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Biochemical and Biophysical Research Communications 345 (2006) 523–529
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Modulation of angiogenesis by tissue inhibitor of metalloproteinase-4 ☆

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Received 15 March 2006
Available online 27 April 2006

Abstract

Despite the importance of MMP activity in the regulation of angiogenesis, relatively little is known about the role of TIMP-4, the most recently discovered endogenous MMP inhibitor, in modulating neovascularization. It has largely been assumed that all TIMPs are capable of inhibiting angiogenesis in vivo. However, it is now widely appreciated that TIMPs-1, -2, and -3 differ significantly in their ability to modulate angiogenic processes in vitro and angiogenesis in vivo. In order to study the effect of TIMP-4 in controlling angiogenesis, we have cloned and expressed TIMP-4 in a *Pichia pastoris* expression system, purified it to homogeneity, and tested its ability to regulate angiogenesis in vivo and in vitro. Our studies demonstrate that TIMP-4 is an inhibitor of capillary endothelial cell migration, but not of proliferation or of angiogenesis in vivo.

Keywords: MMP; Matrix metalloproteinase; TIMP; Tissue inhibitor of metalloproteinase; Angiogenesis; Endothelial cell

TIMP-4, the newest member of the tissue inhibitors of metalloproteinase family, was first cloned in 1996 after having been identified using expressed sequence tag sequencing and homology searching [1]. Unlike other family members, TIMP-4 expression was found to be either absent or present at very low levels in most tissues, with the highest levels detected in the heart. As with other TIMP family members, K_i values for the inhibition of MMPs were in the range of 10–100 nM, with differences being largely attributed to differences in affinity for various MMP family members [2,3]. Although TIMP-4 is a bona fide MMP inhibitor, it has not been shown to inhibit members of the ADAM family of metalloproteinases, as does TIMP-3 [4,5].

Structurally, TIMP-4 is most similar to TIMP-2, sharing approximately 50% sequence similarity and 70% homology. In fact, like TIMP-2, TIMP-4 has been shown to bind to the PEX domain of MMP-2 [6] although it does not promote pro-MMP-2 activation as does TIMP-2 [7]. To date, TIMP-2 is the only TIMP shown to consistently inhibit angiogenesis regardless of the assay system used [8–10]. More recently, two distinct anti-angiogenic activities of TIMP-2 have been characterized, one in the N-terminus and one in the C-terminus. The latter domain, which has no MMP-inhibitory activity, has been shown to inhibit angiogenesis in a variety of in vivo systems. This activity has been further shown to reside in the 24-amino acid domain comprising Loop 6 of TIMP-2 [9]. Given the high degree of sequence similarity between TIMP-2 and TIMP-4, including in the sequences comprising Loop 6, we hypothesized that it might be also possible that TIMP-4 shares this second anti-angiogenic activity with TIMP-2.

Very little is known about the ability of TIMP-4 to directly modulate angiogenesis. In studies where TIMP-4 overexpression in breast cancer cells was reported to decrease tumor growth, a decrease in microvascular density was also observed when compared to control tumors [11]. However, in similar studies in which TIMP-4 was instead delivered by adenoviral transfection in the same tumor system no decrease in tumor growth was observed [12], suggesting that

☆ *Abbreviations:* MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; EC, endothelial cell; CAM, chorioallantoic membrane.

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doi:10.1016/j.bbrc.2006.04.083

the effects previously reported on microvascular density might not be directly due to the activity of TIMP-4. It has been suggested that these studies, along with studies in which overexpression of TIMP-4 inhibited Wilm's tumor growth [13], highlight the potentially different effects that TIMP-4 might exert depending on the tumor system [24], but also suggest that TIMP-4 may not be modulating angiogenesis, a mechanism of tumor growth that is universal to these tumor systems. It has largely been assumed that TIMP-4, as an MMP inhibitor, is also an inhibitor of angiogenesis in vivo, yet no systematic analysis of TIMP-4's ability to regulate angiogenesis has been conducted. The current study focuses on characterizing the anti-angiogenic activities of TIMP-4 in vitro and examines the ability of TIMP-4 to directly inhibit angiogenesis in vivo.

Materials and methods

Cloning and expression of hTIMP-4. Human *TIMP-4* was cloned via PCR of a human fetal heart cDNA library (Clontech, Palo Alto, CA) using primers specific for the mature form of *TIMP-4*. The full-length *TIMP-4* PCR product was then sub-cloned into the yeast expression vector pPICZαA (Invitrogen, Carlsbad, CA) and the sequence verified. A C-terminal His-tag was included in the design of the construct to aid in the purification of the expressed protein. The linearized vector was electroporated into the X-33 strain of the methylotrophic yeast *Pichia pastoris* for expression (Invitrogen), and integrants were selected by culturing on YPDS (2% peptone, 1% yeast extract, 2% glucose, 1 M sorbitol, and 2% agar) plates with 100 μg/ml zeocin (Invitrogen) for three days. Successful insertion of the *TIMP-4* gene into the *Pichia* genome was verified by PCR using *Pichia*-specific primers. Expression conditions were as previously described [9]. Briefly, 25ml overnight cultures were grown at 30 °C in BMGY media (2% peptone, 1% yeast extract, 100 mM potassium phosphate, pH 6.0, 1.34% yeast nitrogenous base, and 1% glucose) containing 100 μg/ml zeocin and cell pellets were collected the next day by centrifugation at 1500*g*. Cultures were induced by re-suspending the cell pellets in 250 ml of methanol-containing media (BMMY: 2% peptone, 1% yeast extract, 100 mM potassium phosphate, pH 6.0, 1.34% yeast nitrogenous base, and 1% methanol) and allowed to grow for 24 h. Media containing the secreted expressed protein were cleared of cell content by centrifugation at 3000*g*.

Purification of recombinant TIMP-4. Histidine-affinity using a Ni–NTA agarose resin (Qiagen, Valencia, CA) was performed as an initial step to purify expressed TIMP-4 from the cleared yeast media. Briefly, expressed protein in 250 ml of cleared media was allowed to bind to 5 ml of resin for 1 h at 4 °C, and then centrifuged at low speed to collect the resin. The resin with the expressed protein bound was then loaded into a 12 ml Bio-Rad (Hercules, CA) glass column by gravity, and the resin was washed with 15 ml of buffer containing 10 mM Imidazole (50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, and 10 mM Imidazole) to reduce non-specific binding. TIMP-4 was then eluted using 10 ml elution buffer containing 100 mM Imidazole (50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, and 100 mM Imidazole), and concentrated by centrifugation using membrane concentrators with 5 kDa molecular weight cutoff (VivaScience, Hannover, Germany). Finally, C4 Reverse Phase HPLC was used to purify TIMP-4 to homogeneity. Separation was carried out over a gradient, from 100% Buffer A (0.05% trifluoroacetic acid in water) to 60% Buffer B (0.05% trifluoroacetic acid in acetonitrile) in 60 min at a flow rate of 1 ml per minute. Purity was confirmed by SDS–PAGE stained with silver.

Peptide synthesis and purification. A 24-amino acid peptide corresponding to Loop 6 of TIMP-4 with sequence ECLWTDWLLERKL YGYQAQHYVCM was purchased from SynPep. Loop 6 of TIMP-2 was synthesized as previously described [9]. Synthetic peptides were further purified by us using C18 Reverse Phase HPLC to remove any truncation products. Briefly, 1 mg of lyophilized peptide was re-suspended in 1 ml of Buffer A (0.05% trifluoroacetic acid in water) and loaded onto the column. Separation was carried out over a gradient, from 100% Buffer A to 60% Buffer B (0.05% trifluoroacetic acid in acetonitrile) in 60 min at a flow rate of 1 ml per minute. Fractions containing the peak of interest were collected by hand and subjected to Mass Spec analysis to confirm identity and purity. Amino acid composition analysis was used to determine yield.

SDS–PAGE and Western blot analysis. Samples containing TIMP-4 were resolved on 12% NuPage gels (Invitrogen) and visualized either by silver or Coomassie blue staining. Once purified to homogeneity, protein identity was verified via Western analysis as previously described [9,14]. Briefly, samples containing purified TIMP-4 were resolved on 12% SDS–PAGE gels and then transferred to nitrocellulose by electroblotting. Membranes were incubated with TIMP-4-specific antibodies (AB816, EMD Biosciences, San Diego, CA) at 1:1000 dilution for 1 h at room temperature, they were then rinsed with TBST and incubated with horseradish peroxidase-labeled anti-rabbit secondary antibodies. Immunoreactive bands were detected using a chemiluminescent substrate (Pierce, Rockford, IL).

MMP-inhibitory activity. MMP-inhibitory activity was assessed using a quantitative ^{14}C-Collagen Film Assay, as previously described by us [9,15]. Briefly, 15 μl of ^{14}C-labeled collagen (10,000 CPM/15 μl) was added to each well of a 96-well plates and allowed to polymerize. To determine inhibitory activity, wells were treated with a known amount of activated type I collagenase plus test sample or with collagenase alone, and the plates incubated at 37 °C for 2.5 h to allow for release of ^{14}C by the enzyme. Supernatants were then analyzed in a Wallac Scintillation Counter, and percent inhibition of collagenolytic activity was calculated. An IC_{50} was defined as the amount of protein necessary to inhibit the proteolytic activity of collagenase by 50%.

Cell culture and capillary endothelial cell proliferation. Capillary endothelial cells (EC), isolated from bovine adrenal cortex, were a kind gift of Dr. Judah Folkman and Catherine Butterfield (Children's Hospital Boston), and were maintained in DMEM (Invitrogen) supplemented with 10% calf serum (HyClone, Logan, UT) and 3 ng/ml bFGF, and grown at 37 °C in 10% CO_2. Capillary EC proliferation was measured as previously reported by us [9,15–18] using a modification of the method of Connolly and coworkers (and verified by cell counting using a Coulter Counter) [19]. Briefly, capillary EC were plated on pregelatinized 96-well plates at a density of 2000 cells per well in DMEM supplemented with 5% calf serum and allowed to attach for 24 h. The next day, cells were treated with fresh media with or without 1 ng/ml bFGF and challenged with TIMP-4 at various concentrations. All samples were tested in duplicate a minimum of three times. Control wells contained cell treated with media alone or media with bFGF. After 72 h, the media were removed and the cells were lysed in buffer containing Triton X-100 and the phosphatase substrate *p*-nitrophenyl phosphate. After a 2-h incubation at 37 °C, NaOH was added to each well to terminate the reaction and cell density was determined by colorimetric analysis using a SpectraMax 190 multiwell plate reader (Molecular Devices, Sunnyvale, CA).

Capillary endothelial cell migration. Capillary EC migration was measured using a two-chamber well system in which the upper and lower chambers are separated by a membrane with 8 μm pores through which the capillary endothelial cells can migrate. The membranes were coated with 100 μl of 10 μg/ml fibronectin and after 1 h 50,000 cells were plated on each of the upper chambers. The cells were allowed to attach to the membrane over 30 min and samples were added at various concentrations to the designated wells. Media containing serum plus bFGF were added to the lower chambers to stimulate migration. After 4 h, the media were aspirated and the cells fixed and stained using the DiffQuik stain kit (Baxter Scientific, McGraw, IL). The membranes were then carefully removed and mounted on glass slides using Permount mounting solution. The total number of migrated cells was determined from a digital image of each membrane. Using NIH Image, a density gradient was adjusted to mark all the cell nuclei in the image and the total number of cells was

determined electronically from the number of particles marked. Each sample was tested in triplicate a minimum of three times.

Chick chorioallantoic membrane assay (CAM). The chick CAM assay was conducted as previously reported by us [9,15–18,20]. Briefly, three-day-old chick embryos were removed from their shells and incubated in plastic Petri dishes for another three days. On embryonic day 6, samples and controls were mixed with 0.45% methylcellulose to create sample-containing discs which were then applied to the surfaces of developing CAMs, above the dense subectodermal plexus. After 48 h of incubation, the eggs were examined for vascular reactions under a dissecting microscope (40×) and photographed. All determinations were made by three independent members of the laboratory, in a double-blinded fashion.

Results

Cloning and expression of TIMP-4

Human *TIMP-4* was cloned from a human heart cDNA library using high-fidelity PCR and TIMP-4 -specific primers (Fig. 1A). The PCR product was then cloned into the PICZαA *P. pastoris* expression vector and the sequence was verified. 10 μg of the linearized plasmid was used to transform X-33 yeast by electroporation. Clones containing the *TIMP-4* gene were selected for as described above and insertion into the *Pichia* genome was verified by PCR analysis. Expression conditions were optimized to those described above and a single clone expressing TIMP-4 was chosen for subsequent studies.

TIMP-4 purification and identification by immunoblot analysis

A two-step purification protocol was designed to optimize protein yield while retaining protein stability. First, expressed protein was affinity purified using a His-binding resin and the elute containing TIMP-4 was then concentrated and subjected to a second round of purification using C4-reverse phase HPLC. A sample chromatogram from the purification of TIMP-4 by C4 reverse phase HPLC is shown in Fig. 1B. Sample purity was assessed by silver-stained SDS–PAGE as previously described (Fig. 1C) and protein identity verified by Western analysis using TIMP-4-specific antibodies (Fig. 1D).

Inhibition of MMP activity by TIMP-4

To verify that the purified protein was biologically active, a radiometric collagen film assay was used to test for inhibition of MMP activity. As expected, TIMP-4

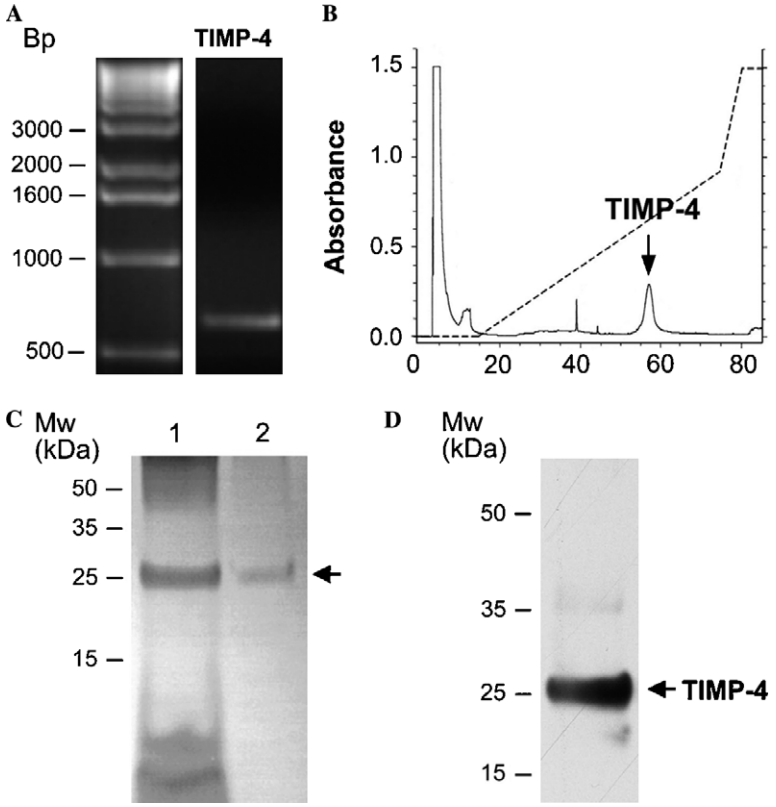

Fig. 1. Cloning, purification, and expression of TIMP-4. Human *TIMP-4* was cloned from a heart cDNA library using specific primers to the mature form of TIMP-4. The PCR product (A) was cloned into the pPICZαA expression vector as described and then inserted into the yeast genome for expression. Expressed TIMP-4 was purified using His-affinity chromatography followed by C4 reverse phase HPLC. A representative HPLC chromatogram is shown in (B). Protein purification was monitored by SDS–PAGE followed by silver staining. A representative example of a silver-stained SDS–PAGE gel of the His-affinity-purified HPLC starting material (lane 1) and the purified TIMP-4 (lane 2) after reverse phase HPLC is shown in (C). Protein identity was verified using TIMP-4-specific antibodies (D).

inhibited MMP activity at nanomolar concentrations, with an IC_{50} of approximately 15 nM. Representative results are shown in Fig. 2A. These results are consistent with previous studies where IC_{50} values are reported to vary from 3 to 83 nM depending on the MMP tested and the system used [2].

TIMP-4 inhibits migration but not proliferation of capillary EC

Given that all TIMPs tested to date (TIMPs-1, -2, and -3) can inhibit the migration of capillary EC, we next tested TIMP-4 in an in vitro migration assay. To do this, a two-chambered well system was employed. The cells were plated in the upper chamber which contained media alone and were then allowed to migrate towards the bottom chamber which contained media supplemented with serum and bFGF. After 4 h, the total number of migrated cells was determined from a digital image of each membrane. TIMP-4 inhibited capillary EC migration with an IC_{50} of approximately 55 nM (Fig. 2B). These results are comparable to those reported for TIMP-4 in other cell lines [2].

Given the high degree of homology between TIMP-2 and TIMP-4 (Fig. 2C), we hypothesized that TIMP-4 might share capillary EC anti-proliferative activity with TIMP-2. TIMP-4 was therefore tested for its ability to inhibit mitogen-driven EC proliferation. The results dem-

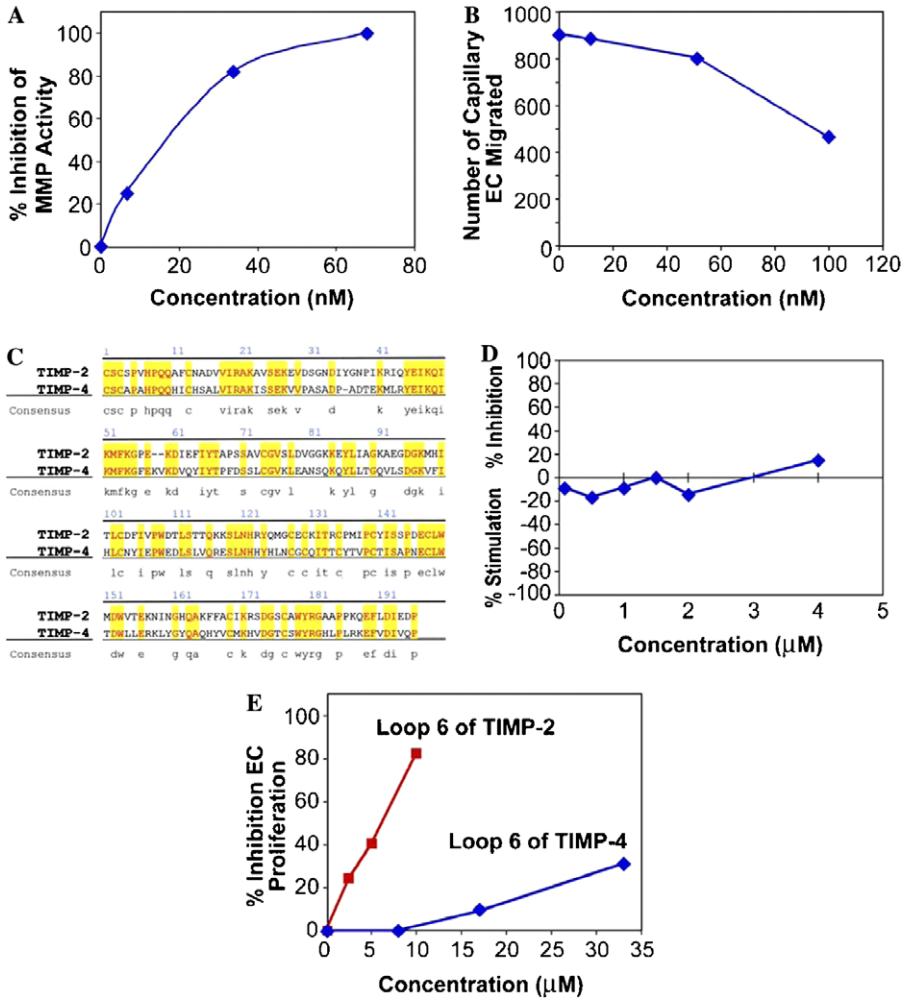

Fig. 2. In vitro anti-angiogenic activities of TIMP-4. To verify that the expressed TIMP-4 retained MMP-inhibitory activity, a radioactive collagen film assay was used. TIMP-4 inhibited MMP activity with an IC_{50} of approximately 15 nM (A). Given that all TIMPs tested to date (TIMPs-1, -2, and -3) have been shown to inhibit capillary EC migration, TIMP-4 was tested in a migration assay (B). The total number of cells migrating in response to bFGF was determined as described in text. Maximal inhibition, achieved at 100 nM TIMP-4, was defined as the dose at which the number of cells migrating in response to bFGF equaled the number of cells migrating without mitogen stimulation. The IC_{50} was approximately 55 nM. (C) Sequence alignment of human TIMP-4 and TIMP-2 illustrate the degree of homology between the protein family members. To determine whether TIMP-4 shares anti-proliferative activity with TIMP-2, TIMP-4 was tested for its ability to inhibit capillary EC proliferation (D). At full-length, TIMP-4 had no significant anti-proliferative activity. Since the anti-proliferative activity of TIMP-2 has been ascribed to the Loop 6 domain, Loop 6 of TIMP-4 was synthesized, purified, and tested for its ability to inhibit capillary EC proliferation (E). Although a modest inhibitory activity could be detected, the dose required was extremely high and approximately 5-fold higher than those required to achieve 50% inhibition when cells were treated with Loop 6 of TIMP-2. Representative assays of each in vitro assay are shown.

onstrate that, contrary to our hypothesis, TIMP-4 did not significantly inhibit EC proliferation, even at doses 20-fold higher than those previously reported for TIMP-2 [8–10]. A representative assay is shown in Fig. 2D. Interestingly, TIMP-4 has been shown to inhibit Wilm's tumor cell growth in vitro at doses as low as 1 nM [13], but not the proliferation of breast tumor cells at any dose tested [11]. These results, along with those reported here, suggest that the growth modulating effects of TIMP-4 may be cell specific.

Since previous work from our laboratory had identified Loop 6 of TIMP-2 as the anti-proliferative site of that molecule, we next examined whether, when isolated from the parent molecule, the same domain of TIMP-4 could share the anti-proliferative activity of Loop 6 of TIMP-2. A synthetic peptide corresponding to Loop 6 of TIMP-4 was synthesized, purified, and tested for its ability to inhibit capillary EC proliferation. Although a modest anti-proliferative effect was observed, the dose required to elicit this effect was very high (20 μg/well), and was almost 10 times higher than the IC_{50} of Loop 6 of TIMP-2 [9]. Representative results are shown in Fig. 2E. Although TIMP-2 and TIMP-4 share approximately 50% overall identity at the amino acid level, Loop 6 of TIMP-4 (T4L6) shares less than 25% similarity with Loop 6 of TIMP-2 (T2L6). Therefore, it is possible that the residues that TIMP-2 and TIMP-4 have in common at Loop 6 are responsible for some of the anti-proliferative effects, but that the difference in amino acid residues results in a reduction in the specific activity of the T4L6 peptide when compared to Loop 6 of TIMP-2.

TIMP-4 does not inhibit embryonic angiogenesis in the CAM

We next tested TIMP-4 for its ability to inhibit angiogenesis in vivo using the chick chorioallantoic membrane assay (CAM). Four different doses (1, 5, 10, and 15 μg per egg) were tested in a minimum of six eggs each. Representative CAMs at 10 μg per egg are shown in Fig. 3. TIMP-4 did not result in any significant inhibition of embryonic angiogenesis (Fig. 3A), with degree of vascularity being more consistent with control CAMs which were treated with methylcellulose discs containing buffer alone (Fig. 3B). In contrast, and as previously reported by us [9], CAMs treated with TIMP-2, used here as positive controls (Fig. 3C), had significantly reduced blood vessel branching, that were characterized by the tortuous appearance of nearby vessels. In addition, and consistent with the results observed in the proliferation assay, Loop 6 of TIMP-4 did not inhibit in vivo blood vessel formation when tested in the CAM (Fig. 3D).

Discussion

One of the most challenging issues in the study of the biological activities of TIMP-4 protein has been related to the low yields and poor stability of expressed TIMP-4 [7]. We therefore designed a protein purification scheme aimed at maximizing the yield of pure, biologically active protein. The two-step purification protocol described above was conducted within 12 h of harvesting the media and the purified TIMP-4 was then quickly lyophilized to reduce protein degradation.

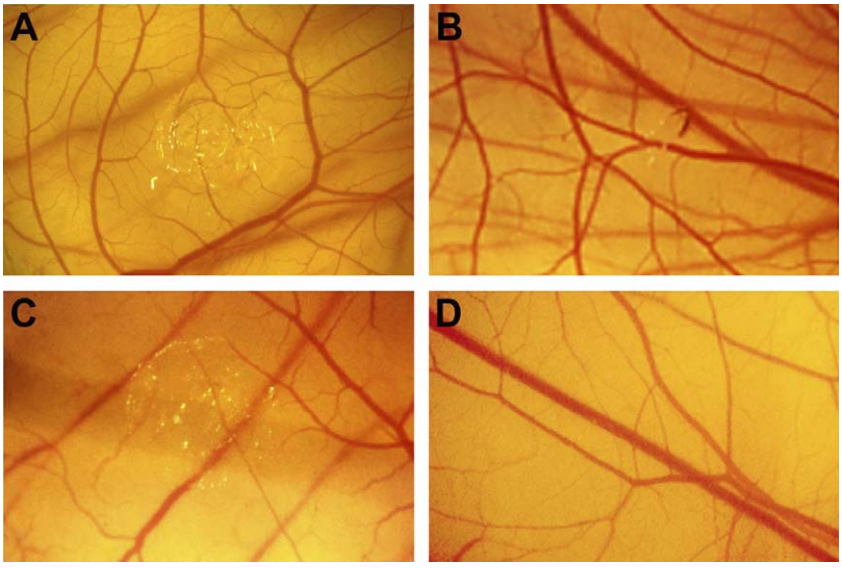

Fig. 3. In vivo anti-angiogenic activity of TIMP-4. TIMP-4 was tested for its ability to inhibit angiogenesis in vivo in the chick chorioallantoic membrane (CAM) assay. TIMP-4 did not result in any significant inhibition of embryonic neovascularization (A) when compared to buffer controls (B). In contrast, and consistent with previous studies, controls treated with TIMP-2 resulted in reduced vessel branching in the vicinity of the methylcellulose disc (C). Additionally, no inhibition was observed in CAMs treated with Loop 6 of TIMP-4 (D).

To date, a majority of the research on TIMP-4 has focused on its role in cardiac pathology based on the observation that TIMP-4 is expressed at relatively high levels in normal heart tissue [1] and that levels decrease after heart failure [21]. It has been suggested that perhaps the tissue-specific expression of TIMP-4 in heart might explain why myocardial tumors are so rare. In a study of the in vitro effects of TIMP-4 on cardiac tumor cells, TIMP-4 was found to stimulate apoptosis of transformed cardiac fibroblasts, while not affecting the growth of normal fibroblasts [22]. Additionally, TIMP-4 has been shown to inhibit platelet aggregation, suggesting that TIMP-4 is involved in the regulation of platelet aggregation and recruitment [23].

Taken together, very little is actually known about the function of TIMP-4 or its possible role in angiogenesis and tumor growth. What is known is that TIMP-4 decreases the migration and invasive potential of cancer cell lines in vitro [2,11,13]. Breast cancer cell lines overexpressing TIMP-4 showed decreased invasive potential in vitro and decreased tumor growth in vivo [11]. However, despite the ability of TIMP-4 to decrease invasion of cancer cells in vitro TIMP-4 did not result in a decrease in metastasis formation. Moreover, when the same tumors were treated with adenovirus-delivered TIMP-4, the tumors actually grew faster and had a decreased apoptotic index [12]. The authors hypothesized that the local concentration of TIMP-4 dictated the net effect, with higher levels leading to inhibition while lower levels result in anti-apoptotic effects. However, in studies where TIMP-4 was delivered by intramuscular adenoviral transfection to mice bearing Wilm's tumors, tumor growth was inhibited [13]. It has been suggested that such seemingly contradictory results may be attributed to the differential effects that TIMPs, in general, might exert on each tumor type [24]. These pleiotropic effects of TIMP-4, and TIMPs in general, also suggest that a universal mechanism of tumor growth inhibition, for example, the inhibition of angiogenesis, is also not common to all TIMPs. It is possible that the observed reduction of microvascular density is due to the global reduction in tumor growth and is therefore secondary to an as of yet defined mechanism. If all TIMPs were direct inhibitors of angiogenesis, then we would expect that all TIMPs would inhibit tumor growth regardless of the tumor system used.

To date, it has been assumed that all MMP inhibitors are inhibitors of angiogenesis, but only TIMP-2 has been consistently shown to be a direct inhibitor of angiogenesis [8–10], with varying effects being reported for TIMPs-1 and -3 [8,25–27]. Although TIMP-3 has been reported to compete for binding of VEGF to its receptor in a genetically modified cell system, the concentration necessary to block VEGF binding is relatively high and has been suggested to be unlikely to play a role in an in vivo setting [27]. RECK, a membrane-bound inhibitor of MMP activity, has been shown to decrease MMP expression (reviewed in [28]), yet MMP inhibition alone has been shown to not be sufficient to inhibit mitogen-driven angiogenesis in vivo [9,10].

The results presented here demonstrate that, like other TIMPs, TIMP-4 is an inhibitor of capillary EC migration. However, to our surprise and despite the sequence similarities to TIMP-2, TIMP-4 did not inhibit capillary EC proliferation. Further studies aimed at comparing Loop 6 of TIMP-4, when isolated from the intact TIMP-4 protein, to Loop 6 of TIMP-2 demonstrated that Loop 6 of TIMP-4 did not have any significant anti-proliferative activity either. Interestingly, the only study to specifically address the possible effects of TIMP-4 on angiogenesis showed that although TIMP-4 could inhibit capillary tube formation in vitro, probably due to its anti-metalloproteinase activity, the dose required to observe this effect was very high (500 nM) [29]. The authors suggest that the high dose required to inhibit tubulogenesis might be due to the presence of MMPs in the growth medium. Interestingly, Ma and co-workers found that corneal wound healing resulted in higher levels of TIMP-4 being expressed, and although it was not determined whether TIMP-4 affects the neovascularization associated with wounding, their results may suggest that TIMP-4 could instead have a pro-angiogenic role [30].

Taken together with our own in vitro studies with TIMP-4, these reports highlighted the importance of assessing the ability of TIMP-4 to inhibit angiogenesis in vivo. We therefore tested TIMP-4 for its ability to inhibit in vivo angiogenesis in the CAM and found that TIMP-4 has no significant effect on neovascularization in vivo. Previous studies from our laboratory have shown that the anti-metalloproteinase activity of TIMP-2, when isolated from the endothelial cell anti-proliferative activity, may not be sufficient to inhibit angiogenesis [9]. Although TIMP-4 could indirectly affect angiogenesis in some systems by virtue of inhibiting members of the MMP family, our studies suggest that TIMP-4, in and of itself, may not be a direct inhibitor of angiogenesis. The results presented here suggest, as previously suggested by us [9,31–33], that MMP inhibition alone may not be sufficient to inhibit the mitogen-driven angiogenesis that is seen in established disease and this may account, at least in part, for the lack of success in the clinic of some of the synthetic MMP inhibitors.

Acknowledgments

This study was supported in part by NIH Grants 2PO1CA45548 and DK065298, and by support from the Simeon Fortin Charitable Foundation and the Advanced Medical Foundation.

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