Generation of Endostatin by Matrix Metalloproteinase and Cathepsin from Human Limbocorneal Epithelial Cells Cultivated on Amniotic Membrane

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Purpose. Cultured human limbocorneal epithelial (HLE) cells secrete endostatin-related molecules that are augmented when the cells are cultivated on denuded amniotic membrane (DAM). This study is to identify mechanisms for enhanced endostatin production by HLE cells cultivated on AM.

Methods. HLE cells were cultured on dish, on intact AM (IAM) or on DAM. Collagen XVIII α1 mRNA was analyzed by real-time quantitative PCR. In HLE/DAM cultures, inhibitors of MMPs (GM-6001; 1,10-phenanthroline), cathepsins (E64; cathepsin B inhibitor II), elastase (elastatinal), and serine proteases (AEBSF; aprotinin) were added. Endostatin in the conditioned medium (CM) was detected by Western blot. MMP-7; MMP-9; and cathepsins B, K, L, and V in the CM were quantitated by ELISA. Exogenous cathepsin B or V was added to the concentrated HLE/DAM CM to see the effect on endostatin production.

Results. The expression of collagen XVIII α1 mRNA in the three groups was similar. Elastatinal, AEBSF, and aprotinin had no effect on endostatin generation. MMP inhibitors inhibited the generation of all the 20- and 28- to 30-kDa endostatin-related fragments, while cathepsin inhibitors inhibited only the 20-kDa endostatin. The level of MMP-7 and cathepsin B but not cathepsin V increased as the culture time increased, and paralleled with endostatin production. However, cathepsins K and L were absent in the CM. Exogenous cathepsins B and V further augmented the generation of endostatin.

Conclusions. MMP-7 and cathepsins B and V are involved in the generation of endostatin by HLE cells. Facilitating endostatin generation may be a novel physiological function of the cornea-specific cathepsin V. (*Invest Ophthalmol Vis Sci.* 2007;48: 644–651) DOI:10.1167/iovs.06-0884

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Normal limbocorneal epithelial cells exhibit the ability to inhibit angiogenesis. This tendency has been documented in vitro[1] and also in vivo by the fact that a successful limbal transplantation suppresses corneal neovascularization induced by limbal stem cell deficiency.[2] Recently, we reported that the angioinhibitory effects of human limbocorneal epithelial (HLE) cells were enhanced when HLE cells were cultured on amniotic membrane (AM), especially on denuded AM (DAM; AM without epithelium) and further cocultured with 3T3 fibroblasts.[3] We also found that endostatin-related antiangiogenic factors may be responsible for such enhanced in vitro antiangiogenic activity. These findings signify the effect of the HLE cell–AM and HLE-cell–3T3 cell interactions on the generation of endostatin. To explore the mechanism, because the secretion of endostatin already increases when HLE cells are cultured on DAM, and to a lesser degree on intact AM (IAM), this study was focused solely on the effect of AM substrate on the generation of endostatin by HLE cells.

Originally found in the murine hemangioendothelioma cell line (EOMA cells),[4] endostatin is the C-terminal proteolytic product of the collagen XVIII α1 chain. Lin et al.[5] reported that the addition of matrix metalloproteinase (MMP)-7 cleaves mouse corneal epithelium–derived collagen XVIII to generate a 28-kDa endostatin-spanning fragment.[5] Recently, Chang et al.[6] reported that MMP-14 also showed similar activity. Nevertheless, to date it remains unknown whether the 18.5- to 22-kDa circulating form of human endostatin is secreted by HLE cells.[7] Also remaining unknown is the molecular mechanism of the increase in endostatin secretion when HLE cells are cultivated on AM. The 20-kDa endostatin has been reported to be generated by cathepsins[8,9] and elastase.[10] However, the observation was based on experiments using EOMA cells, and the mechanism for 20-kDa endostatin generation in normal human cells has not been reported.

In this study, we investigated the mechanism for endostatin generation by HLE cells cultivated on DAM. We found that an increase in protease activity but not the mRNA transcription of collagen XVIII α1 correlated with increased endostatin production. Proteolytic enzymes potentially involved in the process were studied.

Methods

Preparation of Human AM and Cultivation of HLE Cells on AM

The project was approved by the Committee of Medical Ethics and Human Experiment of the Chang Gung Memorial Hospital. The preparation of human AM, and the technique of explant culture of HLE cells from human donor corneoscleral rims have been described,[3] and the procedures adhered to the guidelines in the Declaration of Helsinki. The cells were cultivated on 35-mm dishes rather than on culture inserts.

Real-Time Quantitative PCR for Collagen XVIII α1 mRNA

Before harvesting RNA, 80% to 90% confluent cultures were kept serum- and growth factor–free for 48 hours to avoid the effect of any exogenous growth factors on collagen XVIII transcription. Total RNA from HLE cells cultured on a dish (HLE-only group), on intact AM (HLE/IAM group) and on DAM (HLE/DAM group) was extracted (RNeasy mini kit; Qiagen, Valencia, CA), and the mRNA was reversely transcribed into cDNA (Superscript Pre-amplification System; Invitrogen-Gibco, Grand Island, NY). The reaction mixture was prepared in a 50-μL Eppendorf tube, containing 2× SYBR Green PCR Master Mix (Invitrogen-Gibco), and optimal primers. The samples were amplified and detected with an automatic sequence-detection system (Prism 7000 Sequence Detection System; Applied Biosystems [ABI], Foster City, CA) with the following primer sequences: forward primer, GGCACGCATCTTCTCCTTTGA; reverse primer, CGTCCGCCACGTCTCACA. The thermal cycling parameters were 50°C for 2 minutes, 95°C for 10 minutes, and 40 PCR cycles of 15 seconds at 95°C and 1 minute at 60°C. The detection system measured fluorescence emissions. The point representing the detection threshold of the increase in the fluorescence signal associated with the exponential growth of the PCR product is defined as the cycle threshold (C_T). In this study, the β-actin gene was simultaneously amplified as a normalizer (calibrator or reference). As pilot validation experiments demonstrated that the amplification efficiencies of target and reference in this study were approximately equal, the comparative C_T method ($\Delta\Delta C_T$) was used for relative quantitation of collagen XVIII α1 gene expression.

Because the β-actin gene had a higher expression level than did the collagen XVIII α1 gene (thus, a lower C_T), the calculation for the quantitation starts with getting the difference (ΔC_T) between the C_T values of the two genes: $\Delta C_T = C_T$ (collagen XVIII α1) $- C_T$ (β-actin).

The amount of collagen XVIII α1 mRNA, normalized by β-actin and relative to the control (HLE-only) group is given by:

$$2^{-\Delta\Delta C_T},$$

where $\Delta\Delta C_T = \Delta C_T -$ averaged ΔC_T (reference).

Preparation of Conditioned Medium from HLE Cells Cultivated on AM

At the end of the third week, cultures of HLE cells on a dish, IAM, and DAM were washed twice with PBS, and 2 mL serum- and growth factor–free medium was added. For preparation of conditioned medium (CM), the cultures were maintained for 48 hours, and the medium was collected and stored at −70°C. The procedure was repeated at the ends of the fourth and fifth weeks, and if the viability of the cultures was still good, CM from the 6- and 7-week-old cultures was also collected (collectively, as >5 weeks). Media conditioned by AM kept in a 37°C incubator for 3 weeks (old IAM and old DAM) were also collected.

Western Blot Analysis for Endostatin, Cathepsins, and MMP-7

For the experiments, the CM was thawed and concentrated 10-fold with a centrifugal filter with a molecular size cutoff of 3 kDa (Centricon; Millipore, Bedford, MA). Because it was impossible to use cytosolic proteins such as β-actin to standardize protein concentration of the CM, samples with an equal amount of total protein (usually 4 μg) were added and fractionated on 10% SDS-PAGE gel, and then electrotransferred to polyvinylidene difluoride (PVDF) membrane (GE Healthcare, Piscataway, NJ). After the membranes were blocked with 5% skim milk for 30 minutes and washed three times with 1× PBS, they were incubated with the following primary antibodies: rabbit polyclonal antibody against human endostatin (1:100; Chemicon, Temecula, CA), mouse monoclonal antibody anti-human endostatin (1:100; Oncogene Research Products, San Diego, CA), goat polyclonal antibody against human cathepsin B (1:100; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit polyclonal antibody against cathepsin K (1:100; Calbiochem, La Jolla, CA), goat polyclonal antibody against human cathepsin L (1:100; Santa Cruz Biotechnology), mouse monoclonal antibody anti-human cathepsin V (1: 100, R&D Systems, Minneapolis, MN), and MMP-7 (1: 100; Calbiochem). The membrane was incubated with the primary antibody overnight at 4°C. After three washes, the membrane was incubated at room temperature for 2 hours with horseradish peroxidase (HRP)–conjugated secondary antibodies (1:5000). After another three washes, the membrane was incubated with enhanced chemiluminescent (ECL) substrate of HRP (GE Healthcare) for 1 minute, then wrapped with plastic wrap and exposed to radiograph film (Hyperfilm; GE Healthcare) for 1 to 10 minutes, depending on the intensity of signal. The film was subsequently developed. Negative control was performed similarly, except that no concentrated CM was added.

Inhibition of Endostatin Generation by Protease Inhibitors

The following protease inhibitors were added to determine their effect on endostatin generation by HLE/DAM:AEBSF (a serine protease inhibitor, 0.5–10 mM; Sigma-Aldrich, St. Louis, MO); aprotinin (a serine protease and neutrophil elastase inhibitor, 1–50 μM; Sigma-Aldrich); elastatinal (an elastase inhibitor, 1–100 μM; Sigma-Aldrich); 1,10-phenanthroline (an MMP inhibitor, 1–50 μM; Sigma-Aldrich); GM-6001 (an MMP inhibitor, 1–50 μM; Chemicon); E-64 (a cysteine protease inhibitor, 1–100 μM; Sigma-Aldrich); and cathepsin B inhibitor II (a selective cathepsin B inhibitor, 5–250 μM; Calbiochem). The concentrations of protease inhibitors were chosen according to previous studies[8–10] and suggestions from the manufacturers, to ensure that the cells remained viable during the experiment. Those inhibitors were added to 4- to 5-week-old cultures of HLE/DAM. To avoid interference from endogenous protease inhibitors in the serum, serum-free CM was used, similar to a report by Felbor et al.[8] Forty-eight hours later, the CM was collected and frozen until use in the experiments.

ELISA Assay

ELISA for cathepsin B (R&D Systems), cathepsin L (Calbiochem), MMP-7, and MMP-9 (R&D Systems) was performed to quantitate the proteases related to the generation of endostatin. Serum- and growth factor–free CM of AM alone or HLE cells cultured on a dish, IAM, or DAM were prepared similarly, as previously described.[3] ELISA procedures were performed according to the instructions provided by the vendors. Because no commercial cathepsin V ELISA kit was available, a 96-well tissue culture plate was coated with anti-human cathepsin V monoclonal antibody (R&D Systems) for a direct ELISA procedure with *p*-nitrophenylphosphate (SK-5900; Vector Laboratories, Burlingame, CA) as a substrate for alkaline phosphatase (AK-5000; Vector Laboratories). At least five samples were examined for each condition, and the values were further adjusted to a per protein basis.

Effect of Exogenous Cathepsins on the Generation of Endostatin

CM of HLE/DAM was concentrated 40-fold as just described above. For each 200 μL of concentrated CM, 50 or 250 nM of cathepsin B purified from human liver (Calbiochem), or 10 or 50 nM recombinant human cathepsin V (R&D System) was added, respectively. Reaction condition was in 50 mM sodium acetate, 2.0 mM dithiothreitol (DTT), and 5 mM EDTA (pH 6.0 for cathepsin B and pH 5.5 for cathepsin V). The pH was adjusted by adding 1 M acetic acid. The reaction was sustained at 37°C on an orbital shaker. At 0 minutes; 30 minutes; and 1, 2, 4, and 8 hours, 30 μL of the reactant was extracted, and 0.5 μL protease inhibitor cocktail (Sigma-Aldrich) was added to stop further reaction. Aliquots were later subjected to SDS-PAGE under reducing conditions and then Western blot analysis, as described earlier.

Statistical Analysis

At each time point of every experiment, at least five samples were examined. As the sample size was not large in each group, the Kruskal-

Wallis test (nonparametric test) was made to compare data among groups. Because it is observed that protease concentrations varied at different time points among groups, the Wilcoxon rank-sum test or the Kruskal-Wallis test was again made to compare data among groups within the same time point, when appropriate with a significance level of $P \leq 0.05$.

Results

Expression of 20-, 28-, and 30-kDa Endostatin-Related Fragments by HLE Cells

As is shown in Figure 1, Western blot analysis for endostatin in the CM of HLE cells with a commercial polyclonal antibody (Chemicon) revealed a 20-kDa band and two bands at ~28 to 30 kDa. The signals, especially that of the 20-kDa band increased as the culture time increased. The signals were in general more prominent in the HLE/DAM group than in the HLE/IAM and the HLE-only groups. A similar pattern was seen with another commercial antibody (GF57; Oncogene), but the signals were generally weaker (data not shown). Medium conditioned by old IAM or DAM yielded only negative signals (data not shown), which is consistent with our previous ELISA study.[3] It is unlikely that endostatin increased simply because there were more cells in older cultures, as all samples were adjusted to contain equal amounts of total protein. Because the HLE/DAM group yielded the highest amount of endostatin, HLE cells cultivated on DAM were used for subsequent protease inhibition studies.

Real-Time Quantitative PCR for Collagen XVIII α1 mRNA Expressed by HLE Cells

As is shown in Table 1, the mean ΔC_T for HLE cells cultured on a dish (HLE-only group) was 5.16 ± 0.80 (4.27–6.51; $n = 7$). The ΔC_T for the HLE/IAM and HLE/DAM group was 5.07 ± 0.28 (4.73–5.55; $n = 7$) and 5.10 ± 0.77 (4.15–6.14; $n = 7$), respectively. The $\Delta\Delta C_T$ for the three groups were therefore 0.00, −0.09, and −0.06, respectively. If the relative collagen XVIII α1 mRNA expression ratio in the HLE-only group is defined as 1.00, the ratio in the HLE/IAM and HLE/DAM groups is then 1.06 and 1.04, respectively. The results suggested that the collagen XVIII α1 mRNA transcription level was very similar among the three groups ($P = 0.985$).

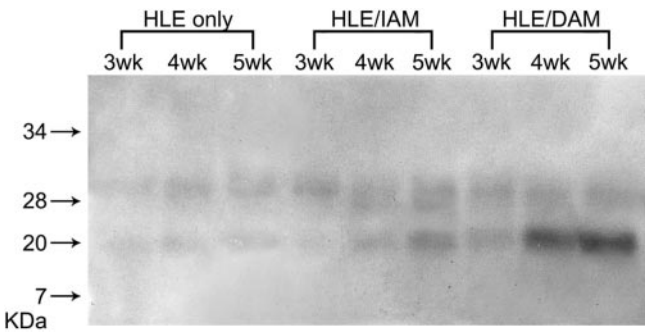

Figure 1. Western blot for endostatin in the CM of HLE cells cultivated on dish (HLE-only), on intact amniotic membrane (HLE/IAM), and on denuded AM (HLE/DAM) at 3, 4, and 5 weeks. Note that in addition to the 20-kDa band representing endostatin, two bands of ~28 to 30 kDa, representing molecules larger than endostatin, were also seen. The band intensities increased as the culture time increased, and were most prominent in the HLE/DAM group, followed by the HLE/IAM and the HLE-only groups.

Table 1. Result of Real-Time Quantitative PCR for Collagen XVIII α1 mRNA

Group	ΔC_T (Mean ± SD)	$\Delta\Delta C_T$	Expression Ratio	P^*
HLE only	5.16 ± 0.80	0.00	1.00	0.985
HLE/IAM	5.07 ± 0.28	−0.09	1.06	
HLE/DAM	5.10 ± 0.77	−0.06	1.04	

Shown are data for mRNA expressed by human limbo-corneal epithelial (HLE) cells cultivated on dish (HLE only), on intact amniotic membrane (HLE/IAM), and on denuded AM (HLE/DAM). $n = 7$ for each group. $\Delta\Delta C_T = \Delta C_T -$ averaged ΔC_T reference (HLE only). The expression ratio $= 2^{-\Delta\Delta C_T}$.

* Kruskal-Wallis test.

Inhibition of Endostatin Generation by MMP and Cathepsin Inhibitors but Not by Elastase or Serine Protease Inhibitors

As is shown in Figure 2, addition of the MMP inhibitor 1,10-phenanthroline (Fig. 2A) and GM-6001 (Fig. 2B) resulted in a dose-dependent inhibition of the generation of all 20-, 28- and 30-kDa endostatin-related fragments in the CM of HLE/DAM. Addition of the cysteine protease inhibitor E-64 (Fig. 2C) and the specific cathepsin B inhibitor II (Fig. 2D) resulted in a dose-dependent inhibition predominantly of the generation of the 20-kDa band. However, addition of the elastase inhibitor elastatinal did not interfere with the generation of all the endostatin-related fragments, even at the highest concentration (Fig. 2E). Likewise, addition of the serine protease inhibitors AEBSF and aprotinin also had no effect on endostatin generation (data not shown), suggesting that these proteases may not be involved in the formation of endostatin.

Quantitation of MMP-7, and -9 and Cathepsin B, L, K, and V in the CM of HLE/DAM

Because our protease inhibition studies revealed that both the MMPs and cathepsins were related to the production of endostatin, we went on to quantitate the concentration of endostatin-generating MMPs and cathepsins in the CM of HLE cell cultures. As is shown in Figure 3A, at 3 weeks, the concentration of MMP-7 was low in the HLE-only, HLE/IAM, or HLE/DAM group. MMP-7 began to increase steadily at 4 weeks, especially in the HLE/DAM group. The MMP-7 concentration in that group continued to increase so that at 5 weeks (92.7 ± 48.8 pg/μg total protein) and longer than 5 weeks (166.8 ± 105.5 pg/μg total protein), the level of MMP-7 in the HLE/DAM group was significantly higher than that in the HLE-only group (at 5 weeks, 53.3 ± 18.8 pg/μg total protein, $P = 0.002$; >5 weeks, 45.7 ± 24.8 pg/μg total protein, $P < 0.001$) and HLE/IAM group (at 5 weeks, 41.8 ± 31.0 pg/μg total protein, $P = 0.026$; > 5 weeks, 78.8 ± 49.1 pg/μg total protein, $P < 0.001$). The presence of MMP-7 in the HLE/DAM CM was also confirmed by Western blot, which showed an ~28-kDa band, corresponding to the proenzyme of MMP-7 (Fig. 3B). Additional bands were seen at ~48 kDa, which may represent the complex of MMP-7 with the tissue inhibitors of metalloproteinases (TIMPs). In contrast, the MMP-9 concentration was highest, especially in the HLE/DAM and HLE-only groups, at 3 weeks. The level dropped dramatically at 4 weeks, and afterward, there was only a small increase in the HLE/DAM group, and a further decline in the other two groups. Nevertheless, at more than 5 weeks, the MMP-9 concentration in the HLE/DAM group (397.5 ± 265.4 pg/μg total protein) was still significantly higher than that in the HLE-only (147.1 ± 75.8 pg/μg total protein, $P < 0.001$) and HLE/IAM (226.5 ± 134.2 pg/μg total protein, $P < 0.001$; Fig. 3C) groups.

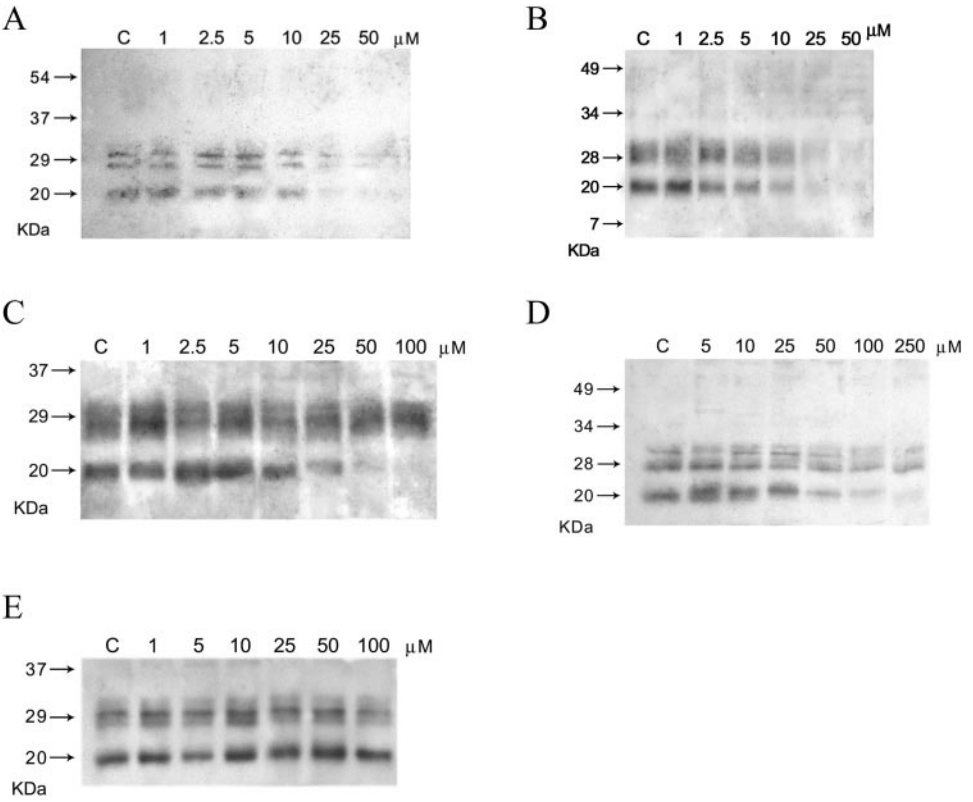

FIGURE 2. Effect of matrix metalloproteinase inhibitors (**A**, **B**), cysteine protease inhibitors (**C**, **D**), and an elastase inhibitor (**E**) on the generation of endostatin in the CM of human limbocorneal epithelial cells cultivated on denuded amniotic membrane. (**A**) Addition of 1,10-phenanthroline. (**B**) Addition of GM-6001. Note the dose-dependent inhibition in the generation of all three endostatin-related molecules. (**C**) Addition of E-64 (cysteine protease inhibitor) (**D**) Addition of cathepsin B inhibitor II (selective cathepsin B inhibitor). Note the dose-dependent inhibition on the generation of 20-kDa endostatin. (**E**) Addition of elastatinal (elastase inhibitor). No obvious effect on endostatin generation was seen. C, control group.

The concentration of cathepsin B in the CM of the HLE/DAM group was initially higher than that in the HLE-only and HLE/IAM groups at 3 weeks, and increased moderately over time (Fig. 4A). Although the difference in cathepsin B concentration between HLE/DAM and HLE/IAM cultures never reached statistical significance, at 5 weeks (3.6 ± 1.6 ng/μg total protein), and >5 weeks (4.4 ± 1.3 ng/μg total protein), the concentration of cathepsin B in the HLE/DAM group was significantly higher than that in the HLE-only group (at 5 weeks, 2.1 ± 0.4 ng/μg total protein, $P = 0.045$; at >5 weeks, 3.0 ± 1.2 ng/μg total protein, $P = 0.019$). The authenticity of the signal was also confirmed by Western blot, which revealed a 37-kDa band, corresponding to the size of procathepsin B (Fig. 4B).

The concentration of cathepsin V in the CM of HLE/DAM was highest in young cultures (at 3 weeks, 50.0 ± 22.7 ng/μg total protein), which was significantly higher than that in the HLE-only group (22.5 ± 16.4 ng/μg total protein, $P = 0.024$) and the HLE/IAM group (14.8 ± 10.0 ng/μg total protein, $P = 0.009$). The concentration of cathepsin V in HLE/DAM CM declined steadily thereafter; however, at 5 weeks (29.5 ± 19.7 ng/μg total protein) and >5 weeks (26.2 ± 15.01 ng/μg total protein), the level was still significantly higher than that in the HLE-only group (at 5 weeks, 16.7 ± 10.4 ng/μg total protein, $P = 0.026$; >5 weeks, 15.8 ± 10.4 ng/μg total protein, $P = 0.036$).

Medium conditioned by old DAM alone contained only a negligible amount of MMPs or cathepsins. Also, in this study, we did not detect either cathepsin K or L in the CM of HLE/DAM by ELISA or Western blot (data not shown).

Generation of Endostatin by Exogenous Cathepsin B and V in Concentrated HLE/DAM CM

The addition of exogenous cathepsin B and V induced a dose- and time- dependent increase in the generation of endostatin in concentrated HLE/DAM CM. This reaction required a weak acidic environment (pH 5.5–6.0), as prior experiments in neutral pH gave no results (data not shown). The addition of 50 nM cathepsin B induced the maximum endostatin generation at 2 hours, and the addition of 250 nM induced the maximum at 1 hour (Fig. 5A). The addition of 10 nM cathepsin V induced maximum endostatin generation at 1 hour, and the addition of 50 nM induced maximum generation at half an hour (Fig. 5B). Note that with extended incubation, the level of endostatin declined again. In Figure 5B at 8 hours, the signal was even weaker than the control, suggesting that the endostatin molecule may eventually be digested by cathepsin V.

DISCUSSION

Felbor et al.[8] and other investigators have reported that the generation of endostatin from the NC1 domain of collagen XVIII requires different sets of proteases: The MMPs cleave and generate fragments of ~28 kDa,[5,6] and cathepsins[8,9] or elastase[10] generate the shorter 20-kDa endostatin fragment. In the cornea, the expression of collagen XVIII[11,12] and the MMP-generated larger fragments have been reported,[5,6] yet previously there has been no report on the secretion of the 20-kDa circulating form of endostatin by the corneal cells.

In this study, we demonstrated that in vitro HLE cells produced the 20-kDa endostatin along with larger MMP-generated fragments. The secretion of endostatin by HLE cells increased as the cultures matured and was further augmented when HLE cells were cultivated on DAM. This increase was not related to increased collagen XVIII α1 mRNA transcription; rather, it was related to the increased MMP-7 and cathepsin B and V concentration in the CM. We found that both MMP-7 and cathepsin B concentrations were increased as culture time increased, whereas MMP-9 and cathepsin V were decreased. Although the MMP-9 level was higher in the HLE/DAM cultures, MMP-9 was reported to be unable to generate cornea-derived 28-kDa endostatin.[5] We also found that elastase activity is not related to

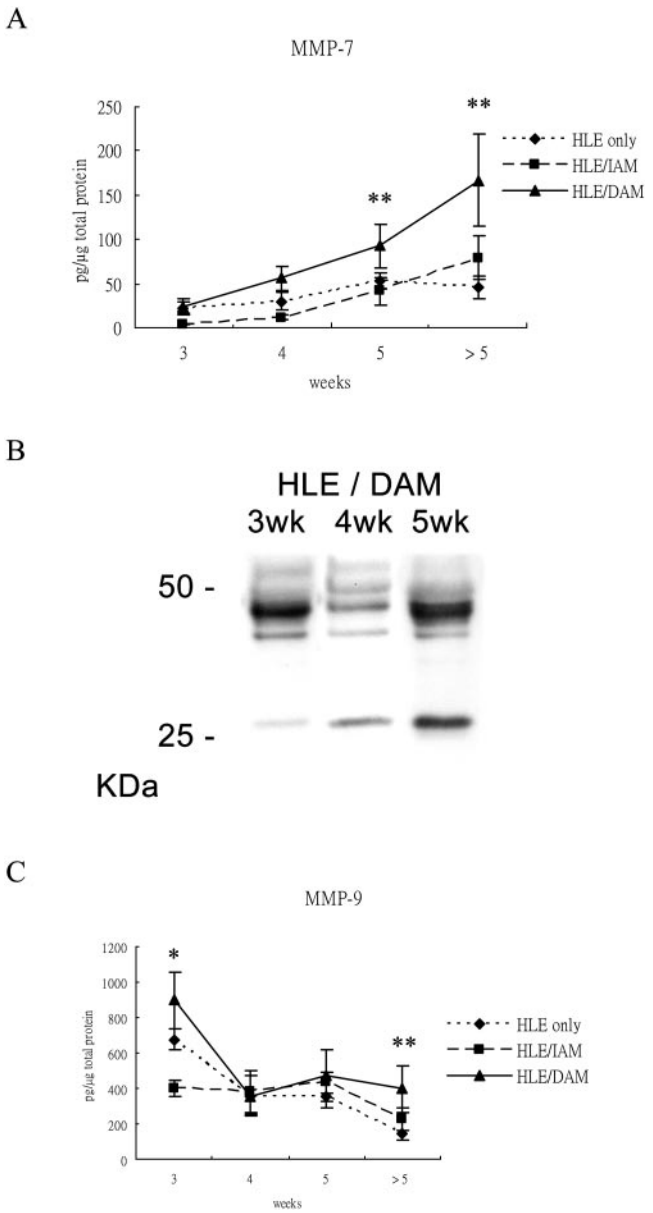

FIGURE 3. ELISA for MMP-7 (**A**) and MMP-9 (**C**) in the CM of human limbocorneal epithelial cells cultivated on a dish (HLE-only), on intact amniotic membrane (HLE/IAM), and on denuded AM (HLE/DAM) at 3, 4, 5, and >5 weeks. The level of MMP-7 increased as the culture time increased and was more prominent in the HLE/DAM group than in the HLE/IAM and the HLE-only groups. The presence of MMP-7 in the HLE/DAM CM was confirmed by Western blot (**B**), which showed an approximately 28-kDa band, corresponding to the proenzyme of MMP-7. **MMP concentration in the HLE/DAM group significantly higher than that in the HLE/IAM and the HLE-only groups. *MMP concentration in the HLE/DAM group significantly higher than that in the HLE/IAM group.

endostatin generation by HLE cells. Because the proteolytic generation of endostatin from the basement membrane collagen XVIII is an extracellular event, protease concentration in the CM rather than in the cell lysate was determined in this study. In addition, the source of endostatin and the proteases in the CM were predominantly from the HLE cells, as old DAM contained very little of either. The role of these proteases in generating endostatin was confirmed by the protease inhibition assay as shown earlier.

The human genome is known to contain at least 11 related but distinct cathepsins.[13] The cathepsins participate in normal protein turnover, antigen processing, and apoptosis[14]; however, their action is not limited in the lysosome. Extracellularly, cathepsins can mediate turnover of the extracellular matrix by direct proteolysis, or by activation of other proteases (e.g., uPA).[15–17] In this study, cathepsins previously known to generate endostatin (cathepsin B, K, and L)[9] were examined. Although there were reports regarding the presence of cathep-

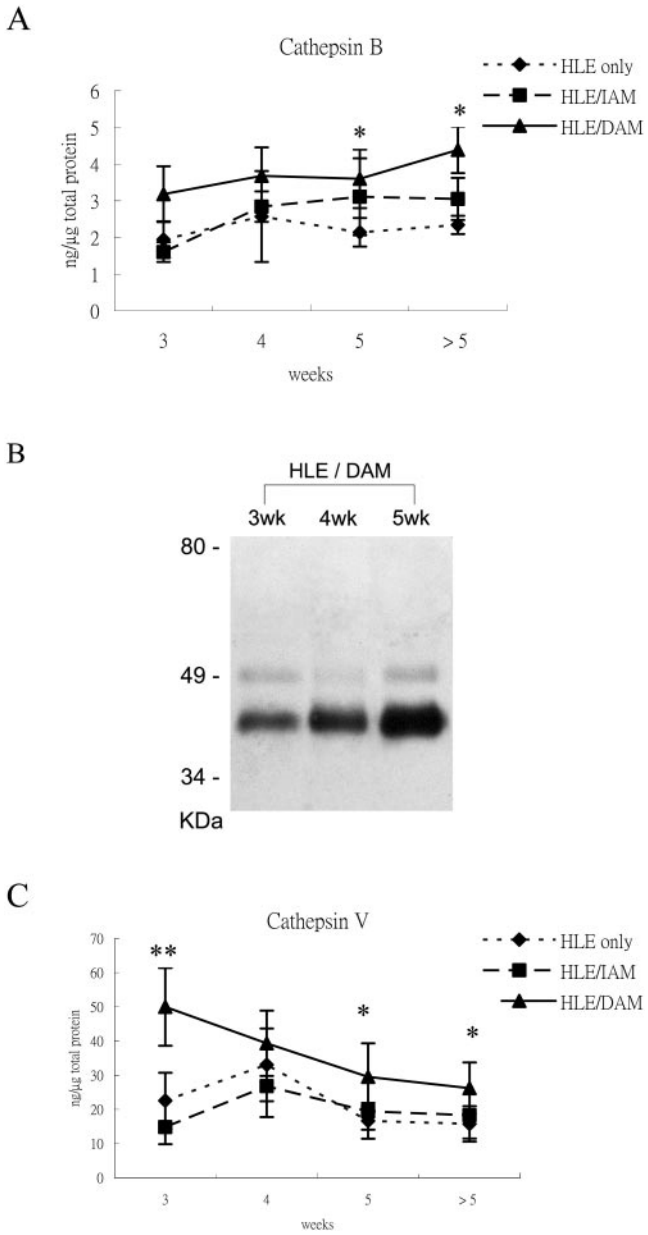

FIGURE 4. ELISA for cathepsin B (**A**) and cathepsin V (**C**) in the CM of human limbocorneal epithelial cells cultivated on dish (HLE-only), on intact amniotic membrane (HLE/IAM), and on denuded AM (HLE/DAM) at 3, 4, 5, and >5 weeks. Note that the level of cathepsin B in the HLE/DAM group and the HLE/IAM group moderately increased as the culture time increased. The presence of cathepsin B in the CM was also confirmed by Western blot (**B**), which revealed a 37-kDa band corresponding to the procathepsin B. **Cathepsin concentration in the HLE/DAM group significantly higher than that in the HLE/IAM and the HLE-only group. *Cathepsin concentration in the HLE/DAM group significantly higher than that in the HLE-only group.

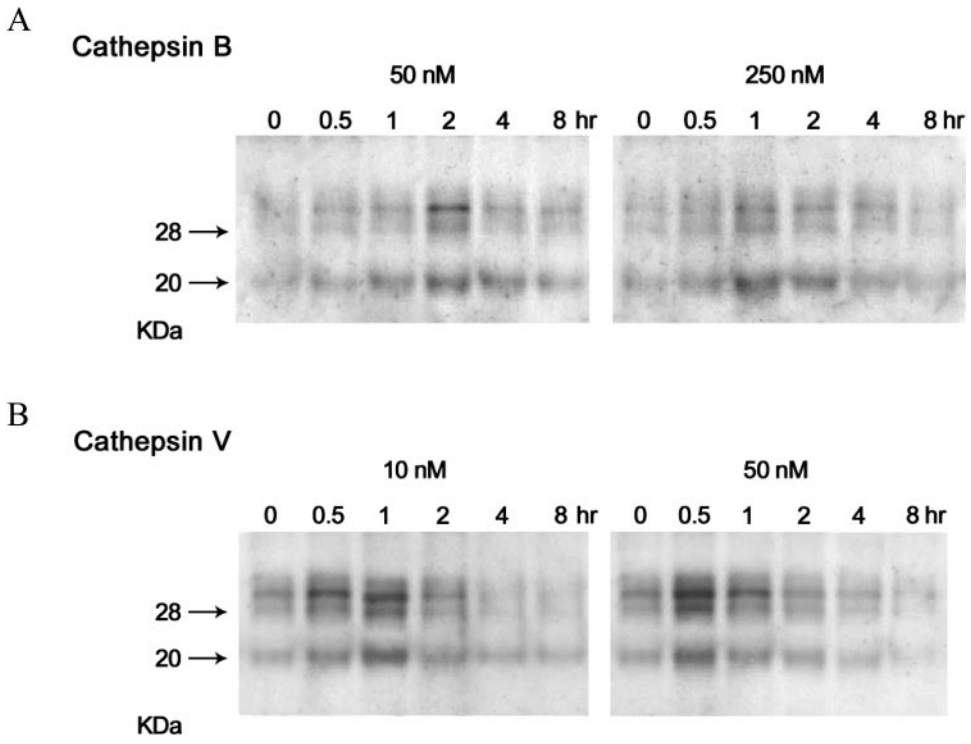

FIGURE 5. Generation of endostatin by exogenous cathepsins. The addition of exogenous cathepsin B (**A**) or V (**B**) to the concentrated CM of human limbocorneal epithelial cells cultivated on denuded amniotic membrane induced a dose- and time-dependent increase in the generation of endostatin. The addition of 50 nM cathepsin B induced maximum endostatin generation at 2 hours, whereas the addition of 250 nM cathepsin B induced maximum generation at 1 hour (**A**). The addition of 10 nM cathepsin V induced maximum endostatin generation at 1 hour, whereas the addition of 50 nM cathepsin V induced maximum generation at half an hour (**B**). Note that with extended incubation, the level of endostatin declined again.

sin K[18] and L[19] in the cornea, neither were found in HLE/DAM CM.

Cathepsin B is the most abundant and widely expressed cathepsin. Oncogenic transformation results in increased cathepsin B (and L) expression, and a relocalization to the plasma membrane adjacent to the underlying basement membrane.[20] The level of cathepsin B may be associated with epithelial differentiation,[21] as we showed that cathepsin B concentration is higher in older HLE/DAM cultures. In the cornea, aberrant cathepsin B expression has been associated with ECM degradation in keratoconus[22,23] or *Pseudomonas* keratitis.[24] In the present study, we showed that cornea-derived cathepsin B may also be involved in the generation of endostatin.

Cathepsins V and L show 78% sequence similarity, consistent with a relatively recent gene duplication of an ancestral cathepsin L gene.[25,26] Although cathepsin L is widely expressed, cathepsin V is expressed in only few tissues, primarily the thymus, testes, and corneal epithelium.[25,27,28] It may be the most abundant peptidase in the corneal epithelium, but its function remains largely unknown.[29,30] A recent study suggested that keratoconus corneas secrete elevated levels of cathepsins V,[31] B, and G.[22,23] This, along with a decreased TIMP-1 level, may play a role in the matrix degradation that is a hallmark of keratoconus corneas.[31]

We estimate that the original cathepsin V concentration in HLE/DAM CM was around 10 to 30 nM, which was similar to that used for in vitro endostatin generation study (10 or 50 nM), whereas the concentration of cathepsin B used in the experiment was at least 10 times higher than that found in the CM (~2–5 nM). This finding suggests that like cathepsin L, cathepsin V is more efficient in generating endostatin than is cathepsin B. However, activation of cathepsins requires a weak acidic environment (pH 5–6 for cathepsin B, and pH 3–6.5 for cathepsin L), but they are unstable at neutral pH.[32] Compared with cathepsin L, cathepsin B is approximately 15-fold more stable at neutral pH.[33] In contrast to the active enzymes, the proenzyme of cathepsin B is stable at neutral pH (which is the form we detected by Western blot). This may explain that although the concentration of cathepsin B is less than that of cathepsin V in the CM, this protease may still play a major role in the generation of endostatin.

In older HLE/DAM cultures, the concentration of MMP-7 and cathepsin B and V was persistently higher than that in HLE-only and HLE/IAM cultures, favoring generation of endostatin. Significant elevation of MMP-7 in matured HLE/DAM cultures is of special interest in that MMP-7 generates not only endostatin but angiostatin as well.[34–36] Although the concentration of cathepsin V is higher in younger than in older cultures, in younger cultures the MMP-7 concentration was very low, thus limiting the production of 28- to 30-kDa fragments and subsequent 20-kDa endostatin.

Recently, Li et al.[37] reported that MMP-9 expression quickly increased in the second week on either HLE/IAM or HLE/DAM, but decreased in the fourth week on HLE/IAM but not on HLE/DAM. It should be pointed out that Li et al. studied basement membrane dissolution and formation and focused on an earlier culture interval (up to 26 days), whereas we quantified MMP-9 expression in more mature cultures (some up to 7 weeks). Nevertheless, we still found that the MMP-9 level was higher in the HLE/DAM cultures at a later stage. However, since MMP-9 declined in older cultures and was reported to be unable to generate cornea-derived 28-kDa endostatin,[5] it seems less likely that MMP-9 plays a major role in generating endostatin in our culture system.

This is a first report describing the generation of endostatin by cornea-specific cathepsin V, which may suggest that by facilitating endostatin formation, this protease probably has an additional physiologic role in the maintenance of corneal avascularity. However, it remains uncertain whether endostatin is the principal antiangiogenic factor regulating corneal avascularity, because to date there has been no study quantitatively comparing the level of endostatin, MMP-7, or cathepsins in corneal and conjunctival epithelial cells.

Using matured HLE/DAM CM rather than the recombinant NC1 domain of collagen XVIII as a substrate, we found that exogenous cathepsin B and V enhanced the production of not only the 20-kDa but also the 28- to 30-kDa fragments. We postulate that soluble collagen XVIII fragments of variable sizes

may present in the CM, and some larger fragments were not readily detected by the antibody we used. Because cathepsins cannot directly generate the 28- to 30-kDa fragments, such an increase was probably due to activation of other proteases by the exogenous cathepsins.[38–40] Nevertheless, with extended incubation, the cathepsins digested all the fragments as previously reported.[9]

In summary, this study signifies the coordinated increase in the secretion of MMPs and cathepsins by HLE cells when cultivated on DAM, which facilitates the generation of endostatin. The enhanced antiangiogenic and anti-inflammatory activity,[41–43] together with the preservation of limbal stem cell population[44,45] justify the clinical transplantation of ex vivo cultivated HLE cells on AM.[46–49] However, whether other newly identified cathepsins in the cornea or the level of endogenous cathepsin inhibitors (e.g., cystatin) may influence endostatin production still awaits further investigation.

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