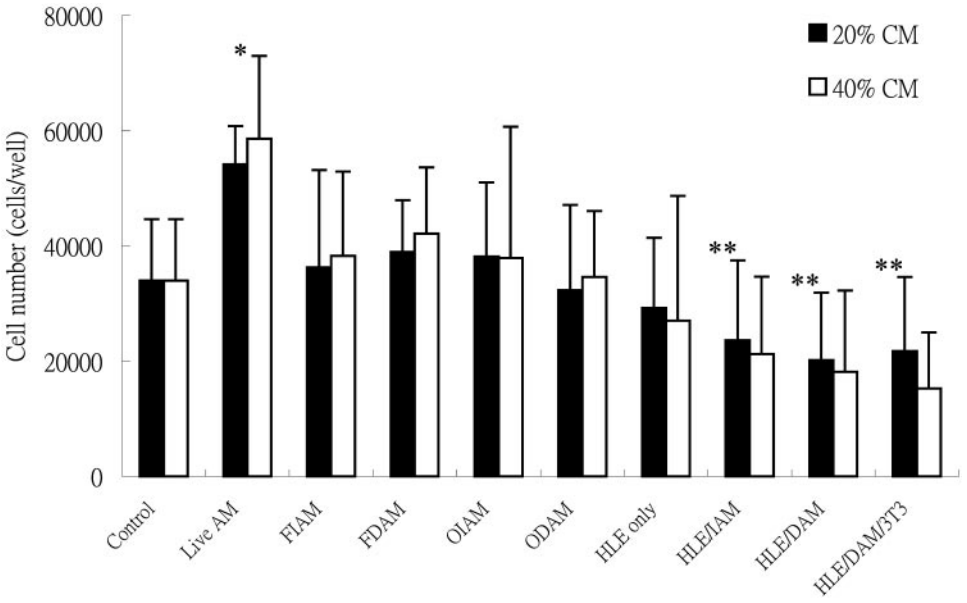

FIGURE 3. Effect of AM or HLE cell CM on the proliferation of vascular ECs. Cells were grown in 24-well cell culture plates for 6 days. When 20% or 40% CM with live AM was added, the number of cells increased to a significantly higher number than in the control cultures ($P < 0.001$). Likewise, the addition of CM from FIAM or FDAM and OIAM or ODAM caused an increase in the number of cells, but the difference did not reach statistical significance. In contrast, a decrease in the number of cells was noted when concentrated CM with HLE cells was added. The decrease was most significant when HLE cells on denuded AM were cocultured earlier with 3T3 fibroblasts ($P < 0.001$). *Significantly higher than control; **significantly lower than control.

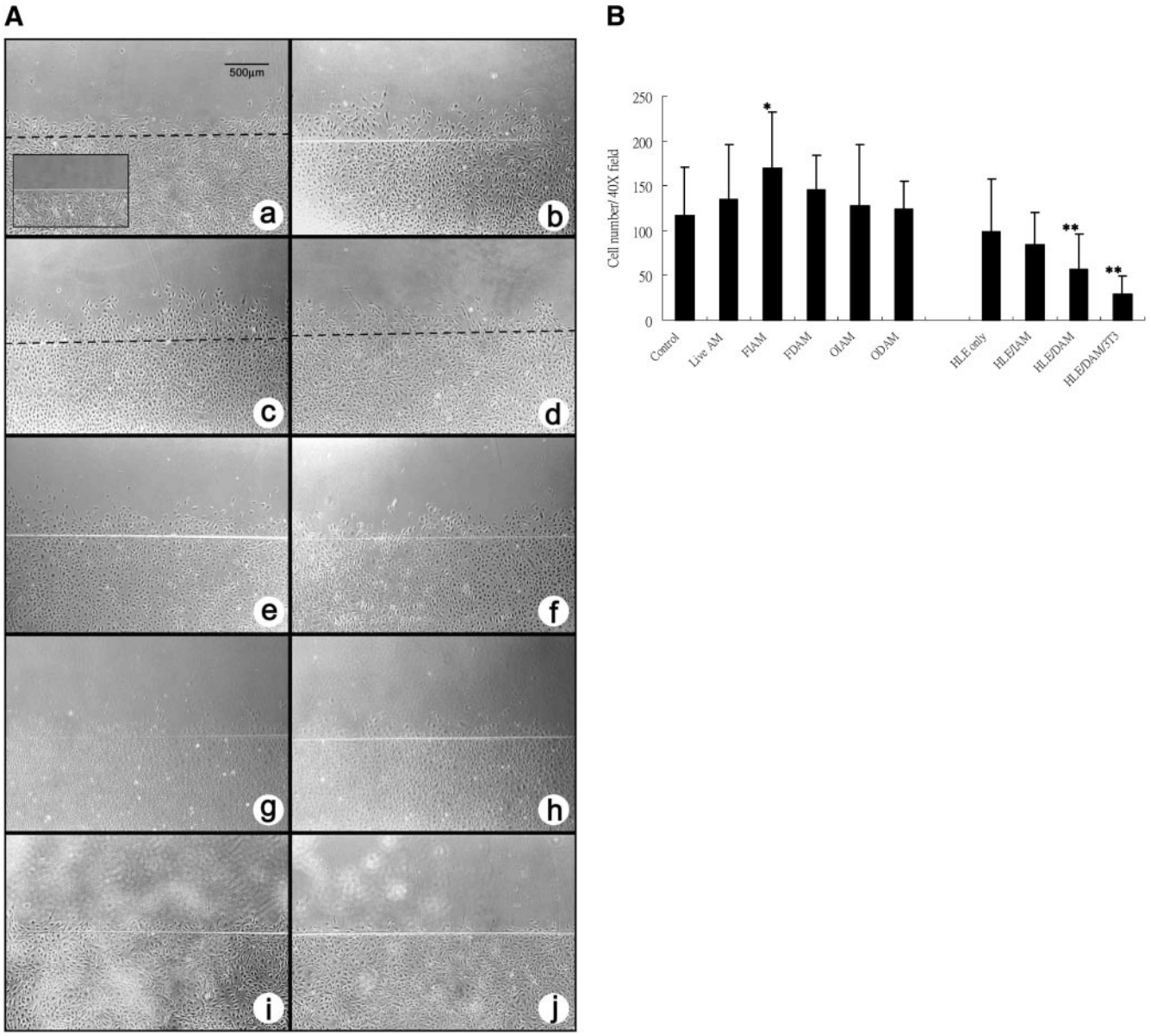

FIGURE 4. (**A**) Effect of AM (**Ab–f**) or HLE cells (**Ag–j**) CM on the migration of vascular ECs. (**Aa**) Control culture 10 hours after wounding. *Inset*: Culture immediately after wounding. When 40% concentrated CM with live AM (**Ab**), FIAM (**Ac**), or FDAM (**Ad**) were added, more ECs migrated across the original line compared with the control. CM of OIAM (**Ae**) or ODAM (**Af**) AM had little effect on EC migration. In contrast, a decrease in the number of migrated cells was noted when HLE CM was added (**Ag**). The decrease was more obvious when HLE cells were cultivated on intact (**Ah**) or denuded AM (**Ai**), and was most significant when HLE cells cultivated on denuded AM were cocultured earlier with 3T3 fibroblasts (**Aj**; $P < 0.001$). (**B**) Number of ECs crossing the original line is expressed as mean ± SD cells per ×40 field. *Significantly higher than control; **significantly lower than control.

endostatin was found to be present in the AM CM, with CM from live AM most abundant and CM from long-preserved AM the least abundant (Fig. 6D).

Effect of Neutralizing Antibody of Endostatin on the Proliferation, Migration, and Differentiation of ECs

To see whether the observed inhibitory effects on EC proliferation, migration, and tube formation by HLE cells is due to the release of endostatin, a neutralizing antibody to endostatin was tested to lessen the inhibition.

The addition of HLE/DAM/3T3 CM at 40% equivalent caused a 51.8% reduction in the number of cells compared with that of the control ($P = 0.003$; Fig. 7A). The addition of 1.0 or 10.0 μg/mL endostatin antibody attenuated the inhibition to 32.1% and 20.1%, respectively ($P = 0.087$ and 0.363 respectively; Fig. 7A). However, the addition of 1.0 or 10.0 μg/mL endostatin antibody did not significantly increase the number of cells compared with that of HLE/DAM/3T3 CM only. To confirm the specificity of the antibody, subsequent studies were conducted using an irrelevant mouse IgG antibody in place of the endostatin antibody. No increase in the number of ECs was noted (data not shown).

The inhibition of EC migration was also attenuated. Addition of HLE/DAM/3T3 CM at 40% equivalent caused a 75.6% reduction in the number of cells that crossed the razor line

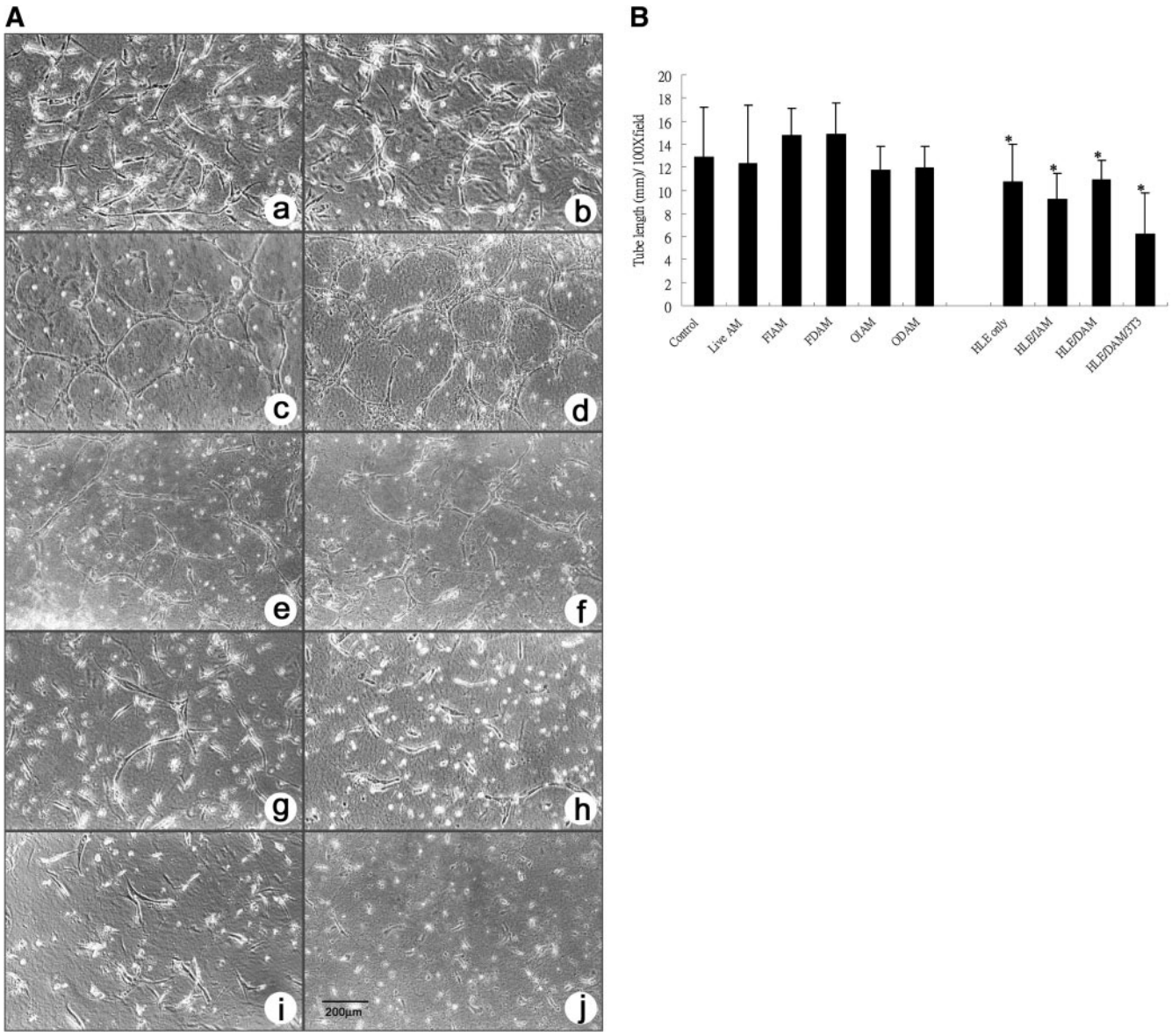

FIGURE 5. (**A**) Effect of AM (**Ab– f**) or HLE cells (**Ag– j**) coculture on the differentiation (tube formation) of vascular ECs. In the presence of exogenous bFGF, the EC in type I collagen gel organized into a delicate capillary network (**Aa**). When cocultured with live AM, there was no obvious change in tube formation (**Ab**). Coculture with FIAM (**c**) or FDAM (**Ad**) caused an increase in tube length, but the difference was not statistically significant. Coculture with OIAM (**Ae**) or ODAM (**Af**) did not significantly reduce EC tube formation. In contrast, coculture with HLE cells significantly reduced the tube length (**Ag**), which was also true of the HLE cells cultivated on intact (**Ah**) or denuded AM (**Ai**). The length of EC tube decreased most significantly when HLE cells on denuded AM were cocultured earlier with 3T3 fibroblasts (**Aj**). (**B**) Total capillary tube length expressed as mean ± SD millimeters per ×100 field. *Significantly lower than control.

compared with that in the control ($P < 0.001$; Fig. 7B). The addition of 1.0 or 10.0 μg/mL endostatin antibody attenuated the inhibition to 53.6% and 31.7%, respectively ($P = 0.002$ and 0.131 respectively; Fig. 7B). Furthermore, the addition of 10.0 μg/mL endostatin antibody increased the number of migrating cells and was significantly different from the response to HLE/DAM/3T3 CM only ($P = 0.002$).

Coculture of ECs with HLE/DAM/3T3 caused a 39.1% reduction in capillary tube formation compared with the control (Fig. 7C). The addition of 1.0 or 10.0 μg/mL antibody attenuated the inhibition to 29.1% and 21.8%, respectively. Under these conditions, however, the average tube length was still significantly less than that in the control (all $P < 0.001$; Fig. 7C), although adding 10.0 μg/mL endostatin antibody caused a significant increase in tube length when compared with the response to HLE/DAM/3T3 only ($P = 0.006$).

Immunoconfocal Microscopy of Endostatin-Containing Collagen XVIII

In live AM, the basement membrane (BM) homogenously was stained positive for endostatin-containing collagen XVIII (Fig. 8A). Patches of positive staining were also observed in the stroma. In FIAM, collagen XVIII staining in the BM was weaker than that in live AM, and, in the stroma, the staining tended to associate with the stromal cells (Fig. 8C). In FDAM, the staining

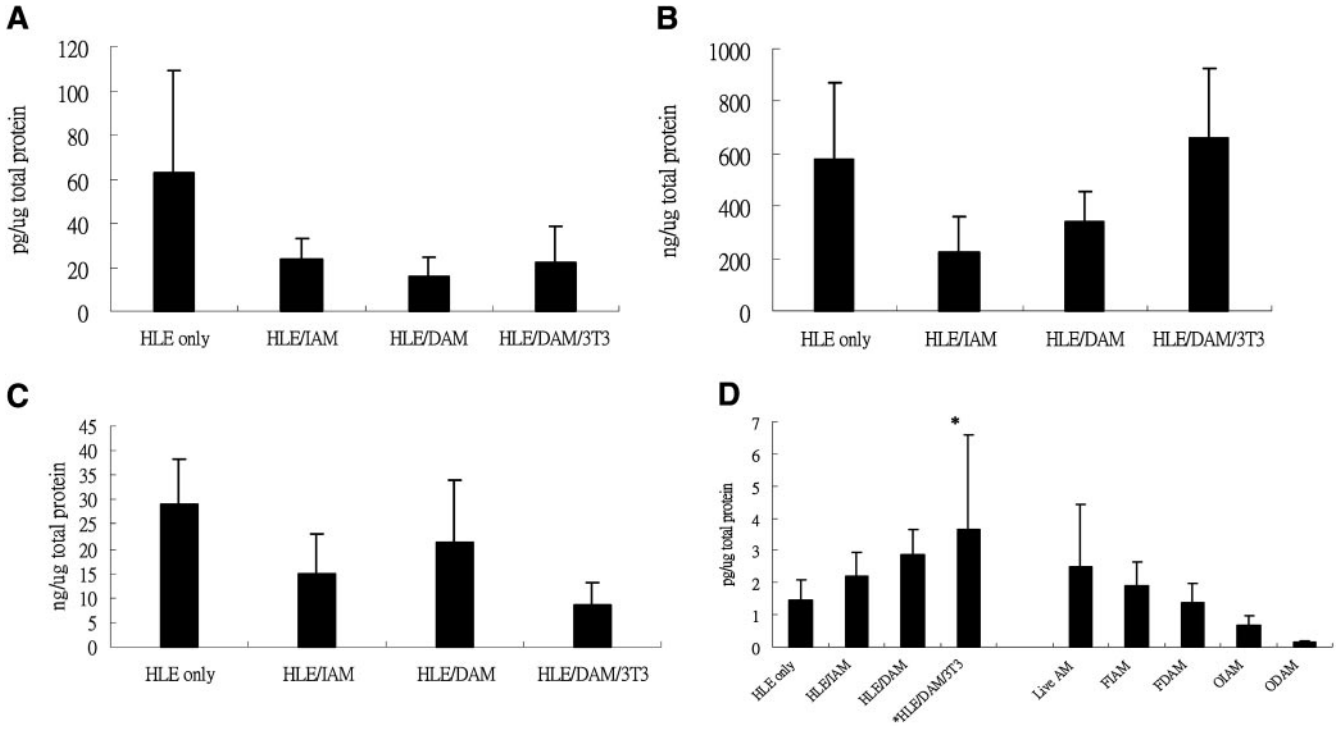

FIGURE 6. ELISA for TIMP-1 (**A**), TSP-1 (**B**), PEDF (**C**), and endostatin (**D**) in CM of HLE cells cultivated on an insert, on intact (IAM) or denuded (DAM) AM, or on DAM and cocultured earlier with 3T3 fibroblasts. Endostatin in CM of live AM, FIAM or FDAM, and OIAM or ODAM was also measured (**D**). *Significantly higher than control.

pattern was similar but was even weaker than that in the FIAM (Fig. 8D). In OIAM (Fig. 8F) and ODAM (Fig. 8H), a weak collagen XVIII staining was observed in the BM and around stromal cells. In contrast, a stronger homogenous BM staining was noted when HLE cells were cultured on intact AM (Fig. 8E), and was even stronger when HLE cells were cultured on denuded AM (Fig. 8G). The staining pattern of the stroma was not changed significantly, and HLE cells cultivated on denuded AM and previously cocultured with 3T3 fibroblasts showed features similar to those of HLE cells cultured on denuded AM only (data not shown).

DISCUSSION

This study clearly showed that factors released from AM and HLE cells acted differently on the ECs. Factors from live or cryopreserved AM promoted the proliferation, migration, and differentiation of ECs. In contrast, factors released from cultured HLE cells inhibited all three angiogenic processes. Earlier studies by others have shown that EC cultured on either the BM side or the stromal side of the AM promotes ECs to form tubelike structures.[34,35] In the present study, we found that coculture with live AM did not inhibit bFGF-induced EC differentiation, and coculture with cryopreserved AM promoted capillary tube formation in addition to the effects of bFGF. Our finding that conditioned medium collected from old AM (3 weeks after 37°C incubation) exerted little effect on EC proliferation and migration suggests that growth factor activities in the AM decreased dramatically during the 37°C cultivation period. Taken together, factors released from the AM, fresh or preserved, do not directly inhibit the angiogenic processes. The in vivo antiangiogenic effect after AM transplantation is therefore more likely to be through its anti-inflammatory activity[21–25] (Kamiya K, et al. *IOVS* 2002;43:ARVO Abstract 2262). However, it should be stressed that certain antiangiogenic factors, such as TIMP-3, are matrix bound and are not secreted into the medium. Because the design of this project allowed only the study of the effects of soluble antiangiogenic factors, it is beyond the study's scope to determine the antiangiogenic activity caused by the matrix-bound factors.

In contrast to the effect of AM, factors released from HLE cells inhibited the proliferation, migration, and differentiation of ECs. Such inhibitory activities were augmented and became significantly different from the control when HLE cells were cultured on intact or denuded AM and were most significant when HLE cells cultivated on denuded AM were further cocultured with 3T3 fibroblasts. Because the preserved AM itself did not inhibit the angiogenic processes, such enhanced inhibitory activities are most likely derived from HLE cells. The results thus highlight the significance of cell–matrix (by the AM) and cell–cell (by the 3T3 cells) interactions in modulating the antiangiogenic activity expressed by HLE cells.

In an attempt to identify factors responsible for the antiangiogenic activity in HLE CM, we first selected TIMP-1, TSP-1, PEDF, and endostatin for study, as they represent the major categories of angiogenic inhibitors found in the cornea ever published.[36–41] We found that only the level of endostatin correlated with enhanced antiangiogenic activities. The role of endostatin was further confirmed by the observation that neutralizing endostatin activity significantly abrogated the inhibitory effect on EC proliferation and migration.[42] Similar observations were reported by Chang et al. (Chang JH, et al. *IOVS* 2002;43:ARVO Abstract 1867; Chang, JH, et al. *IOVS* 2003;44: ARVO E-Abstract 832), who suggested that endostatin may play a major role in the HLE cell–induced antiangiogenic effect. That the addition of endostatin antibody exerted less effect on

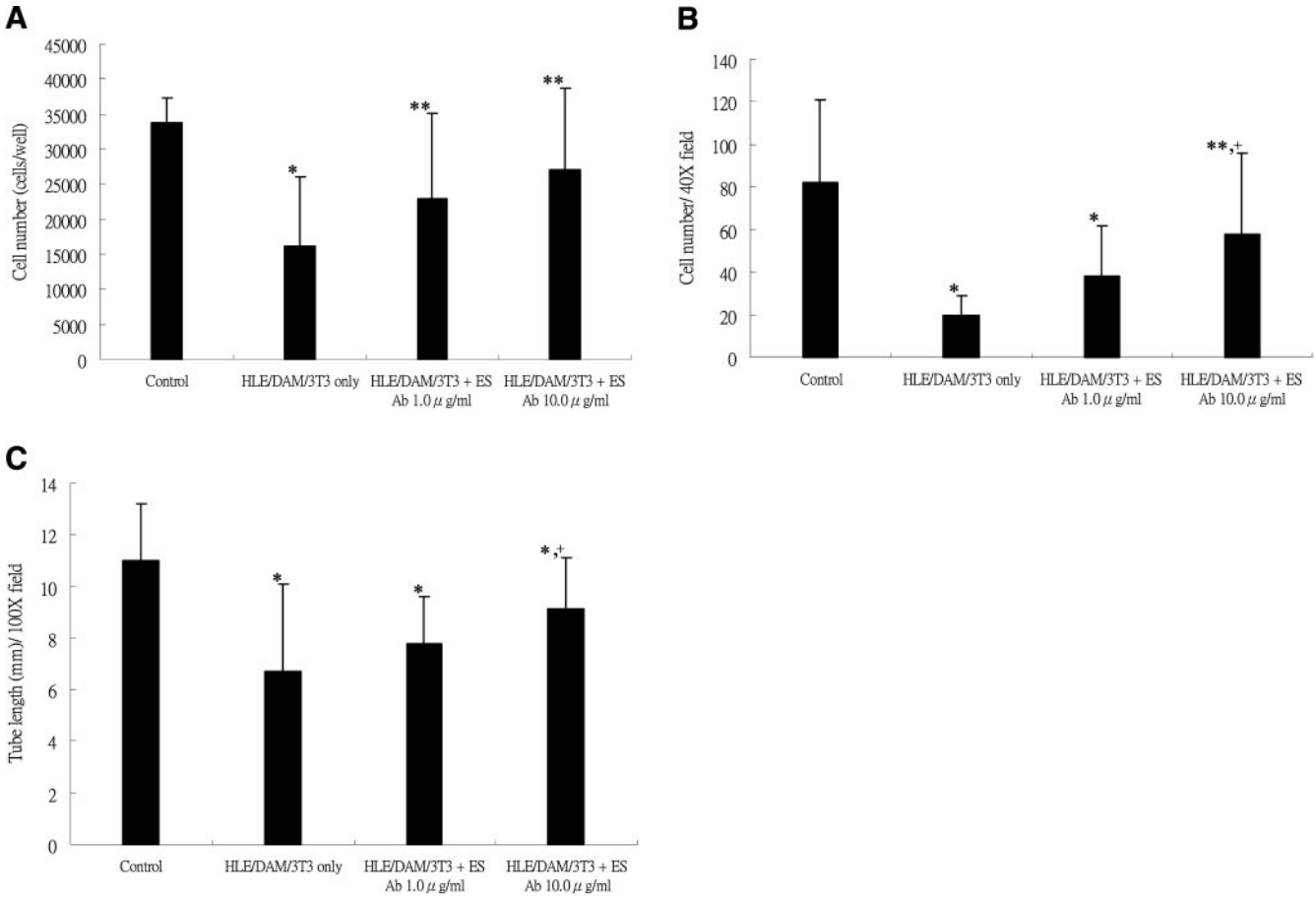

FIGURE 7. Effect of 1.0 or 10.0 μg/mL endostatin antibody (ES Ab) on attenuation of the in vitro antiangiogenic effect of HLE cells cultivated on denuded AM and cocultured earlier with 3T3 fibroblasts (HLE/DAM/3T3). Culture supernatant conditioned by HLE/DAM/3T3 was used for a vascular EC proliferation (**A**) and migration (**B**) assay. HLE/DAM/3T3 were cocultured with ECs in type I collagen gel to assay the effect on capillary tube formation (**C**). *Significantly lower than control; **not significantly lower than control; $^{+}$significantly higher than HLE/DAM/3T3 only.

the HLE cell–induced inhibition of EC differentiation is compatible with a previous report showing that endostatin is essential for the maintenance of the capillary tube structures.[43] Although fresh AM is rich in endostatin content (Figs. 6D, 8A), it is interesting that neither live AM nor cryopreserved AM inhibited in vitro angiogenesis, suggesting the concomitant presence of high levels of proangiogenic factors, such as VEGF,[44–50] placental growth factor,[50,51] and FGF[52,53] in the AM, which promoted the in vitro angiogenic processes.

The mechanism(s) by which endostatin content was increased in the CM after cultivation of HLE cells on AM is intriguing. Collagen XVIII is an inherent component of basement membrane[54] and is present in Bowman's membrane, Descemet's membrane of the cornea,[38,40] and AM.[21] Endostatin content can be increased either by increased synthesis of collagen XVIII or by increased proteolytic cleavage of the collagen by proteases secreted by HLE cells.[38] Immunoconfocal microscopy revealed that the old intact or denuded AM showed only very faint staining for endostatin-containing collagen XVIII but showed prominent staining in the basement membrane zone of HLE cells cultivated on AM, suggesting that new endostatin molecules may have been produced. Nevertheless, it is still possible that the increased endostatin is merely due to an increased number of HLE cells cultured on AM. To clarify, we intentionally did not air-lift the HLE cells, and quantitated the endostatin concentration by ELISA on a per cellular protein basis, and found that there was still a significant elevation of endostatin-related protein. It has been reported that angiostatin secreted by corneal epithelial cells was augmented through epithelial-fibroblast interaction (Savage JM, et al. *IOVS* 2001;42:ARVO Abstract 2592), which might also be true of endostatin.

As for the size of the endostatin-related protein in HLE CM, our preliminary Western blot data show that bands at ~20 to 21 and ~26 to 28 kDa were both found (Yao JY, et al. *IOVS* 2004;45:ARVO E-Abstract 4812). Whether HLE cells secrete the exact size of endostatin protein (approximately 20 kDa) in addition to the reported 28-kDa fragment[38] needs further clarification. However, because the addition of endostatin antibody did not completely block the antiangiogenic activity of HLE cells, it appears that other major antiangiogenic factors, especially those derived from a proteolytic process, such as angiostatin,[55] or collagen IV–derived antiangiogenic factors may also play a role.[56–61]

In summary, we report that factors released from HLE cells inhibited the proliferation, migration, and differentiation of ECs. Such inhibitory activities were further augmented by cultivating HLE cells on AM and coculturing with 3T3 fibroblasts. The findings justify the clinical application of combined AM and limbal transplantation or the transplantation of cultivated limbal epithelial cells on AM. The findings in our in vitro study suggest that endostatin may be responsible for the antiangiogenic effect. However, the mechanisms for increased

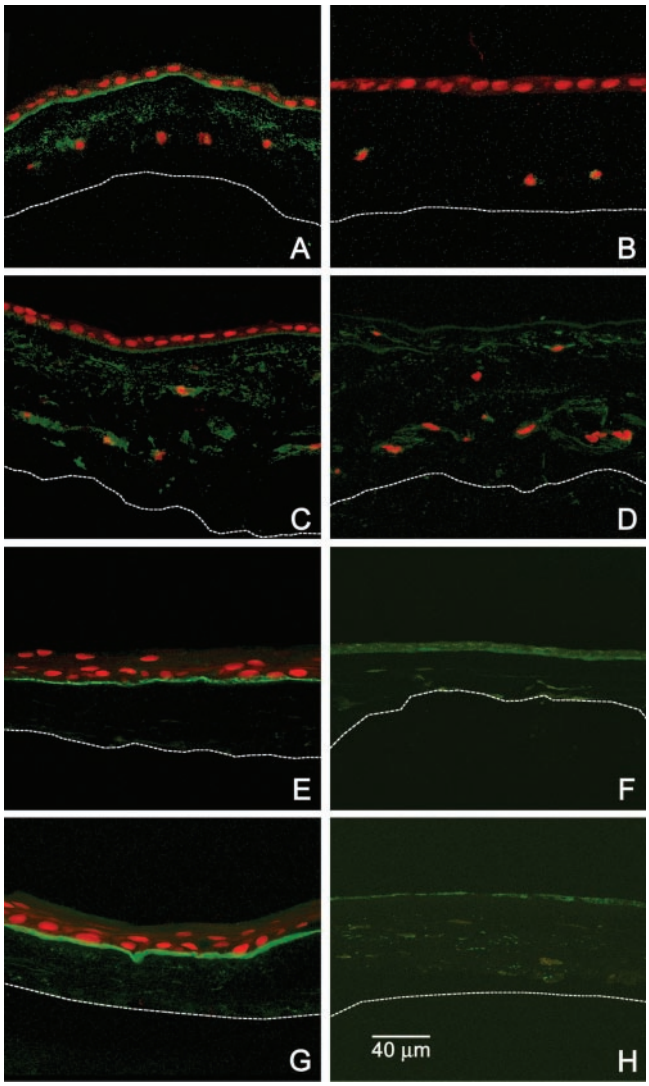

FIGURE 8. Immunoconfocal microscopy for endostatin-containing collagen XVIII expressed by HLE cells cultivated on AM. In live AM, a homogenous BM staining was evident (**A**). Patches of stromal staining were also seen. Negative control eliminating the primary antibody generated no signal, and only the counterstained nuclei of AM epithelial and stromal cells were seen (**B**). In FIAM, collagen XVIII staining in the BM was weaker than that in the live AM, and in the stroma the staining tended to associate with the stromal cells (**C**). In FDAM, the staining pattern was similar but was even weaker than that in the FIAM (**D**). In OIAM (**F**) and OFAM (**H**), weak collagen XVIII staining was seen in the BM and around stromal cells. In contrast, distinct and homogenous BM staining was visible when HLE cells were cultured on intact AM (**E**); the staining became more prominent when HLE cells were cultured on denuded AM (**G**). In both conditions, there was only negligible stromal staining. *Dotted lines*: lower margin of the AM.

endostatin secretion by HLE cells cultivated on AM await further investigation.

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