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MINI REVIEW

Tissue Inhibitors of Metalloproteinases and Their Cell Growth-Promoting Activity

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Prologue.

Members of the matrix metalloproteinase (MMP) family, represented by interstitial collagenase, have been recognized to participate in various kinds of important biological phenomena such as ovulation, morphogenesis, angiogenesis, tissue remodeling, involution, and wound healing as physiological events, and inflammation, tumor invasion and metastasis as pathological events (1).

On the other hand, it has been recognized that various tissue explants and cells synthesize and release intrinsic metalloproteinase inhibitors. The first such inhibitor found was evaluated as having the ability to inhibit interstitial collagenase, and so was formerly termed *collagenase inhibitor*. Once it was clarified that this inhibitor is effective against all members of the MMP family, it was redesignated as *tissue inhibitor of metalloproteinases*, abbreviated TIMP. This classical TIMP is now specified as TIMP-1, since a second TIMP, TIMP-2, was recently found.

In this review, I would first like to describe the structures and major properties of TIMPs and then discuss a completely new aspect of TIMPs, that is, their cell growth-promoting activity, which is independent of its MMP inhibitory activity.

1. What are TIMPs?

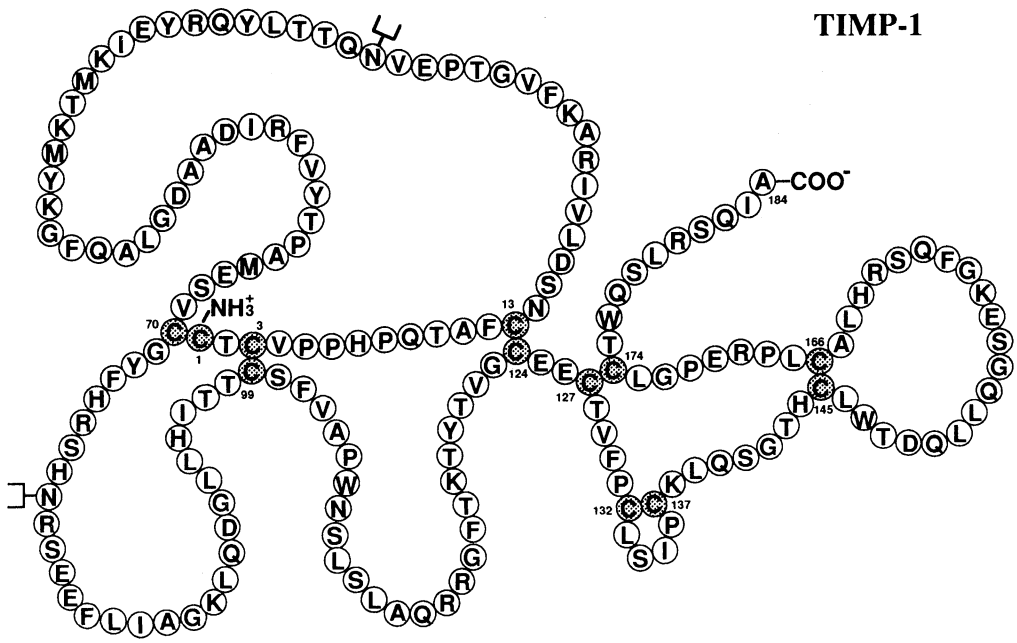
TIMP-1 is a glycoprotein having a molecular weight of around 30 K, which inhibits collagenase derived from tadpoles to human beings, but not membrane-bound procollagen peptidase, bacterial collagenase, or thermolysin, the latter of which are also metalloenzymes. TIMP-1 interacts with interstitial collagenase with the same stoichiometry and an apparent Kd of 1.4×10^{-10} M and with stromelysin 1 with a 1 : 1 stoichiometry and a Kd of 3.8×10^{-10} M. These complexes do not dissociate by gel filtration, indicating a very tight binding. β_1-Anticollagenase in serum is now recognized to be identical to TIMP-1. TIMP-1 is produced by various kinds of explanted tissues like aorta, cartilage, embryonic bone, tendon, dental pulp, gingiva, synovium, uterus, and by cultured cells like fibroblasts, epithelial cells, endothelial cells, osteoblasts, chondrocytes, smooth muscle cells, platelets, monocytes/macrophages, and many tumor cells. TIMP-1 has been found in every human body fluid examined, suggesting it to be a fundamental and ubiquitous protein in human beings (2).

The primary structure of human TIMP-1 has been clarified by the isolation of its cDNA (Fig. 1). TIMP-1 core protein consists of 207 amino acids of which the first 23 represent a signal sequence that is cleaved off on secretion. Mature TIMP-1 has a calculated molecular weight of 20,685. Glycosylation at the two conserved sites shown in Fig. 1 probably accounts for the molecular weight of about 30 K of the actual secretion form. The TIMP-1 sequence contains 12 cysteine residues, all of which have recently been assigned to six disulfide bonds. The MMP inhibitory activity of TIMP-1 resides in three N-terminal loops. TIMP-1 easily loses its inhibitory activity by reductive alkylation, but is fairly stable in various protein-denaturing agents such as 8 M urea, 6 M guanidine-HCl, and 4 M KSCN and is also resistant to treatment with proteases such as trypsin.

The primary structure of human TIMP-2 was clarified first by peptide analysis, and then by cDNA cloning (Fig. 1). The mature form consists of 194 amino acid residues and shares 43% amino acid sequence homology with TIMP-1. TIMP-2 also contains 12 highly conserved cysteine residues. Based on these properties TIMP-2 is considered to be a member of the TIMP family. Recently, Shimizu *et al.* (3) revealed the primary structure of mouse TIMP-2 and found it to have extraordinarily high homology (97%) compared to human TIMP-2. As mouse and human TIMP-1 amino acid sequences have been reported to show less homology (74%), the high homology of TIMP-2 between species presumably indicates a fundamental biological role of the protein. TIMP-2 inhibits all MMPs as does TIMP-1. For half-maximal inhibition, however, TIMP-2 is more than 10- and 7-fold more effective than TIMP-1 at inhibiting gelatinases A and B, respectively, whereas interstitial collagenase is inhibited by TIMP-1 more than twofold more effectively than it is by TIMP-2.

TIMP-2 inhibits the four major MMPs, that is, interstitial collagenase, gelatinases A and B, and stromelysin 1 in a 1 : 1 stoichiometry, but has no effect at all on trypsin, plasmin, and bacterial collagenase. Other properties such as heat stability, susceptibility to reductive alkylation, and resistance to treatment with trypsin or 4-aminophenylmercuric acetate (APMA) are quite similar to those of TIMP-1. Neither do the TIMPs show any immunological cross-reactivity with the other. TIMP-2 is clearly distinguished from TIMP-1 by having no ability to bind to a Con A-Sepharose column. This lack of binding thus indicates TIMP-2 to be an unglycosylated protein.

Recently, another TIMP, ChIMP-3 (chicken inhibitor of metalloproteinases), produced by chicken embryo fibroblasts, was reported and has been designated as TIMP-3 (4). Its cDNA encodes a 24-residue signal peptide, followed by a 188-residue mature inhibitory domain. ChIMP-3 also has 12 highly conserved cysteine residues like the other two TIMPs. As with TIMP-2,



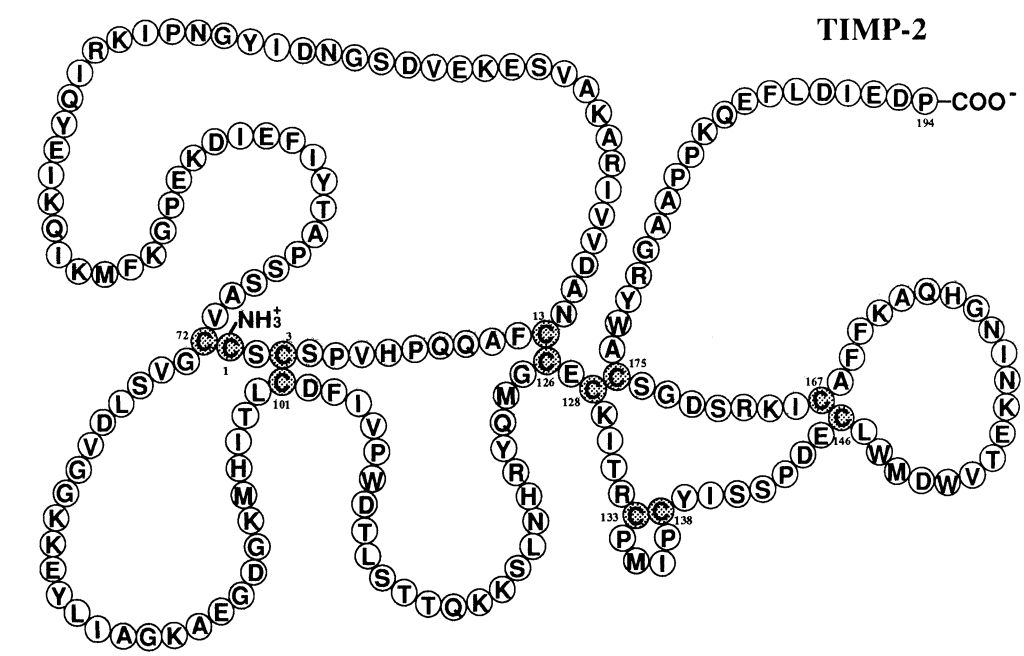


Table I. TIMP FAMILY.

	TIMP-1	TIMP-2	TIMP-3 (chicken)
mRNA	0.9 kb	1.0, 3.5 kb	0.63 kb
gene structure	5 exons & 4 introns (4.3 kb)	?	?
amino acids			
signal peptide	23	26	24
mature form	184	194	188
sequence homology	43%	42%	
	28%		
	all have 12 Cys residues, the positions of which are well conserved		
molecular weight	25–36 K (20,685*)	20–24 K (21,600*)	22 K (21,825*)
N-linked oligosaccharide	+	—	—
immunological cross-reactivity	—	—	—
distribution in cell culture system	culture medium	culture medium	ECM
other features	forms a complex with progelatinase B and regulates the auto-activation	forms complexes with interstitial procollagenase and progelatinase A and regulates the autoactivation	promotes the detachment of transforming cells from the ECM and accelerates the morphological changes associated with cell transformation

* calculated from their amino acid sequences

ChIMP-3 seems to be unglycosylated, and it shows 28% and 42% amino acid homology with TIMP-1 and TIMP-2, respectively. ChIMP-3 does not immunologically cross-react with either TIMP-1 or TIMP-2. ChIMP-3 is clearly distinguished from the other two TIMPs by its presence not in the culture medium but in the extracellular matrix (ECM). Besides having MMP inhibitory activity, ChIMP-3 promotes the detachment of transforming cells from the ECM and also appears to accelerate the morphological changes associated with cell

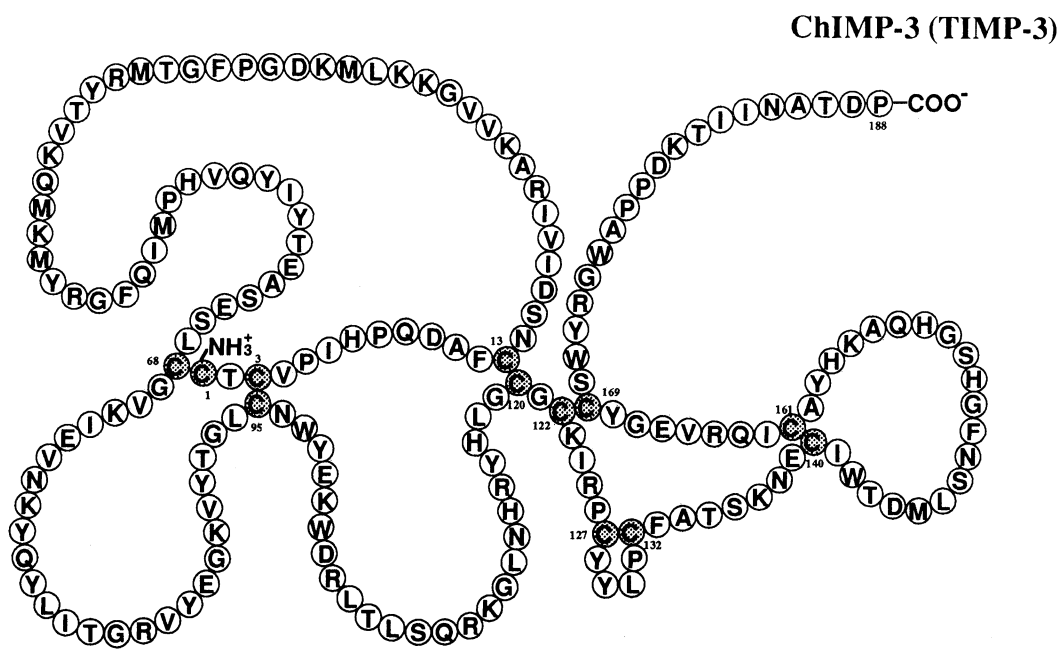


Fig. 1. Two-dimensional representation of TIMP family structures. Denotes oligosaccharide chains.

transformation (5). Major properties of the TIMP family are summarized in Table I.

Another feature of TIMP-1 and TIMP-2 is that, in addition to inhibiting MMP activity by binding to the active form of the enzymes, TIMP-1 can bind to progelatinase B and TIMP-2 can bind to progelatinase A, in a 1 : 1 stoichiometry and in a non-covalent fashion. The physiological significance of these complexes has been revealed, that is, TIMP-2 inhibits the autoactivation of either interstitial procollagenase or progelatinase A. TIMP-2 binds to the C-terminal domain of progelatinase A with a Kd of 0.42 nM. It was also suggested that the C-terminal domain of TIMP-1 participates in the complex formation with progelatinase B. Recently, Okada *et al.* (6) demonstrated that TIMP-1 complexed with progelatinase B inhibited the autoactivation of progelatinase B. TIMP complexed with its corresponding progelatinase still has a capability to inhibit active MMPs suggesting the formation of tertiary complex.

2. Cell growth-promoting activity of TIMPs

The amino acid sequence of TIMP-1 deduced from its cDNA analysis is known to be identical to that of erythroid potentiating activity (EPA) which stimulates the growth of erythroid precursors (BFU-E and CFU-E). We demonstrated that TIMP-1 produced by human bone marrow stromal cell line KH-102 stimulated the colony formation of BFU-E and CFU-E (7).

We recently reported that human TIMP-1 has a potent growth-promoting activity for a wide range of human and bovine cells; actually all the cells examined, such as fibroblasts, epithelial cells, endothelial cells, smooth muscle cells, chondrocytes, SV 40-transformed human lung cells, breast adenocarcinoma cells, lymphoblasts, K-562 cells, HL 60 cells and 3 Burkitt lymphoma cell lines (Raji, Daudi and Ramos), were maximally stimulated at around 100 ng/ml (8). Fig. 2 shows the effect of TIMP-1 on Raji cells. As Raji cells secrete neither MMPs nor TIMPs, TIMP-1 seems to stimulate the cells independently of its inhibitory activity. However, in the case of adherent cells such as gingival fibroblasts, which produce both matrix components and MMPs, there is the possibility that TIMP-1 also stimulates cell proliferation indirectly as an inhibitor through the maintenance of ECM integrity. The cell growth-promoting activity of TIMP-1 is suggested to be a direct cellular effect mediated by a cell surface receptor. Avalos *et al.* (9) demonstrated the presence of the EPA receptor (actually identical to the TIMP-1 receptor) on K-562 cells. It had a Kd of 62 (50–152) nM, and a receptor number of 60,000 sites/cell was calculated. They also identified a 32-kDa receptor protein using a cross-linker, disuccinimidyl suberate. Recently, Bertaux *et al.* (10) reported that human keratinocytes have a TIMP-1

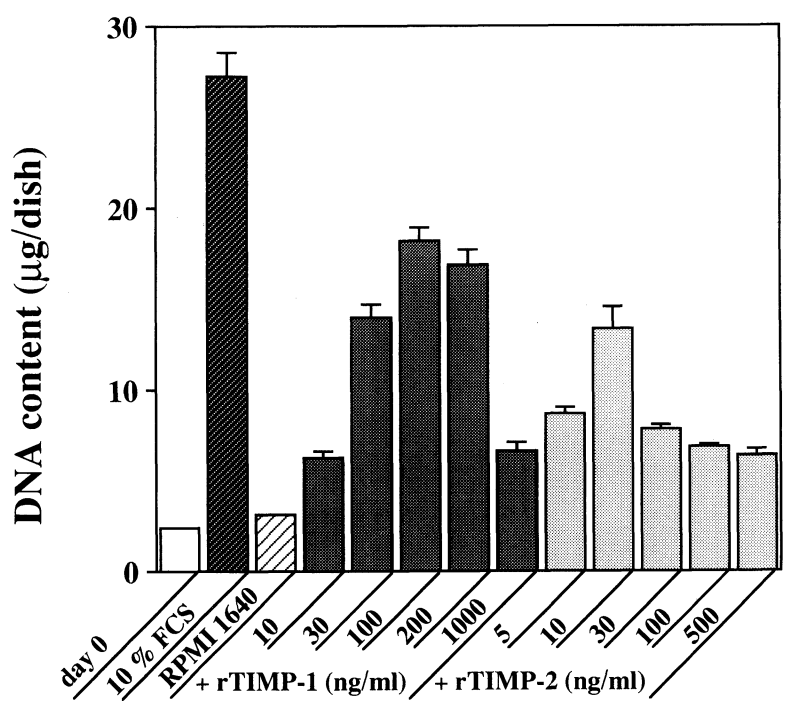


Fig. 2. Effect of the addition of different concentrations of TIMP-1 and TIMP-2 on the proliferation of Burkitt lymphoma cell line Raji cells in serum-free basal medium. DNA content at day 3 was determined. Results are the mean of three experiments, along with standard deviation. RPMI 1640 is a basal culture medium containing amino acids, vitamins and minerals together with D-glucose, and mainly used for suspension culture. rTIMPs denote human recombinant TIMPs.

receptor with a Kd of 8.7 nM and a receptor number of 135,000 sites/cell.

Human TIMP-2 also has a potent growth-promoting activity for a wide range of human, bovine, and mouse cells, having an optimal concentration of 10 ng/ml (0.46 nM) that is, ten-times lower than that of TIMP-1 (11). Fig. 2 also shows the effect of TIMP-2 on Raji cells. Under steady-state conditions at 4°C, high-(Kd =0.15 nM) and low-(35 nM) affinity binding sites for TIMP-2 were identified on Raji cells, with receptor numbers of 20,000 and 1.4×10^5 sites/cell, respectively. Either high- or low-affinity binding of ^{125}I-TIMP-2 to Raji cells was competitively inhibited by unlabeled TIMP-2 but not by unlabeled TIMP-1, thus suggesting the presence of a receptor for TIMP-2 independent from that of TIMP-1.

Either progelatinase B-TIMP-1 or progelatinase A-TIMP-2 complex, both of which have fully inhibitory activity against active MMPs showed no cell growth-promoting activity at all. On the contrary, both reductive-alkylated TIMPs, having no MMP inhibitory activity, significantly stimulated cell proliferation. These facts again clearly indicate that the cell-proliferating activity of TIMPs is independent of their MMP inhibitory activity. It was demonstrated that [^{3}H]thymidine was significantly incorporated into Raji cells as early as 3 h after the addition of either TIMP-1 or TIMP-2, indicating the direct stimulation of DNA synthesis by these TIMPs. Recently, a growth-stimulating protein from SV40-transformed human fibroblasts was suggested to be identical to TIMP-2 (12).

ChIMP-3 or TIMP-3, which has been proposed to be a new, matrix-bound TIMP, as already mentioned, was reported to stimulate the proliferation of growth-retarded, non-transformed cells maintained under low-serum conditions (5). These findings lead us to conclude that cell growth-promoting activity is a common feature of members of the TIMP family.

Now we know that both TIMP-1 and TIMP-2 are constitutive components in human serum and also in fetal calf serum (FCS). We demonstrated that cell proliferation was suppressed remarkably in TIMP-free FCS and was significantly recovered by the addition of TIMPs (Fig. 3), suggesting that both TIMPs are new potent growth factors in serum (11). Although several chemically defined synthetic media have been developed, the requirement for serum still remains for many vertebrate cell lines, hinting that one or more unknown serum factors may play key roles in regulating *in vivo* cell maintenance and proliferation. TIMPs may be such factors. The growth dependency on both TIMPs in FCS was, however, different from one cell line to another (11).

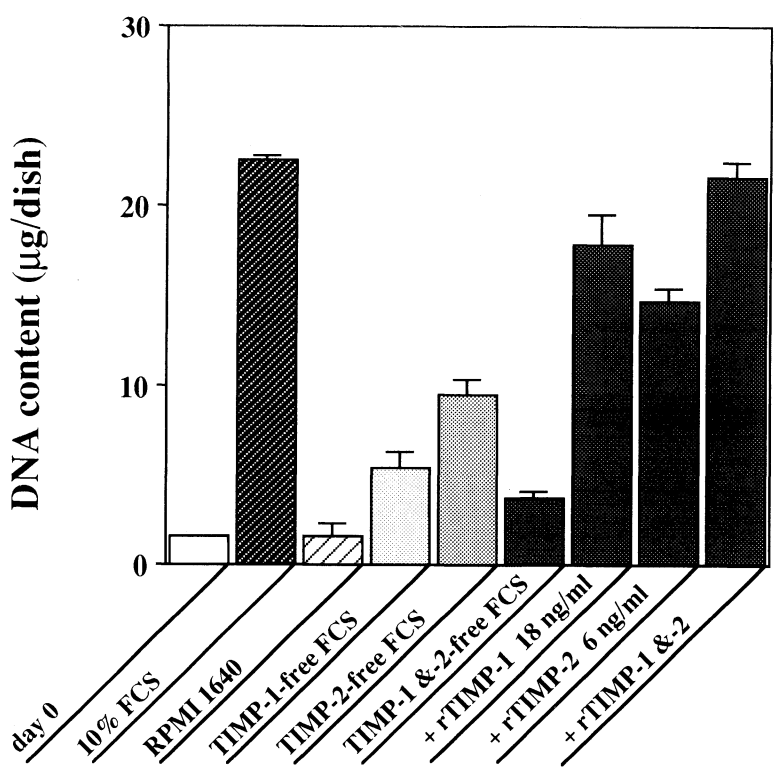


Fig. 3. TIMP-dependent proliferation of Raji cells. The 10% FCS originally contained 18 ng/ml TIMP-1 and 6 ng/ml TIMP-2. Results are the mean of three experiments, with standard deviation.

Growth might depend on the amount of TIMPs that those cell lines produce by themselves, the cell proliferation of which TIMPs would stimulate by an autocrine mechanism. It might also depend on the characteristics of the receptors of each cell line.

We demonstrated that TIMP-2 also stimulated the proliferation of K-562 cells as TIMP-1 did (11). This is consistent with a previous report indicating that human rTIMP-2 has EPA activity (13). Recently, it has been demonstrated that EPA activity seems to be the joint work of both erythropoietin and TIMP-1 and that TIMP-1 appears to participate in this event exclusively as a growth factor toward erythroid progenitor cells (14).

Epilogue.

Both TIMP-1 and TIMP-2 have two distinctive functions, that is, MMP inhibitory and cell growth-promoting activities. Toward reconciling these seemingly unrelated functions, it has been recognized that the interaction between cells and their environment is indispensable for the differentiation and proliferation of cells. A good example is the hematopoietic microenvironment for the development of blood cells. TIMPs participate, on the one hand, in the maintenance of ECM integrity and, on the other hand, in the proliferation of cells. In this sense, it would seem logical that TIMPs have both activities.

Among other bifunctional molecules having both protease inhibitory and cell growth-promoting activities such as pancreatic secretory trypsin inhibitor/tumor-associated trypsin inhibitor (PSTI/TATI) (15), epidermal growth factor/urogastrone (EGF/URO) (16), and urinary glycoprotein protease inhibitor (16), EGF/URO is quite comparable to TIMPs in many respects. Both factors are fundamental and ubiquitous proteins in human beings, and stimulate a wide range of cells. EGF/URO, the amino acid sequence of which is homologous with that of PSTI/TATI (17), actually has potent protease inhibitory activity.

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