Structural and Functional Uncoupling of the Enzymatic and Angiogenic Inhibitory Activities of Tissue Inhibitor of Metalloproteinase-2 (TIMP-2)

LOOP 6 IS A NOVEL ANGIOGENESIS INHIBITOR*

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Tissue inhibitors of metalloproteinases (TIMPs) regulate tumor growth, progression, and angiogenesis in a variety of experimental cancer models and in human malignancies. Results from numerous studies have revealed important differences between TIMP family members in their ability to inhibit angiogenic processes in vitro and angiogenesis in vivo despite their universal ability to inhibit matrix metalloproteinase (MMP) activity. To address these differences, a series of structure-function studies were conducted to identify and to characterize the anti-angiogenic domains of TIMP-2, the endogenous MMP inhibitor that uniquely inhibits capillary endothelial cell (EC) proliferation as well as angiogenesis in vitro. We demonstrate that the COOH-terminal domain of TIMP-2 (T2C) inhibits the proliferation of capillary EC at molar concentrations comparable to those previously reported for intact TIMP-2, while the NH2-terminal domain (T2N), which inhibits MMP activity, has no significant anti-proliferative effect. Interestingly, although both T2N and T2C inhibited embryonic angiogenesis, only T2C resulted in the potent inhibition of angiogenesis driven by the exogenous addition of angiogenic mitogen, suggesting that MMP inhibition alone may not be sufficient to inhibit the aggressive neovascularization characteristic of aberrant angiogenesis. We further mapped the anti-proliferative activity of T2C to a 24-amino acid peptide corresponding to Loop 6 of TIMP-2 and show that Loop 6 is a potent inhibitor of both embryonic and mitogen-stimulated angiogenesis in vivo. These findings demonstrate that TIMP-2 possesses two distinct types of anti-angiogenic activities which can be uncoupled from each other, the first represented by its MMP-dependent inhibitory activity which can inhibit only embryonic neovascularization and the second represented by an MMP-independent activity which inhibits both normal angiogenesis and mitogen-driven angiogenesis in vivo. In addition, we report, for the first time, the discovery of Loop 6 as a novel and potent inhibitor of angiogenesis.

Matrix metalloproteinases (MMPs),1 the multigene family of zinc-dependent endopeptidases, have been implicated in a number of important physiological events, both normal and pathological (1). Among these is angiogenesis, the formation of new capillaries from the pre-existing vasculature. Regulation of MMP activity is achieved at the transcriptional level (1) as well as by a family of endogenous inhibitors, the tissue inhibitors of metalloproteinases (TIMPs). Shifts in the proteolytic balance in favor of MMP activity have been shown to play a crucial role in regulating angiogenesis as well as tumor growth and metastasis (2–9). Historically, the potential therapeutic value of endogenous, as well as of synthetic inhibitors of metalloproteinases, has been predicated on their ability to inhibit MMP activity. More recently, attention has been focused on the ability of TIMPs to regulate angiogenesis.

Since it was first demonstrated that TIMPs could inhibit angiogenesis, numerous studies have sought to characterize the specific angiogenic processes effected by MMP inhibitors (2, 10–13). It is now widely appreciated that although all three TIMPs (TIMP-1, -2, and -3) tested to date inhibit MMP activity and the migration of endothelial cells stimulated by angiogenic mitogens, these inhibitors differ in their ability to regulate other angiogenic processes (4, 11, 13). For example, TIMP-2 has the ability to inhibit the proliferation of capillary endothelial cells driven by angiogenic mitogens, while TIMP-1 has been reported to be a modest stimulator of capillary EC growth (11, 14). Although TIMP-3 has recently been reported to inhibit the proliferation of genetically modified aortic endothelial cells that overexpress the vascular endothelial growth factor receptor KDR (15), previous studies have reported that TIMP-3 actually had no effect on capillary endothelial cell proliferation (13). TIMP-4, the latest member of the TIMP family to be cloned, has yet to be rigorously tested. Moreover, neither Batimastat (BB-94), a synthetic MMP inhibitor with potent MMP-inhibitory activity, nor immunoneutralizing antibodies to MMP-2, inhibit capillary EC proliferation (11). In fact, Batimastat has been shown to stimulate the outgrowth of capillary vessels, another process required for successful angiogenesis (16). It has been suggested that the pleiotropic effects that MMP inhibitors have on specific angiogenic processes, such as the inhibition of endothelial cell proliferation by TIMP-2, may

1 The abbreviations used are: MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; EC, endothelial cell(s); CAM, chorioallantoic membrane; T2N, NH2-terminal domain of TIMP-2; T2C, COOH-terminal domain of TIMP-2; EA-T2N, MMP inhibition-deficient mutant of T2N; HPLC, high performance liquid chromatography; bFGF, basic fibroblast growth factor.
be independent of their metalloproteinase inhibitory activity.

The goal of the current study was to identify and characterize the structural domains of TIMP-2 responsible for its endothelial cell growth-modulating activity and its anti-angiogenic activity in vivo. Previous reports have shown that the NH$_2$-terminal domain of TIMPs house the MMP inhibitory activity and that the amino acid sequence of this inhibitory site is highly conserved throughout the TIMP family (17–22). We therefore hypothesized that the unique anti-proliferative activity of TIMP-2 would be located in the more variable COOH-terminal domain (Fig. 1A). To test this hypothesis, we have conducted a series of structure-function studies to identify the structural determinants of the anti-angiogenic activity of TIMP-2. We have independently expressed and purified both the NH$_2$-terminal domain (T2N) and the COOH-terminal domain (T2C) of TIMP-2 as defined by the biochemical and three-dimensional structural information available (17–19, 22–25) and have systematically analyzed these domains in a variety of in vitro and in vivo angiogenesis assays as well as in MMP activity assays (2, 7, 26–28).

In this report, we demonstrate, for the first time, that TIMP-2 possesses two distinct anti-angiogenic activities which can be dissociated from each other, both in terms of their structure and their angiogenesis-modulating activities in vitro. The NH$_2$-terminal domain, T2N, which inhibits MMP activity but not capillary EC growth, suppresses embryonic neovascularization in vitro but not the neovascularization driven by an angiogenic mitogen. These results suggest that inhibition of MMP activity alone may not be sufficient to inhibit the mitogen-stimulated neovascularization that is characteristic of pathologic angiogenesis. In contrast, the COOH-terminal domain of TIMP-2, T2C, which is deficient in MMP-inhibitory activity but which inhibits capillary EC proliferation, is a potent inhibitor of both embryonic and mitogen-stimulated angiogenesis in vitro. Further structural mapping of this activity using synthetic peptides demonstrates that the anti-angiogenic activity of T2C can be found in Loop 6. In summary, we have uncoupled the MMP-dependent and MMP-independent angiogenesis-modulating activities of TIMP-2, and in doing so, have identified a novel, potent, small molecular weight inhibitor of angiogenesis.

EXPERIMENTAL PROCEDURES

Cloning and Expression of hTIMP-2 and hTIMP-2 Domains—Human TIMP-2 was cloned via PCR of a human fetal heart cDNA library (Clontech, Palo Alto, CA) using primers specific for the mature form of TIMP-2. Two separate TIMP-2 domains were produced using PCR primers designed to yield two fragments of TIMP-2 which encode for either the three NH$_2$-terminal loops (Cys$^1$–Glu$^{127}$), designated T2N, or the three COOH-terminal loops (Cys$^{128}$–Pro$^{194}$), designated T2C. A fourth construct, designated EA-T2N, was designed to produce an inactive mutant of T2N using PCR to add two amino acid residues, a 90-amino acid peptide corresponding to Loop 6, and a 24-amino acid peptide corresponding to Loop 7 with sequence ECLWMDWTERNINGHQAAPKFFACI, and a 19-amino acid peptide corresponding to the carboxy-terminal tail with sequence AWRYGAAPQKFGFLDIEPD. A fourth peptide of sequence VIRAK corresponding to a conserved sequence in the NH$_2$-terminal domain of all TIMPs was also synthesized for use as a control peptide. All four peptides were synthesized via Fmoc (9-fluorenylmethoxycarbonyl) solid phase synthesis on Advanced Peptide (Clayton, CA) using C18 reverse phase HPLC to remove any truncation products. The purified peptides were further purified by us using C18 reverse phase HPLC to remove any truncation products. Briefly, 1 mg of lyophilized peptide was reconstituted 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 20% Buffer B to 60% Buffer B in 60 min at a flow rate of 1 ml/min. Fractions containing the peak of interest were collected by hand and subjected to mass spectroscopy to confirm identity and purity of the peptides. Yield was determined by amino acid composition.

Purification of Recombinant TIMP-2 and TIMP-2 Domains—Expression conditions were as follows: 25 ml overnight cultures were grown at 30°C in BMGY medium (2% peptone, 1% yeast extract, 100 mM potassium phosphate pH 6.0, 1.34% yeast nitrogen base, 1% glucose) containing 100 μg/ml zeocin, and cell pellets were collected the next day by centrifugation at 1500 rpm. Cultures were induced by resuspending the cell pellets in 250 ml of methanol-containing medium (BMMY: 2% peptone, 1% yeast extract, 100 mM potassium phosphate, pH 6.0, 1.34% yeast nitrogen base, 1% methanol), and allowed to grow for 24 h. Medium containing the secreted expressed protein was cleared of cell content by centrifugation at 30,000 × g for 1 h. The cleared supernatant was then loaded onto a 12-ml Bio-Rad glass column by gravity, and the resin was washed with 15 ml of buffer containing 10 mM imidazole (50 mM Na$_2$HPO$_4$, pH 8.0, 300 mM NaCl, 10 mM imidazole) to reduce nonspecific binding. Protein was then eluted using 10 ml of elution buffer containing 100 mM imidazole (50 mM Na$_2$HPO$_4$, pH 8.0, 300 mM NaCl, 100 mM imidazole) and concentrated by centrifugation using membrane concentrators with 3-kDa molecular mass cutoff (Centrifrep, Amicon, Beverly, MA). Concentrated protein was further purified to homogeneity by C4 reverse phase HPLC. 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/min. Purity was confirmed by silver staining of SDS-PAGE gels and amino acid composition.

SDS-PAGE Electrophoresis and Protein Sequencing—Proteins were resolved on 12% NuPage gels (Invitrogen) run at 200 V for 1 h and visualized either by silver or Coomassie Blue staining. Once purified to homogeneity, protein identity was verified via NH$_2$-terminal amino acid sequencing. Briefly, proteins to be sequenced were blotted onto polyvinylidene difluoride using a Bio-Rad Transblot apparatus for 1 h at 100 V, stained with Amido Black, and excised from the membrane. NH$_2$-terminal sequence was determined by Edman degradation using an Applied Biosystems 477A protein sequence (Dana Farber Microsequencing Facility, Boston, MA).

Peptide Synthesis and Purification—Peptide sequences were designed to represent various structural domains of the carboxyl terminus of TIMP-2. These include: a 10-amino acid peptide corresponding to Loop 5 with sequence TRCPMPCYL, a 24-amino acid peptide corresponding to Loop 6 with sequence ECLWMDWTERNINGHQAAPKFFACI, and a 19-amino acid peptide corresponding to the carboxy-terminal tail with sequence AWRYGAAPQKFGFLDIEPD. A fourth peptide of sequence VIRAK corresponding to a conserved sequence in the NH$_2$-terminal domain of all TIMPs was also synthesized for use as a control peptide. All four peptides were synthesized via Fmoc (9-fluorenylmethoxycarbonyl) solid phase synthesis on Advanced Peptide (Clayton, CA) using C18-reverse phase HPLC to remove any truncation products. Synthetic peptides were further purified by us using C18 reverse phase HPLC to remove any truncation products. Briefly, 1 mg of lyophilized peptide was reconstituted 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 20% Buffer B to 60% Buffer B (0.05% trifluoroacetic acid in acetonitrile) in 60 min at a flow rate of 1 ml/min. Fractions containing the peak of interest were collected by hand and subjected to mass spectroscopy to confirm identity and purity of the peptides. Yield was determined by amino acid composition.

MMP Inhibitory Activity—MMP inhibitory activity was assessed using a quantitative [14C]collagen film assay, as described previously by us (2). Briefly, 14C-labeled collagen was added to 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 1 h. After incubation, the enzyme-supernatants were then analyzed in a Wallac scintillation counter, and percent inhibition of collagenolytic activity was calculated. An IC$_{50}$ unit 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 EC were isolated from bovine adrenal cortex, were a kind gift of Dr. Judah Folkman (Children’s Hospital, Boston) and were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% calf serum (HyClone) and 3 mg/ml basic fibroblast growth factor (bFGF), and grown at 37°C in 10% CO$_2$. Capillary EC proliferation was measured as
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Cloning, expression, and purification of TIMP-2 and TIMP-2 domains. A, the amino acid sequences comprising the COOH-terminal domains of human TIMPs 1–4. Residue 1 in each case is the first cysteine of the COOH-terminal domain, which corresponds to Cys127 of TIMP-1, Cys128 of TIMP-2, Cys129 of TIMP-3, and Cys130 of TIMP-4. Conserved residues are highlighted in yellow and indicated below the sequence alignment. The locations of the synthetic peptides of TIMP-2 used in this study are underlined. B, we cloned human TIMP-2 from a human heart cDNA library using high-fidelity PCR with specific primers to the mature sequence of TIMP-2. Subsequent PCR was performed to yield two TIMP-2 truncation products encoding the NH2 terminus (T2N) and the COOH terminus (T2C) of TIMP-2, as well as a PCR product encoding the mutant form of T2N, EA-T2N. Expressions were then purified by C4 reverse phase HPLC. As an example, a representative chromatogram of the purification of T2C by reverse phase HPLC is depicted in C. Protein purification was monitored by SDS-PAGE. An example of the His affinity-purified HPLC starting material of T2C (lane 1) and the purified T2C (lane 2) after reverse phase HPLC resolved on an SDS-PAGE gel and stained with silver is shown in D. E, representative silver-stained SDS-PAGE analysis of TIMP-2 and TIMP-2 domains. Amounts loaded in each well were as follows: T2C, 0.5 μg; T2N, 1 μg; EA-T2N, 5 μg; TIMP-2, 0.5 μg.

RESULTS

Cloning, Expression, and Purification of TIMP-2 and TIMP-2 Domains—Human TIMP-2 was cloned from a human heart cDNA library using high fidelity PCR and primers designed to produce full-length TIMP-2, a 3’-deletion fragment and a 5’-deletion fragment (Fig. 1B). Cloning of these gene fragments result in the expression of two deletion mutants of TIMP-2 as distinct peptides. The first is composed of the three NH2-terminal disulfide-bonded loops (Cys1–Glu127) designated T2N (~15 kDa), and the second is composed of the three COOH-terminal disulfide-bonded loops (Cys128–Pro194) designated T2C (~8.5 kDa). In addition, following recent reports suggesting that the addition of amino acid residues to the NH2-terminal Cys1 results in the abrogation of MMP-inhibitory activity (30, 31), a mutant form of T2N that encodes for a form of T2N with additional glutamic acid and alanine residues at the NH2 terminus of the protein, and designated EA-T2N, was designed, expressed, and purified for use as a control for TIMP-2 as distinct peptides. The first is composed of the three NH2-terminal disulfide-bonded loops (Cys1–Glu127) designated T2N (~15 kDa), and the second is composed of the three COOH-terminal disulfide-bonded loops (Cys128–Pro194) designated T2C (~8.5 kDa). In addition, following recent reports suggesting that the addition of amino acid residues to the NH2-terminal Cys1 results in the abrogation of MMP-inhibitory activity (30, 31), a mutant form of T2N that encodes for a form of T2N with additional glutamic acid and alanine residues at the NH2 terminus of the protein, and designated EA-T2N, was designed, expressed, and purified for use as a control for MMP inhibition. All expressed proteins were NH2-terminally sequenced to verify either native or mutant forms. All proteins, as well as intact human TIMP-2, were expressed using the Pichia pastoris yeast expression system, which has been shown to successfully produce other disulfide-bonded proteins (32, 33).
Expressed proteins with incorporated COOH-terminal His-tags are secreted into the growth media and purified to homogeneity by histidine affinity chromatography followed by C4 reverse phase HPLC. A sample chromatogram of the purification of T2C by reverse phase HPLC is shown in Fig. 1C. Sample purity was monitored by SDS-PAGE run under reducing conditions followed by silver-staining as previously reported (2, 28). Fig. 1D shows a representative example of a silver-stained gel of both the HPLC starting material (lane 1) and purified T2C (lane 2). An example of a silver-stained gel of each of the purified TIMP-2 and TIMP-2 domains is shown in Fig. 1E. Typical yields of each sample protein were as follows: 1.2 mg/liter for T2N, 240 μg/liter for T2C, 15 mg/liter for EA-T2N, and 300 μg/liter for intact TIMP-2.

T2N, but Not T2C, Inhibits Metalloproteinase Activity—To demonstrate that the NH2-terminal domain of TIMP-2, T2N, retains MMP-inhibitory activity as reported previously (17, 18, 22–25) and to validate the ability of this expression system to produce biologically active protein, we tested T2N, the TIMP-2 domains, and an MMP inhibition-deficient form of T2N, EA-T2N, for their ability to inhibit MMP activity using a standard quantitative radiometric MMP assay (2). As expected, T2N inhibited MMP activity at concentrations comparable to standard quantitative radiometric MMP assay (2). As expected, T2N, EA-T2N, for their ability to inhibit MMP activity using a TIMP-2 domains, and an MMP inhibition-deficient form of tested at the same doses (data not shown). Although T2N is responsible for inhibition of MMP activity, it had no significant effect on capillary EC proliferation. These results demonstrate that the inhibitory effect of TIMP-2 on capillary EC proliferation is independent of MMP inhibition and that these bioactivities can be dissociated from each other. This unique anti-proliferative activity of T2C represents a second anti-angiogenic domain within TIMP-2.

T2C and T2N Inhibit Angiogenesis in Vivo in the Chick CAM Assay—Given that MMP inhibitors have been shown to inhibit angiogenesis and that T2C independently inhibits capillary endothelial cell proliferation, we next tested both T2C and T2N for their ability to inhibit embryonic, unstimulated angiogenesis in vivo in the CAM assay. Approximately 120 eggs were tested in these experiments. The representative CAMs shown in Fig. 2 were treated with equivalent amounts of each of the proteins tested at the lowest dose in which inhibition should be achieved for T2N. The addition of either T2C or T2N to the chorioallantoic membrane resulted in a decrease in neovascularization (Fig. 2, C and D). T2C produced avascular zones at doses as low as 112 pmol per egg, while T2N inhibited at doses 5-fold higher. EA-T2N, which is deficient in MMP and capillary EC inhibitory activity, did not inhibit neovascularization (Fig. 2E). Intact TIMP-2 was used as a control (Fig. 2F).

T2C Inhibits bFGF-driven Neovascularization in the Mouse Corneal Pocket Assay—We next assessed the ability of T2C to inhibit angiogenesis in a second and more complex in vivo system, the mouse corneal pocket assay, where the neovascularization is stimulated by the addition of an exogenous angiogenic mitogen. T2C (5 μg/eye) resulted in an 87% reduction of bFGF-driven corneal neovascularization when compared with the contralateral control eye treated with only bFGF (Fig. 3, A and B). Surprisingly, treatment of the corneas with T2N resulted in only modest inhibition of angiogenesis (Fig. 3C) and did not differ significantly from the inhibition observed when corneas were treated with the MMP inhibition-deficient form of T2N, EA-T2N (Fig. 3, D and F). In contrast, intact TIMP-2 resulted in potent inhibition of corneal neovascularization (Fig. 3E), which was statistically the same as that obtained with T2C (Fig. 3F). The average percent inhibition of corneal neovascularization obtained from separate experiments of 18 corneas per treatment group is shown in Fig. 3F.

Loop 6, a 2.9-kDa Domain of T2C, Inhibits Capillary EC Proliferation—To further map the anti-proliferative activity of TIMP-2 within T2C, three peptides corresponding to various smaller domains of T2C were synthesized, purified, and tested for their ability to inhibit bFGF-stimulated capillary EC proliferation in vitro. These domains and their amino acid sequence are depicted in Fig. 4A. One peptide, corresponding to Loop 6 of intact TIMP-2 with amino acid sequence ECLWMDVWTEKNINGHQRFFACI, significantly inhibited capillary EC proliferation, while a peptide corresponding to the carboxyterminal tail (T2-Tail), AWYRGAAPKQEFLDIEDP, had no effect. A third peptide corresponding to Loop 5 of intact TIMP-2, TRCPMPFCYI, stimulated the proliferation of capil-
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It is widely appreciated that TIMPs are multifunctional proteins with respect to cell growth, apoptosis, angiogenesis, and other bioactivities (1, 34). Although some of these differences in TIMP activities can be attributed to differences in their affinity for various MMPs and/or their role in MMP activation, other functions appear to be entirely MMP-independent. For example, both TIMP-1 and TIMP-2 have been shown to possess erythropoietin-potentiating activity (35, 36). TIMP-3 has been reported to inhibit tumor necrosis factor-α-converting enzyme (37) as well as inducing apoptosis (38–40), while TIMP-1 can actually inhibit apoptosis (41–43). TIMP-2 has been shown to induce apoptosis in some systems (44, 45) but to have no effect in others (40). Of particular interest is the fact that only TIMP-2 has been shown to inhibit mitogen-driven capillary EC proliferation (11, 13).

Given that all TIMPs can inhibit MMP activity, the ability of TIMP-2 to inhibit capillary EC proliferation has been suggested to constitute a second function of TIMP-2, independent of its MMP-inhibitory activity (11, 31). Based on these findings, we hypothesized that there must be a structural entity unique to TIMP-2 that might be responsible for the inhibition of mitogen-driven capillary EC proliferation, thereby representing a second anti-angiogenic site within the molecule. Until now, no direct evidence of independent TIMP structural elements responsible for growth inhibition has been demonstrated (11, 15, 31, 46). In this structure-function study, we isolate and characterize the MMP-dependent and MMP-independent anti-angiogenic effects of TIMP-2 and demonstrate, for the first time, that these activities are structurally independent. In doing so, we have identified a novel inhibitor of angiogenesis, Loop 6.

Our first series of experiments demonstrated that TIMP-2 possesses two anti-angiogenic activities, one that is associated with MMP inhibition (T2N) and one that is not (T2C). These in vitro studies showed that the unique ability of TIMP-2 to inhibit capillary EC proliferation is housed in the MMP inhibitory-deficient carboxyl-terminal domain, T2C. In addition, T2C inhibited angiogenesis in both the chorioallantoic membrane assay (Fig. 2C) and in the mouse corneal pocket assay at a level comparable to that obtained with intact TIMP-2 (Fig. 3E). We further mapped the anti-proliferative activity of T2C to the 24-amino acid sequence of Loop 6 (Fig. 4). Importantly, as with T2C, Loop 6 significantly inhibited angiogenesis in two different in vivo models (Fig. 4).

It is interesting to note that the morphology of the vessels in the CAMs treated with either T2C or Loop 6 was qualitatively different from those treated with T2N. CAMs treated with T2C or Loop 6 had much larger avascular zones and the few remaining vessels had a tortuous appearance reminiscent of vessels undergoing regression (47). The inhibition of neovascularization in the vicinity of the methylcellulose disc containing T2N, however, could be characterized by a dissolution of CAM vessels. Given that numerous studies have demonstrated the requirement for increased MMP activity at the migrating edge of growing vessels during angiogenesis (48–50), the appearance of the CAMs treated with T2N is consistent with the direct inhibition of MMPs.

Given that neither T2C nor Loop 6 inhibit MMP activity, our results demonstrate that TIMP-2 has an additional anti-angiogenic domain that is independent of direct MMP inhibition. Interestingly, a recent study has found that T2C can inhibit the activation of pro-MMP-2, presumably by sequestering pro-MMP-2 away from the activating complex (51). This study further demonstrates that this activity is mediated by the binding of the carboxyl-terminal tail of TIMP-2 (T2-Tail) to the PEX domain of MMP-2, an interaction that is required for cell surface activation of pro-MMP-2. These findings with respect to
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T2-Tail, along with our own that demonstrate that T2-Tail has no anti-proliferative or anti-angiogenic activity (Fig. 4B), suggest that the ability of TIMP-2 to inhibit capillary EC proliferation, which we have now experimentally attributed to Loop 6, is not only independent of its ability to directly inhibit MMP activity, but is also most likely to be independent of its involvement in pro-MMP activation. It remains to be determined whether or not the anti-proliferative activities of T2C or its smaller angi-inhibitory domain, Loop 6, are operative whether or not the anti-proliferative activities of T2C or its smaller peptide, Loop 6, proved to be inhibitors of both normal and mitogen-stimulated angiogenesis in vivo. These data suggest that the inhibition of MMP activity may be sufficient to inhibit physiologic angiogenesis as represented by the embryonic vasculature of the chick chorioallantoic membrane, but alone may not be sufficient to inhibit the neovascularization which is characteristic of pathological conditions. This angi-inhibitory limitation may explain the less than successful results of clinical testing of synthetic MMP inhibitors (52), whose activity is dependent solely on enzymatic inhibition.

In this study, we identify Loop 6 as a new inhibitor of angiogenesis found within the COOH terminus of TIMP-2. It is now widely appreciated that small molecular weight inhibitors such as Loop 6 may exhibit more desirable therapeutic potential both in terms of ease of administration and targeting as well as increased bioavailability. Loop 6, as we have demonstrated in this study, is particularly amenable to production by synthetic means as a function of its amino acid composition and small size. Moreover, since these small peptides are derived from naturally occurring proteins, they may possess the feature of reduced toxicity or other side effects (53).

There remain a number of critical yet unsolved questions with respect to the TIMPs and their important yet diverse biological functions (1, 34). Among these questions, Nagase and co-workers (1) have highlighted the critical need for a better understanding of the structural relationship(s) between MMP inhibition, cell growth-stimulating and growth-inhibiting activities, and the inhibition of angiogenesis. The current report addresses these questions and establishes the structural determinants responsible for capillary endothelial cell growth-inhibition and the inhibition of angiogenesis in vivo by uncoupling these activities from the inhibition of MMP activity. In doing so, we have discovered Loop 6, a novel, potent inhibitor of angiogenesis, which as a function of its biocompatibility, may serve a clinically useful role in the treatment of diseases characterized by dysregulated angiogenesis.
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