Original Paper

Journal of Biomedical Science

J Biomed Sci 2003;10:526–534
DOI: 10.1159/000072380

Received: March 20, 2003
Accepted: May 25, 2003

Inhibition of Fibroblast-Induced Angiogenic Phenotype of Cultured Endothelial Cells by the Overexpression of Tissue Inhibitor of Metalloproteinase (TIMP)-3

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Key Words
Limbal fibroblast · Endothelial cell · Angiogenesis · Tissue inhibitor of metalloproteinase-3 · Recombinant adenovirus

Abstract
In this study, we examined the effect of overexpression of tissue inhibitor of metalloproteinase (TIMP)-3 on the angiogenic phenotype expressed by vascular endothelial cells (ECs). ECs were infected with a recombinant adenovirus carrying the TIMP-3 gene at various multiplicities of infection, and TIMP-3 expression by transfected cells was confirmed by Western blotting and reverse zymography. At transfection doses of 6.25, 12.5, 25, 50 and 100 multiplicity of infection, EC migration was reduced to 66, 45, 25, 17 and 5%, respectively, of that of the control. At the multiplicity of infection of 20, capillary tube length was reduced by 80% compared to that of the control. Thus, expression of TIMP-3 by ECs effectively inhibited EC migration and tube formation. Overexpression of TIMP-3 by ECs may be considered a gene therapy strategy for the treatment of pathological angiogenesis such as cancer and diabetic retinopathy.

D.H.-K.M. and J.-I.C contributed equally to this work.

Introduction

Angiogenesis is the growth of new capillaries from preexisting venules. It is essential for embryogenesis and development, and in adults, it is also important for reproductive function and wound healing [17]. Angiogenesis is also implicated in the pathogenesis of a variety of disorders, including proliferative diabetic retinopathy, corneal neovascularization, rheumatoid arthritis, psoriasis, and tumor growth and metastatic spread [20, 25]. The angiogenic phenotype of a tissue is dependent upon the local balance of angiogenic factors and inhibitors. Angiogenic factors, including vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), are produced by a number of cells, including vascular endothelial cells (ECs), smooth muscle cells, leukocytes, tumor cells and fibroblasts [11, 21, 24, 44]. During wound repair, interaction of fibroblasts and ECs is believed to modulate the formation of granulation tissue. Fibroblasts

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have been shown to induce an angiogenic phenotype of ECs in a collagen gel coculture system [36, 38, 44]. In rat mesentery, it was found that fibroblasts are closely associated with capillary sprouts during the initial stages of capillary growth, and myofibroblasts isolated from rat epididymal adipose tissue have been shown to stimulate angiogenesis [42]. These observations indicate that pericapillary fibroblasts may modulate angiogenesis through a paracrine mechanism [39]. In addition, the hypoxia-related angiogenesis induced by fibroblasts via growth factor secretion (e.g. VEGF) is considered an important cause of tumor angiogenesis [30].

The response of ECs to angiogenic factors includes digestion of the vascular basement membrane, migration toward the source of the signal, proliferation and elongation to form capillary tubes. During angiogenic processes, there is a transition of ECs from a proteolytic state to an antiproteolytic state through regulation of production of metalloproteases and inhibitors [31]. In the initial steps, basement membrane and stroma proteolysis is mediated by components of the plasminogen activator-plasmin system and members of the matrix metalloprotease (MMP) family [35]. MMP activity is tightly regulated at multiple levels, including transcriptional control, proenzyme activation and inhibition of activated enzymes by nonspecific inhibitors or by tissue inhibitors of metalloproteases (TIMPs) [6, 14, 19].

TIMPs are a group of zinc-binding, cysteine-containing proteins, and so far, 4 homologous forms (TIMP-1 to -4) have been identified. Because of the potential of antiangiogenesis in cancer treatment, the roles of MMPs/TIMPs in regulating the angiogenic phenotype of ECs during angiogenic processes have now become the focus of intensive research. TIMP-3 [5] is unique among the 4 TIMPs in that it binds tightly to the extracellular matrix (ECM) and induces apoptosis in various cells, including tumor cells [1, 2, 7, 9, 10, 43], vascular smooth muscle cells [8, 18] and retinal pigment epithelial cells [34]. It is therefore considered a candidate for cancer gene therapy, for treatment of restenosis following bypass surgery, Sorsby's fundus dystrophy and age-related macular degeneration. However, at present, little is known about the expression of TIMP-3 by ECs and its effect on angiogenesis [3].

In a previous report, we showed that fibroblasts isolated from the human ocular surface (cornea, limbus and conjunctiva) secreted VEGF and bFGF to promote differentiation of ECs into capillary tubes in collagen gel, suggesting that fibroblasts may be considered 'feeder cells' for studying EC morphogenesis [32]. Morphological changes in ECs require well-controlled MMP/TIMP activity. To further understand the antiangiogenic activity of TIMP-3 and the effect of TIMP-3 overexpression on the phenotype of ECs, we transfected the TIMP-3 gene into ECs and cocultured transfected cells with limbal fibroblasts to study changes in their phenotypes. We show that human limbal fibroblast-induced expression of angiogenic phenotypes of vascular ECs is inhibited by TIMP-3 overexpression in ECs.

Materials and Methods

Materials

MCDB 107 medium was purchased from JRH Biosciences (Lenexa, Kans., USA). Dulbecco's modified Eagle's medium (DMEM), Ham's F-12 nutrient mixture and fetal bovine serum (FBS) were from Gibco BRL (Gaithersburg, Md., USA). TIMP-3 polyclonal antibody and peroxidase-conjugated secondary antibody were from Santa Cruz Biotechnology (Santa Cruz, Calif., USA). All other chemicals were from Sigma Chemical Co. (St. Louis, Md., USA).

Cell Culture

ECs were isolated from human umbilical cord veins by type V collagenase as previously described [13]. Cells were cultured in MCDB 107 medium supplemented with 2% FBS and a fibroblast growth factor-enriched fraction of porcine brain extract (1 μg/ml). Cells were incubated at 37°C in a humidified incubator containing 95% air and 5% CO_2, and were used below the fifth passage.

Human limbal fibroblasts were grown from tissue explants in HSE medium (25% Ham's F-12, 75% DMEM, 5×10^{-9} *M* sodium selenite, 20 m*M* Hepes, 1.2 g/l sodium bicarbonate, 0.4 g/l histidine and 5×10^{-5} *M* 2-aminoethanol), supplemented with 10% FBS. Cells were used between passages 3 and 5. At least 3 replicates were performed for each experiment.

Generation of Recombinant Adenovirus Carrying the TIMP-3 Gene

Recently, He et al. [23] reported a simplified method (AdEasy system) using highly efficient homologous recombination machinery present in bacteria to significantly decrease the time required to generate usable viruses. We applied this system to generate the recombinant adenovirus (RAd) for TIMP-3 gene transfer. The starting plasmids, pAdEasy-1 and pAdTrack-CMV, were kind gifts from Dr. Tong-Chuan He (Howard Hughes Medical Institute, John Hopkins University). The full-length human TIMP-3 cDNA sequence was obtained by RT-PCR from epithelial cells of the human amniotic membrane [22]. The 5′-primer was CAG CAG GGG CAA TGA CCC CTT G, and the 3′-primer was TCT GGC GCT CAG GGG TCT GTG G, encompassing a 656-bp sequence. The PCR product was ligated with an amplifyng vector (PCR Script, Stratagene), then transfected into DH10B cells. Colonies with inserts were picked out and further purified. Inserts with the correct orientation were picked out, grown and sequenced. The PCR Script-TIMP-3 plasmid was then ligated with the pAdTrack-CMV plasmid. Possession of the correct insert was confirmed by *Hin*dIII digestion. PAdTrack-CMV-TIMP-3 and pAdEasy-1 plasmids were then linearized and electro-

porated into BJ-5183 cells, where homologous recombination took place. Colonies with recombined plasmid were grown, and MiniPrep plasmid DNA was prepared and confirmed by *Bam*H1 digestion. The PAdTrack-CMV-TIMP-3-pAdEasy-1 genome was then transfected into DH10B cells. Plasmid DNA was purified and transfected into 293 cells by a liposome method (LipofecTAMINE, Gibco BRL). Virus production was evidenced by lytic zone formation and green fluorescence protein (GFP) expression. The virus was purified, mass-produced and titrated by plaque assay.

Infection Protocol

To determine the infection efficiency of ECs by the viral vector, 1×10^5 ECs were plated and incubated in 24-well culture plates overnight. Then, cells were washed twice with phosphate-buffered saline (PBS) and infected with RAdGFP or RAdTIMP-3 at multiplicities of infection of 12.5, 25, 50, 100, 250 and 500 in serum-free medium. After incubation for 2 h, fresh medium containing 2 × supplements was added. Cells were further incubated for 48 h, and the intensity of the GFP was examined using a fluorescence microscope.

Preparation of Cell Lysate, Conditioned Medium and Matrix Fractions

Subconfluent cells were infected with RAdGFP or RAdTIMP-3 at 20 plaque-forming units (PFU)/cell, or were uninfected. Twenty-four hours later, cells were lysed with 1 × immunoprecipitation buffer [1% Triton X-100, 150 m*M* NaCl, 10 m*M* Tris-HCl, pH 7.4, 1 m*M* EGTA, 0.2 m*M* sodium vanadate (Na_3VO_4), 0.2 m*M* PMSF and 0.5% NP-40] and centrifuged at 14,000 rpm for 15 min at 4°C in a microcentrifuge to remove insoluble materials. The supernatant was collected and stored at −20°C until use.

The conditioned medium (CM) and matrix fraction were prepared 72 h after infection. CM was collected and centrifuged to remove floating cells, and was concentrated by Amicon ultrafiltration using a YM-10 membrane (molecular weight cutoff, 10 kD; Amicon, Danvers, Mass., USA). For matrix fraction preparation, cell layers were washed with cold PBS, incubated with 2% Triton X-100 in PBS for 10 min on ice and rewashed several times with cold PBS. The material which remained on the culture surface was scraped off and collected with Laemmli sample buffer.

Immunoprecipitation and Western Blotting

Aliquots of cell lysate or CM equivalent to the desired cell number were immunoprecipitated with polyclonal TIMP-3 antibody. The immunocomplex was precipitated with protein A-sepharose and washed with TBS buffer (25 m*M* Tris-HCl, pH 7.4, 150 m*M* NaCl and 0.1 m*M* Na_3VO_4). The pellets were resuspended in 2 × electrophoresis sample buffer, boiled and centrifuged. The supernatants were fractionated on 12% SDS-PAGE. The resolved protein bands were electrophoretically transferred to nitrocellulose membranes and blotted with a 1:10,000 dilution of a goat anti-human TIMP-3 antibody. Blots were further incubated with a 1:10,000 dilution of a peroxidase-conjugated anti-goat IgG, and the immunoreactive protein bands were visualized by the ECL system (Amersham Pharmacia Biotech, Piscataway, N.J., USA).

Reverse Zymography

To assay metalloproteinase inhibitor activity after RAdTIMP-3 infection, samples were fractionated on 12.5% SDS polyacrylamide gels containing 1 mg/ml swine skin gelatin and collagenase, referred to as the protease/substrate gel, and electrophoresed. After electrophoresis, SDS in the gel was removed by incubation with 2 changes of 2.5% (v/v) Triton X-100 for 1 h. The gel was then incubated overnight in substrate buffer (50 m*M* Tris, pH 8.0, 50 m*M* NaCl, 10 m*M* $CaCl_2$, and 0.05% Brij 35) at 37°C. After incubation, the gel was stained with 0.1% Coomassie brilliant blue. Protection of the gelatin digestion in the gel by the presence of TIMP-3 led to the production of dark bands against a lighter background.

Cell Migration Assay

Endothelial cell migration was measured by the razor wound method [37]. Cells with or without prior infection with RAdGFP or RAdTIMP-3 were plated in 35-mm collagen-coated dishes and grown to confluence. Cultures were wounded by gently pressing a razor through the cell layer into the plastic wall to mark the origin, then drawing the razor on 1 side through the monolayer to remove the cells. The medium was replaced with fresh medium twice to remove floating cells. Cell migration was permitted for up to 10 h and was stopped with 4% formaldehyde in PBS. The number of cells that had traversed across the original line was counted.

In vitro EC Tube Formation

ECs and fibroblasts were separately suspended in collagen gels and cocultured using the Falcon transwell system (Becton Dickinson, Franklin Lakes, N.Y., USA), with fibroblasts in the outer well and ECs in the insert. Details for suspending cells in collagen gels have been described previously [32]. One million fibroblasts in 1 ml of collagen gel were cultured for 24 h, and the medium was replaced with the same medium. Subconfluent ECs were harvested 24 h after RAdGFP or RAdTIMP-3 infection (both at 20 PFU/cell). Cells at 5×10^5 were suspended in 1 ml of collagen solution (1 mg/ml) and added to the insert. The EC-collagen mixture was allowed to gel at room temperature, and the insert was placed to allow interactions between ECs and fibroblasts. Cultures were maintained for 3 days, and then ECs were fixed with 4% paraformaldehyde in PBS. Endothelial tubes were quantitated using a computerized image analysis system connected to a phase-contrast microscope (IMT-2, Olympus). Total capillary tube length in 5 random fields per insert was computed and expressed as millimeters per insert.

Statistics

Total capillary tube lengths in the two groups were compared using a 2-sample unpaired t test or the Mann-Whitney test when appropriate. All p determinations were 2 sided and were considered significant when $p < 0.05$.

Results

Characterization of RAdTIMP-3-Infected ECs

ECs were infected with RAdGFP or RAdTIMP-3 at various PFU/cell, and infected cells were identified by positive GFP expression (fig. 1). At 50 PFU/cell, close to 100% of cells were infected and showed strong green fluorescence (fig. 1C, G). At this level, the morphology and viability of uninfected and RAdGFP- or RAdTIMP-3-infected cells were similar. Figure 2A shows that the growth rates of uninfected, and RAdGFP- or RAdTIMP-

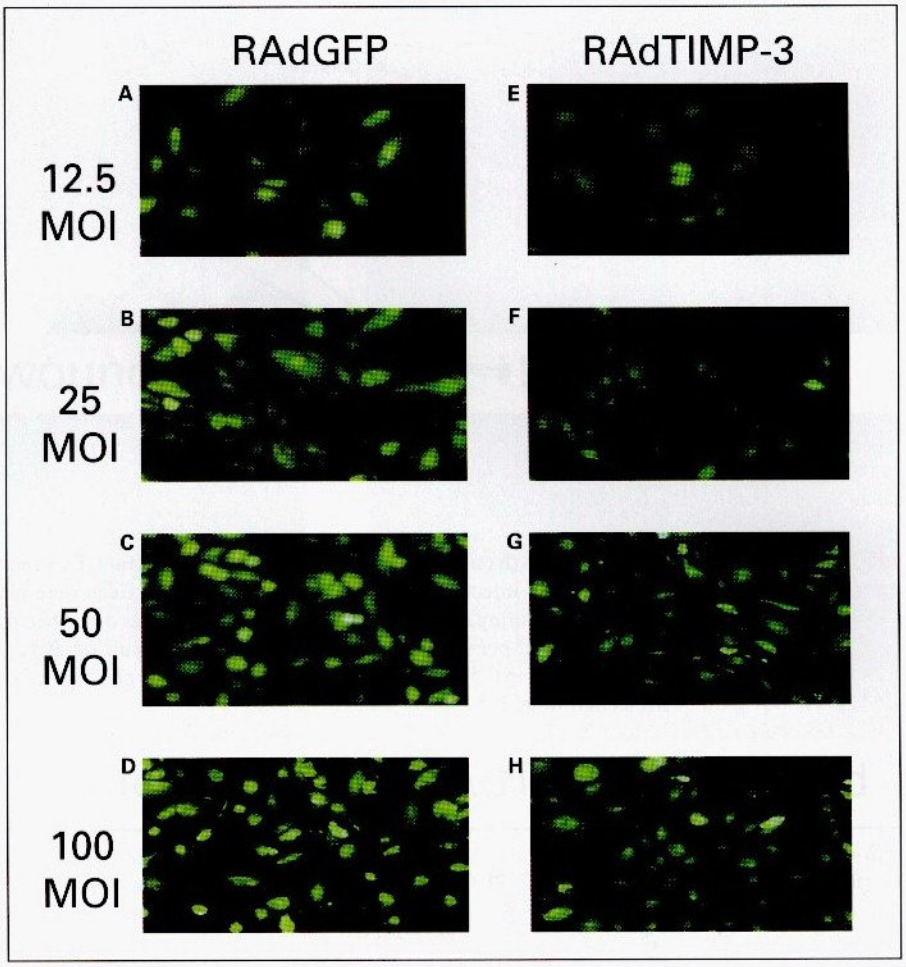


Fig. 1. Evaluation of transfection efficiency. Vascular ECs at 1×10^5 were infected with increasing multiplicities of infection of RAdGFP (**A–D**) or RAdTIMP-3 (**E–H**). The multiplicity of infection (MOI) used was 12.5 for panels **A** and **E**, 25 for **B** and **F**, 50 for **C** and **G** and 100 for **D** and **H**. Original magnification ×100.

3-infected (20 PFU) cells did not significantly change. No cell apoptosis was observed in infected cells as judged by agarose gel fractionation of genomic DNA (fig. 2B). At 20 PFU/cell, about 80% of ECs were infected and showed moderate green fluorescence. To avoid any detrimental effect due to overexpression of the GFP, we chose to infect ECs with RAdGFP or RAdTIMP-3 at 20 PFU/cell for the rest of the experiment.

To see if RAdTIMP-3-infected ECs express TIMP-3 protein, cell lysate, CM and matrix fractions were prepared, fractionated on 12% SDS-PAGE and probed with anti-human TIMP-3 antibody. Figure 3 shows that a 21-kD protein band was detected in the matrix fraction, but not in the cell lysate or CM fractions of RAdTIMP-3-infected cells. The 21-kD protein band was not detected in cell lysate, CM or matrix fractions of the control nor of the RAdGFP-infected cells. The MMP-inhibitory activity of this 21-kD protein in the matrix fraction of RAdTIMP-3-infected cells was examined by gelatin-collagenase reverse zymography. Figure 4 shows that collagenase-inhibitory activity with a molecular mass of around 21 kD was present in the matrix fraction of RAdTIMP-3-infected, but not of uninfected nor of RAdGFP-infected cells. These results indicate that functional TIMP-3 protein is expressed in RAdTIMP-3-infected cells.

Effect of TIMP-3 Overexpression on EC Migration

Exogenous TIMP-3 has been shown to inhibit EC migration in culture [3]. In this study, we examined the effect of RAdTIMP-3 overexpression on the migratory activity of ECs. As shown in figures 5 and 6, transfecting cells with RAdTIMP-3 markedly reduced cell migration in a titer-dependent manner. At multiplicities of infection of 6.25, 12.5, 25, 50 and 100, the number of ECs migrating across the original line was reduced to 66, 45, 25, 17 and 5%, respectively, of that of the control. Cells infected with RAdGFP also exhibited reduced migratory activity, however, to a lesser extent (fig. 6).

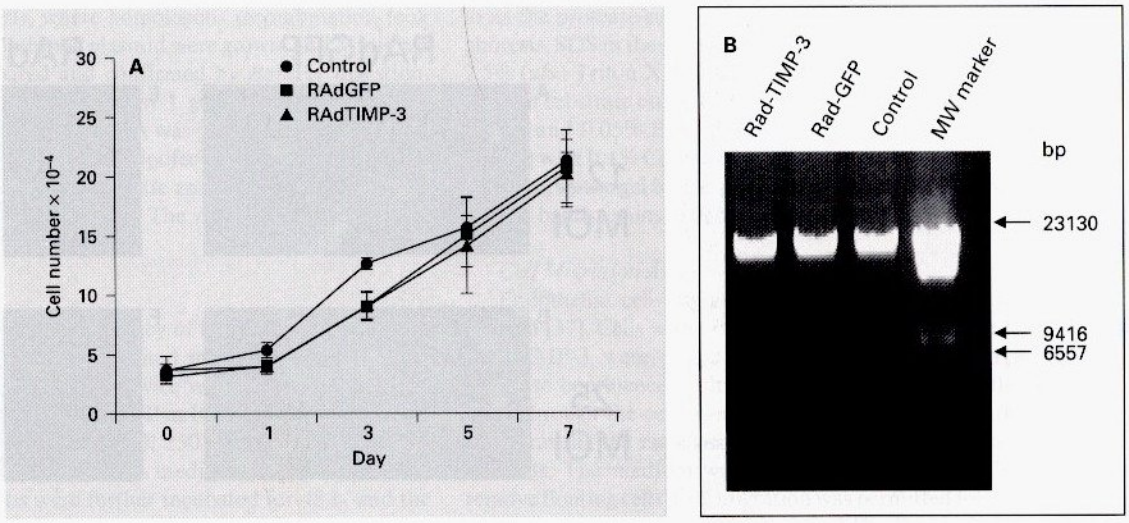
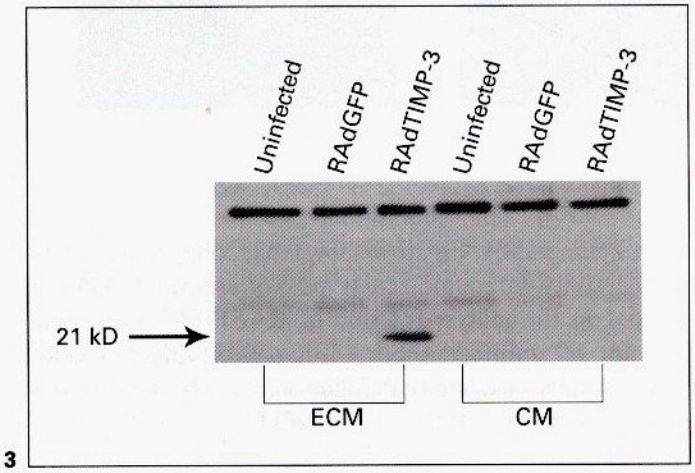


Fig. 2. A Growth curve of ECs with or without transfection. ECs were transfected with RAdGFP or RAdTIMP-3 at a multiplicity of infection of 20, or were untransfected. Cells were plated at 2,500 cells per 1-cm well in MCDB 107 medium containing 2% FBS and porcine brain extract as described in Materials and Methods. On the days indicated, the cell number per well was determined by Coulter counting. **B** Total cell DNA was prepared and fractionated on a 1% agarose gel in 0.5 × TBE. No DNA degradation was observed in the untransfected and transfected samples. MW = Molecular weight.



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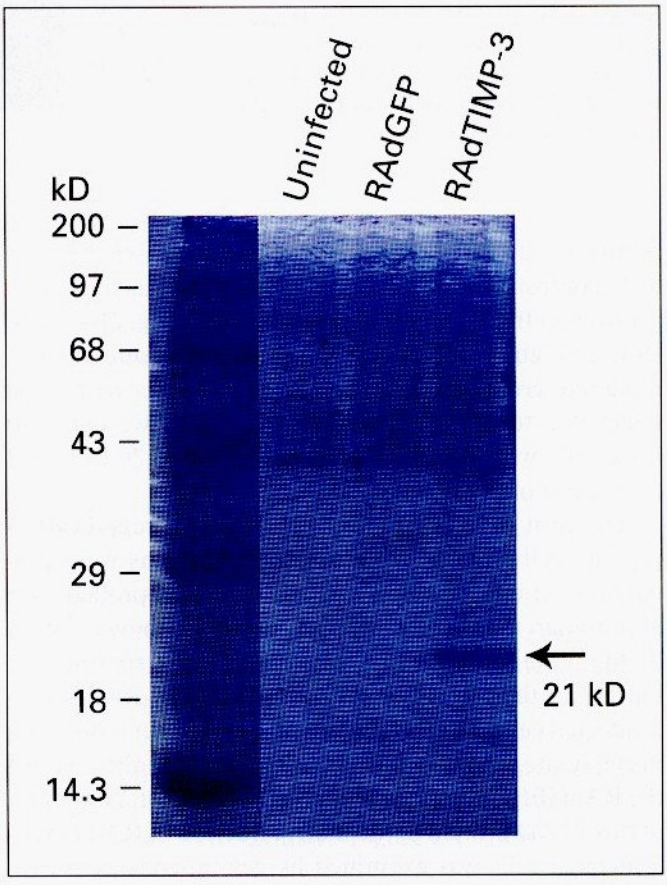

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Fig. 3. Immunoblot analysis of the presence of TIMP-3 in the ECM and CM of ECs. The ECM fraction and concentrated CM were prepared as described in Materials and Methods and were immunoprecipitated with anti-human TIMP-3 antibody. The precipitated materials were loaded at an equivalent of 1×10^6 cells per lane.

Fig. 4. Metalloprotease-inhibitory activity of TIMP-3 produced by RAdTIMP-3-infected ECs. Reverse zymography of the ECM fraction showed an inhibitory activity at a molecular mass of around 21 kD. No activity was detected in the ECM fractions of RAdGFP-infected cells (lane 2), nor in the control (uninfected) cells (lane 1).

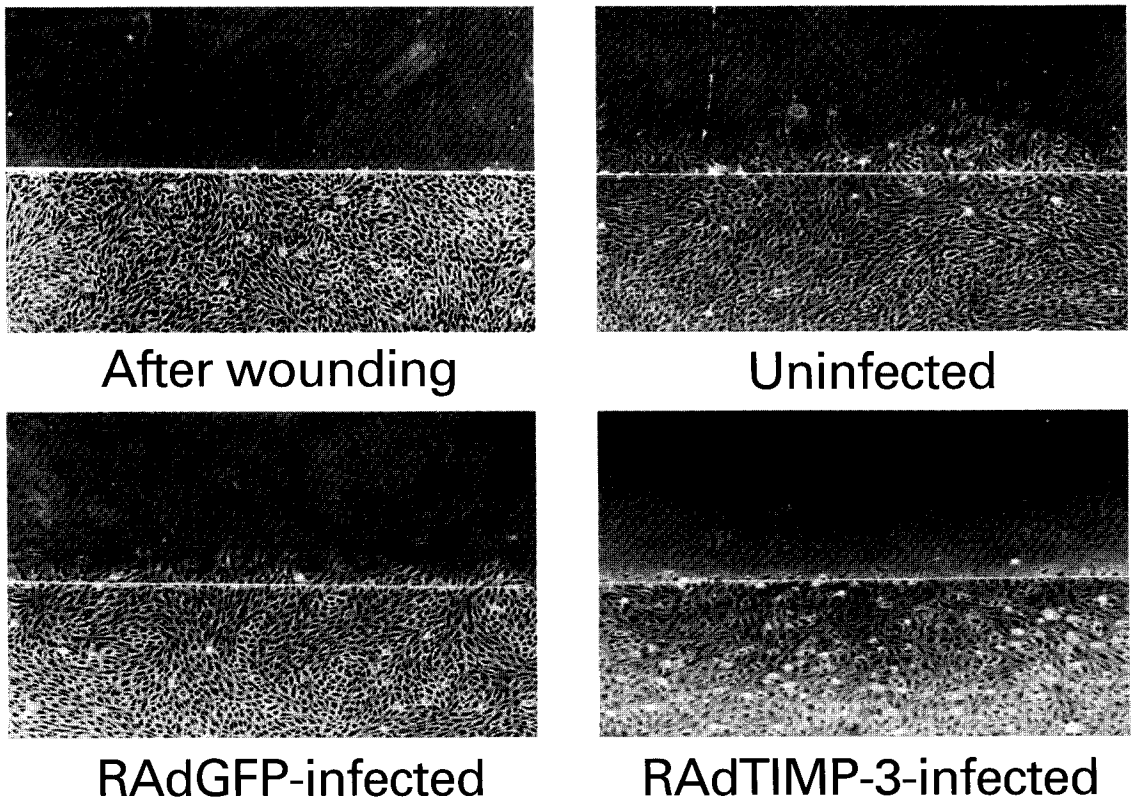

Fig. 5. Effects of TIMP-3 overexpression on endothelial cell migration. Cells were plated on collagen-coated 35-mm dishes and were grown to confluence. Cultures were infected with RAdTIMP-3 or RAdGFP at a multiplicity of infection of 12.5, then were wounded 24 h later with a razor (**A**) and further incubated for 10 h to examine cell migration across the original line. **B** Uninfected cells. **C** RAdGFP-infected cells. **D** RAdTIMP-3-infected cells. Original magnification × 40.

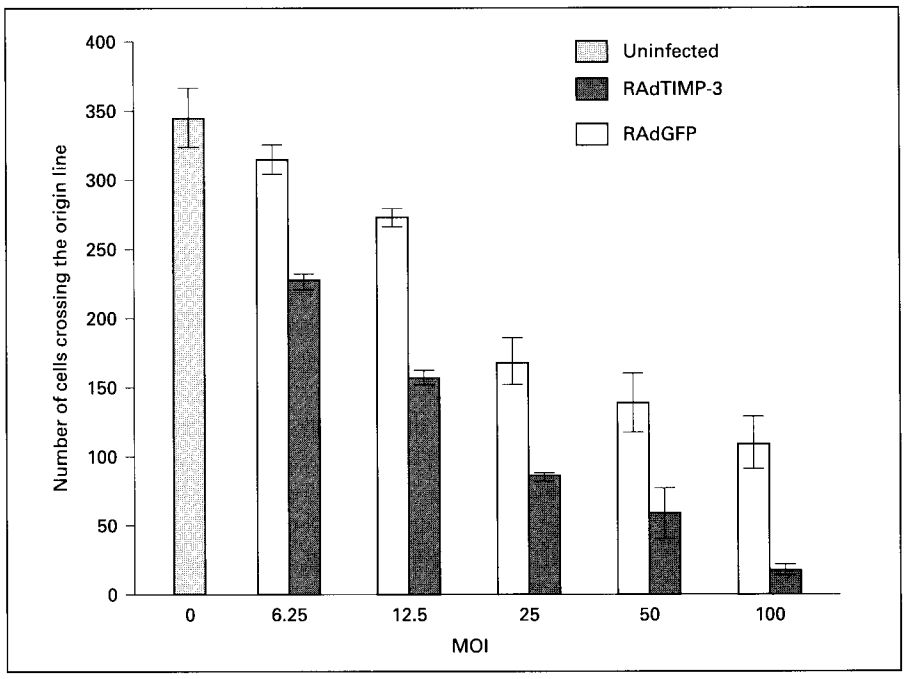

Fig. 6. Effects of TIMP-3 overexpression on endothelial cell migration. Cells were infected with RAdTIMP-3 or RAdGFP at a multiplicity of infection (MOI) of 6.25, 12.5, 25, 50 or 100, or were not infected (0; control). The number of cells crossing the original line 10 h after wounding is expressed as the mean ± SD.

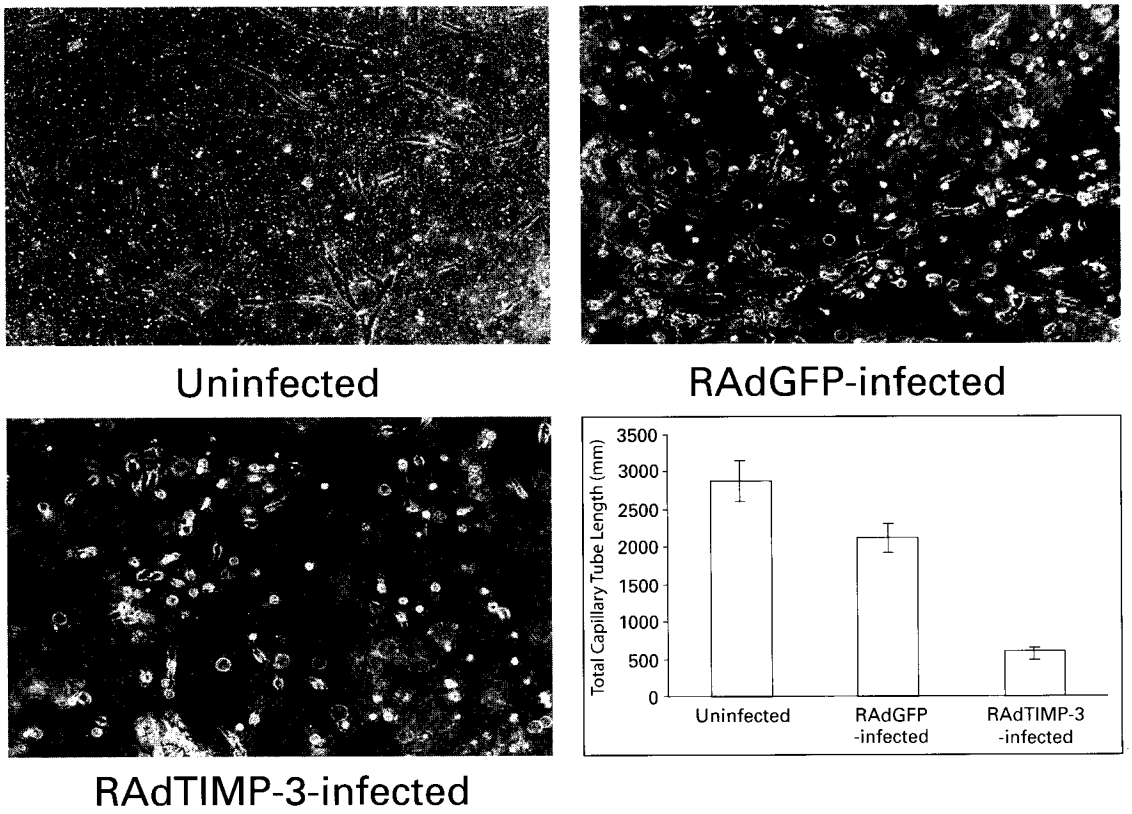

Fig. 7. Effects of TIMP-3 overexpression on fibroblast-stimulated endothelial cell tube formation. ECs were either uninfected or infected with RAdGFP or RAdTIMP-3 at a multiplicity of infection of 20. After infection, cells were suspended in collagen gel and cocultured with 1×10^6 limbal fibroblasts. On day 3, cultures were fixed and photographed. Total tube length per insert was calculated and is expressed as the mean ± SD. Original magnification × 100.

Effects of TIMP-3 Overexpression on EC Capillary Tube Formation

In a previous report [32], we showed that most of the fibroblast-induced angiogenic activity is due to elaboration of VEGF and bFGF by fibroblasts. To determine whether deposition of TIMP-3 in the ECM affect the fibroblast-induced angiogenic phenotype of ECs, control, RAdTIMP-3- or RAdGFP-infected ECs were separately cocultured with human limbal fibroblasts for 3 days, and their tube-forming activities were compared. As shown in figure 7, RAdTIMP-3-infected ECs exhibited reduced capacity to form capillary-like tubes. The tube length formed by uninfected cells was 2,871 ± 281 mm/insert. At a multiplicity of infection of 20, the tube lengths formed by RAdTIMP-3- and RAdGFP-infected cells were 575 ± 81 mm/insert (an 80% reduction) and 2,114 ± 182 mm/insert (a 26.4% reduction), respectively. The total tube length in the RAdTIMP-3 group was significantly shorter than that in the control group ($p = 0.0003$) and in the RAdGFP group ($p = 0.011$).

Discussion

In a previous study, we showed that human limbal fibroblasts produce VEGF and bFGF to stimulate EC tube formation [32]. In the present study, we show that fibroblast-stimulated tube formation by ECs is suppressed by TIMP-3 overexpression in ECs. Fibroblasts have also been shown by others to express VEGF, fibroblast growth factor, platelet-derived growth factor and insulin-like growth factor and to induce EC tube formation [40]. In the same previous study [32], we found that all 3 types of ocular surface fibroblasts, i.e. conjunctival, limbal and corneal fibroblasts, induced prominent capillary tube formation in vitro, an indicator of the proan-

giogenic phenotype. In contrast, limbal, but not conjunctival, epithelial cells inhibited the fibroblast-induced capillary morphogenesis, suggesting a unique role of limbal epithelial cells (progenitors of corneal epithelial cells) in inhibiting corneal neovascularization [32]. On the basis of this study, we suggest that EC-fibroblast coculture offers a system for analyzing the effects of cell-cell and cell-matrix interactions in EC angiogenic phenotype expression.

To date, all 4 isoforms of TIMPs have been identified in the cornea, and are thought to be involved in the maintenance of corneal avascularity [29, 33]. Although TIMP-3 has been reported to inhibit chemotaxis, collagen gel invasion and capillary morphogenesis of ECs in vitro, and to inhibit bFGF-induced angiogenesis in vivo [4], little is known about the expression of TIMP-3 in ECs and the antiangiogenic activity produced by TIMP-3 overexpression.

Recently, Bungo et al. [12] reported that murine brain microvascular ECs constitutively expressed a high level of TIMP-3 but not TIMP-1 mRNA. The expression of TIMP-1 was dramatically upregulated by proinflammatory cytokines like IL-1β and TNF-α, while the level of TIMP-3 mRNA was significantly decreased following treatment with these two cytokines [12]. Because TIMP-3 specifically binds to ECM components of the basement membrane [45], the presence of TIMP-3 may protect the vascular basement membrane from proteolytic degradation, prohibiting vascular sprout formation in the initial step of angiogenesis. Unlike murine microvascular ECs, we showed that normal human umbilical vein ECs and RAdGFP-infected ECs do not produce detectable amounts of TIMP-3 protein, and it was only after RAdTIMP-3 transfection that they produced detectable amounts of TIMP-3. Microvascular ECs have been reported to differ from macrovascular ECs in their expression of MMP/TIMP [27], and species differences could be another cause for such discrepancies.

Inhibition of EC migration has previously been reported following adenovirus-mediated TIMP-1 gene transfer [15]. In the present study, we show that adenovirus-mediated TIMP-3 gene transfer inhibits both EC migration and tube formation in a dose-dependent manner. We also show that infection with the control vector, RAdGFP, also inhibits cell migration and tube formation, however, to a much lesser extent. Since RAdGFP-infected cells produced no detectable activity of the metalloprotease inhibitor when analyzed by reverse zymography and Western blotting, the reduced cell migration and tube formation exhibited by RAdGFP-infected cells is probably due to the predominant production of viral and GFP proteins driven by the strong CMV promoter. In contrast to inducing tumor and vascular smooth muscle cell apoptosis by RAdTIMP-3 [1, 2, 7–10, 18], in this study, we showed that RAdGFP- or RAdTIMP-3- transfected cells showed no sign of apoptosis. Further, a level of infection as low as 20 PFU/cell was used to maintain the viability of infected cells and to avoid possible detrimental effects due to overexpression of the GFP.

The mechanism by which TIMP-3 inhibits angiogenesis remains to be elucidated. Previous studies have shown that TIMP-3 might directly inhibit the EC signaling cascade required for angiogenic morphogenesis, or inhibit the matrix proteases elaborated by ECs that are essential for their migration and matrix remodeling [41]. Alternatively, by preventing matrix proteolysis, TIMP-3 may prevent the release of angiogenic factors sequestered in the ECM, which are required for the angiogenic switch of ECs. Because of its unique ability to bind to the ECM, we speculate that the inhibition of EC migration and tube formation by TIMP-3 shown in the present study is most likely due to its inhibition of matrix proteolysis at the onset of EC migration and morphogenesis. The results of the present study suggest that overexpression of TIMP-3 may be considered as a gene therapy strategy for the treatment of pathological angiogenesis such as that found in cancer and diabetic retinopathy.

Acknowledgements

This work was supported by grants from the National Science Council, Taiwan, to J.-K.C. (NSC89-2320-B182058) and to D.H.-K.M (NSC90-2314-B-182A-143).

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