Eur Respir J, 1994, 7, 2062–2072
DOI: 10.1183/09031936.94.07112062
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European Respiratory Journal
ISSN 0903 - 1936

REVIEW

The role of matrix metalloproteases and their inhibitors in tumour invasion, metastasis and angiogenesis

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The role of matrix metalloproteases and their inhibitors in tumour invasion, metastasis and angiogenesis. J.M. Ray, W.G. Stetler-Stevenson. ©ERS Journals Ltd 1994.

ABSTRACT: One critical event of tumour invasion that signals the initiation of the metastatic cascade is thought to be interaction of the tumour cell with the basement membrane. Basement membranes may also pose as barriers to tumour cell invasion at multiple points later in the metastatic cascade, including during the processes of vascular infiltration and extravasation. Thus, an important proteolytic event in the metastatic cascade, and also angiogenesis, appears to be degradation of basement membrane components.

A specific class of extracellular matrix degrading metalloenzymes, the matrix metalloproteases, and their endogenous inhibitors, the tissue inhibitors of metalloproteases, are thought to have a role in the creation of the proteolytic defect in basement membrane type IV collagen. We will review the evidence which indicates that matrix metalloproteases and tissue inhibitors of metalloproteases are essential for tumour cell invasion and angiogenesis. The regulation of matrix metalloproteases will be discussed, including gene activation and transcription, messenger ribonucleic acid (mRNA) stability, binding of proenzymes to cell membranes and/or matrix components, proenzyme activation, and inactivation by endogenous inhibitors. We will also discuss the mechanism for tissue inhibitor of metalloproteases–mediated inhibition of tumour invasion and angiogenesis. This appears, at least in part, to be through inhibition of protease activity required for cellular invasion, although recent observations suggest that tissue inhibitors of metalloproteases affect other distinct groups of biological activities through mechanisms other than matrix metalloprotease inhibition.

Eur Respir J., 1994, 7, 2062–2072.

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Keywords: Angiogenesis
cancer
invasion
matrix metalloproteases
metastasis
tissue inhibitors of metalloproteases

Received: July 5 1994
Accepted for publication July 8 1994

Tumour invasion and metastases are the major causes of morbidity and death for cancer patients. The exact mechanisms responsible for the formation of metastases are not fully understood. However, several general principles have been elucidated. The critical event of tumour invasion that signals the initiation of the metastatic cascade is thought to be interaction of the tumour cell with the basement membrane [1, 2]. There are at least three critical steps involved in this process. The first is attachment to the extracellular matrix (ECM), which may be mediated by pre-existing or newly formed contact sites. The second is creation of a proteolytic defect in the ECM. The final phase is migration through the proteolytically modified matrix.

Tumour cell interactions with the ECM, and in particular with the basement membrane, occurs at multiple stages throughout the metastatic cascade. No matter how extensive the architectural disorganization, benign disorders are always characterized by a continuous basement membrane separating the "tumorigenic" epithelium from the stroma. In contrast, invasive carcinomas possess a discontinuous basement membrane with zones of matrix loss surrounding the invading tumour cells. Thus, a critical proteolytic event early in the metastatic cascade appears to be the degradation of basement membrane components. Basement membranes may also pose as barriers to tumour cell invasion at multiple points later in the metastatic cascade, including vascular infiltration and extravasation. Basement membranes contain type IV collagen as well as laminin and heparan sulphate proteoglycan as major components. Studies suggest that type IV collagen may form the basement membrane scaffolding on which laminin, heparan sulphate proteoglycan and minor components of the basement membrane are assembled [3]. Much attention has focused on the ability of metastatic tumour cells to degrade type IV collagen.

There is abundant evidence, both direct and by correlation, which implicates the specific class of extracellular matrix degrading metalloenzymes known as the matrix metalloproteases (MMPs) in the creation of the proteolytic defect in basement membrane type IV collagen that appears essential for cellular invasion [4, 5].

The matrix metalloprotease family

Many biological processes involving ECM turnover have been linked with expression of matrix metalloprotease

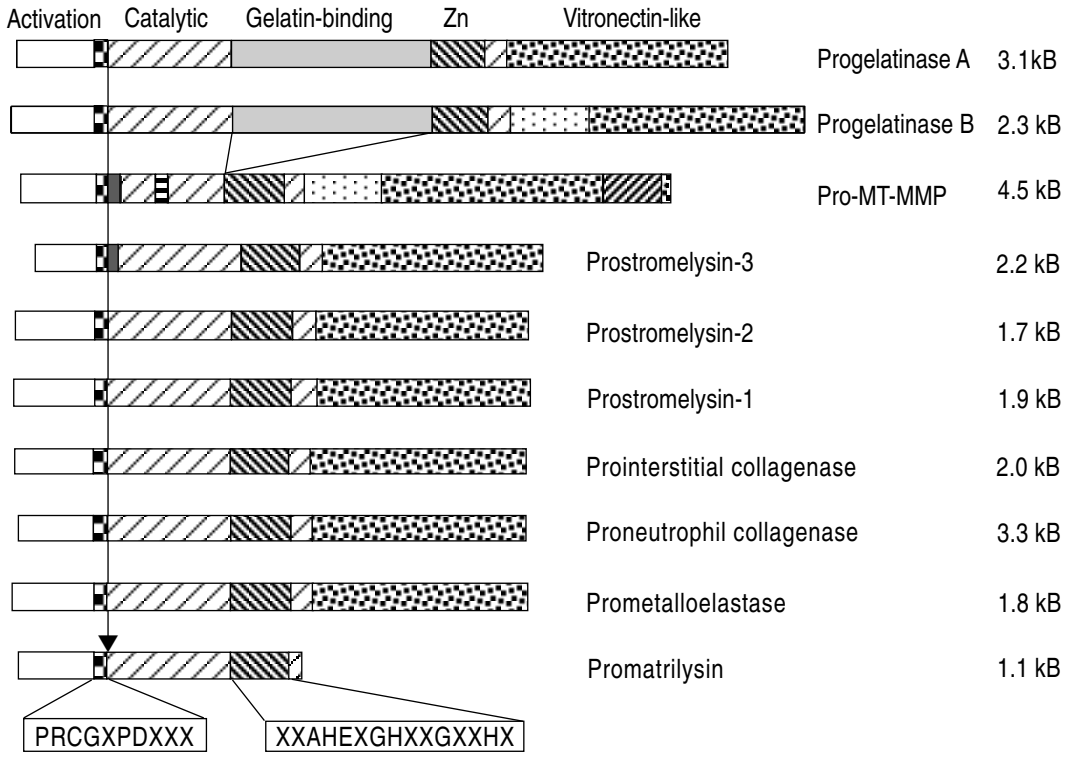

Fig. 1. – The domain structure of the matrix metalloproteinase family. Progelatinase A and B, pro-MT-MMP, prostromelysin-3, prostromelysin-2 and prostromelysin-1, prointerstitial collagenase, proneutrophil collagenase, prometalloelastase, and promatrilysin are represented diagrammatically and aligned to show regions of protein sequence homology [7–14]. Procollagenase-3, the newest number of this family is not shown. The various patterns represent different protein sequence domains encoded by separate exons. The figure demonstrates the relative size and the conservation of domains between the family members. There are four functional domains: ▭: signal peptide domain; ▬: propeptide domain; ▭ : catalytic domain; ▬ : haemopexin/vitronectin domain. Gelatinase A and B contain a cysteine-rich gelatin-binding domain (▬) which shows homology to fibronectin, but is absent from the other matrix metalloprotease enzymes. MT-MMP and stromelysin-3 contain a 10 amino acid insertion ▬ , which has homology to recognition sequences for furin-like enzymes. Gelatinase B and MT-MMP both have an insert that is similar to the type V collagen α-chain [15]. MT-MMP also contains a small, unique insertion inside the catalytic domain and a potential transmembrane domain. Below are the conserved amino acid sequences for the activation locus and the metal-binding atom domain of the active site. X: any amino acid; P: proline; R: arginine; C: cysteine; G: glycine; D: aspartic acid; H: histidine; E: glutomic acid; A: alanine.

(MMP) enzymes. The MMPs are a family of zinc atom-dependent endopeptidases with specific and selective activities against many components of the extracellular matrix [2, 4, 6–9]. This family currently consists of 11 enzymes, which are secreted as zymogens that must be activated extracellularly. They have been classified into three subgroups based on substrate preference: the interstitial collagenases, stromelysins and gelatinases (type IV collagenases); although, all of the enzymes have overlapping substrate specificity. The primary amino acid structure of the family consists of five modular domains, including a signal sequence; a profragment activation locus; a Zn atom-binding, catalytic domain; a proline-rich hinge region; and a haemopexin- or vitronectin-like C-terminal domain (fig. 1). The gelatinases contain an additional fibronectin-like gelatin-binding domain immediately upstream of the Zn-binding domain [9]. MT-MMP and stromelysin-3 contain a 10 amino acid insertion, which has homology to recognition sequences for furin-like enzymes [10]. Gelatinase B and MT-MMP both have an insert that is similar to the type V collagen α-chain [15]. MT-MMP also contains a small, unique insertion inside the catalytic domain and a potential transmembrane domain [16].

Regulation of MMPs

Malignant tumour cells exhibit repeated attachment and release from the ECM, as well as enhanced proteolysis and migration through matrix barriers that results in a sustained invasive capacity. In order to maintain the balance of the components necessary for optimal invasion, these processes must be tightly regulated both temporally and spatially. The role of MMPs in ECM degradation can be regulated at many stages, including gene activation and transcription, messenger ribonucleic acid (mRNA) stability, translation and secretion of latent proenzymes, binding of proenzymes to cell membranes and/or ECM components, proenzyme activation, inactivation by endogenous inhibitors and degradation or removal of active or inactive enzyme species [17].

At the level of transcription, many of the MMPs appear to be regulated by similar mechanisms. MMPs are responsive to cytokines and growth factors and hormones. In general, most of the MMPs are induced by interleukin-1β (IL-1β), tumour necrosis factor–α (TNF–α), platelet derived-growth factor (PDGF), transforming growth factor-a (TGF–α), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF) and nerve growth

factor (NGF) and are repressed by transforming growth factor-β (TGF–β) (reviewed in [9]). Interleukin-1α (IL-1α) appears to be acting as an obligatory intermediate regulator for stimulation of interstitial collagenase expression [18]. Sensitivity of individual MMPs to these factors varies from enzyme to enzyme, and is tissue-specific. Specificity is maintained by induction and repression of distinct MMP family members. Also, many factors must be integrated to elicit a response that is cell-specific. MMP production has also been found to be regulated by the pericellular environment, cell matrix interactions, and components of the ECM [19–26]. For example, a number of diverse agents have been found to stimulate production of interstitial collagenase by macrophages or epidermal keratinocytes, including calcium influx [27], ultraviolet light [28], and cell shape [29]. IL-1α and other cytokines have also been shown to stimulate interstitial collagenase release from fibroblasts in response to interaction between extracellular matrix and cell surface receptors [22, 30]. Interestingly, gelatinase A exhibits only a slight response to cytokine and growth factors [31–33], and calcium influx suppresses gelatinase A mRNA and protein synthesis [11]. This may indicate a unique role for gelatinase A in matrix homeostasis.

Activation of MMPs

Under some conditions, transcriptional activation of the MMP genes may be a requirement for ECM turnover. Current evidence also suggests that transcriptional activation alone may not be sufficient. Activation of proenzyme forms of these proteases is required for initiation of matrix degradation and acquisition of the invasive phenotype. The balance of activated proteases and endogenous inhibitors is crucial for determining the extent of ECM turnover [4, 17].

The biochemistry of MMP activation has been well-characterized through many *in vitro* studies. The mechanism for mammalian MMP activation is referred to as the "cysteine switch". In this mechanism, an unpaired cysteine residue in the profragment co-ordinates with the active site zinc atom and maintains the latency of the enzyme [34]. When this cysteine-zinc atom interaction is interrupted by chemical or physical means a conformational change occurs and subsequent proteolytic cleavage of the amino-terminal profragment ensues (reviewed in detail in [9]).

An understanding of the biological activation of the MMPs has not yet been fully elucidated, although a growing body of work has been put forth to understand the cellular and *in vivo* mode of activation. So far, two possible pathways for *in vivo* activation of MMPs have been proposed. Latent interstitial collagenase, stromelysin-1 and gelatinase B may be activated by the plasmin cascade. These events occur either at the cell surface *via* urokinase plasmin activator (uPA) receptor or distant from the site of secretion of the enzyme [35, 36]. Briefly, the zymogen plasminogen is cleaved by uPA or tissue plasmin activator (tPA) into plasmin. PA may be inhibited by an endogenous PA inhibitor (PAI) [37]. Plasmin cleaves 84 amino-terminal amino acids from latent fibroblast stromelysin to form activated stromelysin, and cleaves 81 amino-terminal amino acids from latent interstitial collagenase to form partially activated interstitial collagenase. Activated stromelysin may sub-sequently increase the activity of the partially activated interstitial collagenase 5–8 fold by clipping an additional 15 amino acids from its carboxy-terminus [36].

In contrast, activation of gelatinase A appears to occur in a cell-mediated fashion, although the exact mechanism of this activation is controversial. Plasmin/PA-mediated activation of insoluble gelatinase A has been reported [38, 39], whilst others have reported that purified progelatinase A and progelatinase A/tissue inhibitor of metalloprotease-2 (TIMP-2) complex are degraded, not activated, by plasmin [40]. A third series of publications demonstrate that serine proteases, such as plasmin and uPA, are not responsible for cell-mediated activation of gelatinase A [41–43]. In these experiments, serine protease inhibition failed to block cell-mediated activation of progelatinase A. Phorbol-ester or concanavalin A treatment of tumour cells enhances the expression of a cell membrane-associated activator of gelatinase A. Activation of latent gelatinase A or gelatinase A/TIMP-2 complex can be inhibited by addition of exogenous TIMP-2 or by chelating agents. Carboxy-terminally truncated gelatinase A is not activated by a similar mechanism, and therefore the carboxy-terminus appears to be necessary for activation [42]. The cell-surface associated activator has recently been isolated and shown to be an integral membrane matrix metalloprotease, referred to as MT-MMP [16]. Immunohistochemical studies have localized this MT-MMP to the surface of invasive tumour cells, but not surrounding fibroblasts. Further characterization of the role of this protease in tumour cell invasion is ongoing.

Tissue inhibitors of metalloproteases

The MMP family is further defined by the fact that all members are inhibited by a group of related endogenous inhibitors known as the tissue inhibitors of metalloproteases (TIMPs) [4–6]. The balance between the levels of activated MMPs and free TIMPs determines the net MMP activity. Altering this equilibrium affects the process of cellular invasion. Three distinct TIMP molecules have been isolated, cloned and characterized from several species [44–52].

TIMP-1 is a 28.5 kDa glycoprotein that preferentially forms a 1:1 complex with activated interstitial collagenase, stromelysin-1, and both the latent and active forms of the 92 kDa type IV collagenase (progelatinase B) [15, 53, 54]. Interaction of TIMP-1 with activated gelatinase B results in protease inhibition, whereas interaction with latent gelatinase B blocks stromelysin-mediated activation of this enzyme [55]. In addition to its role as a MMP inhibitor, TIMP-1 is also considered to function as a metastasis suppressor gene [56, 57].

TIMP-2, a nonglycosylated 21 kDa protein, has a high affinity for progelatinase A [58], and will form a 1:1 complex with either the latent or activated forms of the enzyme. TIMP-2 has also demonstrated inhibitory activity against other members of the MMP family. TIMP-2 inhibits both the type IV collagenolytic and gelatinolytic activities of gelatinase A [59], and blocks the hydrolytic activity of all activated MMPs [58–61].

TIMP-3, the newest member of the TIMP family, was initially isolated from SV-40 transformed chick embryo fibroblasts [62]. Recently, the murine and human *timp-3* complimentary deoxyribonucleic acids (cDNAs) have also been cloned [49, 50, 52]. Unlike the other family members, which are secreted from cells and remain soluble, TIMP-3 is localized to the ECM and shows preferential binding to ECM components.

Although they have overlapping inhibitory activities, the proteins are immunologically distinct, they are encoded by genes located on different chromosomes, and their expression is independently regulated [45, 47, 60, 61, 63, 64]. The *timp-1* gene has been mapped to the p11 region of human chromosome X [64, 66]. The *timp-2* gene is located on chromosome 11 in the mouse and on human chromosome 17q25 [64, 67]. The *timp-3* gene is localized on human chromosome 22 [49]. Northern blot analysis of *timp-1* reveals a single 0.9 kb transcript, while there are two *timp-2* transcripts of 3.5 and 1.1 kb, and a strong 2.4 kb *timp-3* transcript, as well as other transcripts at 5, 2.7, 1.6 and 1.1 kb for this inhibitor. Comparison of the human TIMP-1 and TIMP-2 amino acid sequences shows 37% identity and 66% homology (fig. 2a). There is 40% identity between human TIMP-1 and TIMP-3, and 45% identity between TIMP-2 and TIMP-3. The 12 cysteine residues are conserved among all members of the family [52], and similar disulphide bridges forming six peptide loops and two knots are expected for TIMP-1 and TIMP-2 (fig. 2b) [68]. These loops are thought to be responsible for inhibiting MMP activity, and residues beween cysteine-3 and cysteine-13 are critical for inhibition of MMP activity [69–71].

Studies on the regulation of individual timp gene expression indicates that each inhibitor may have specific and distinct physiological functions. Transcription of *timp-1* is responsive to the tumour promoter phorbol 12-myristate 13-acetate (PMA) [72, 73], hormones, [74, 75], and a variety of cytokines [72, 76]. Mouse and chicken *timp-3* transcription are also inducible by a variety of agents, including cytokines, tumour promoters and anti-inflammatory agents [50, 57]. In contrast, *timp-2* gene expression is primarily constitutive, and when sensitive reacts in a manner opposite to that of *timp-1* [47, 51, 78].

In addition to their role as inhibitors of MMPs, the TIMPs may also have other functions critical for regulation of the ECM. The binding of TIMP-1 and TIMP-2 to progelatinase B and A, respectively, may be important for controlling the activation of the latent enymes [15, 60, 79–81]. TIMP-1 has been found to be a serum mitogen *in vitro* [82, 83]. Chicken TIMP-3 (ChIMP-3) promotes detachment of cells that are acquiring the transformed phenotype from the ECM, and has growth stimulatory activity under low serum conditions [84]. TIMP-3 is thought to be involved in tissue specific, acute matrix remodelling [50, 52].

MMPs and cancer

A variety of studies have indicated a role for MMP enzymes in tumour invasion and metastasis. A number of methods have been utilized for assessing the presence of MMPs in human tumour tissues and serum from cancer patients. These include localization of MMPs in human tumour tissue by immunoperoxidase staining (IPS) of tissue sections, Northern blot analysis of MMP transcripts in riboneucleic acid (RNA) samples isolated from human tumour samples, localization of MMP transcription in tissue sections by *in situ* hybridization (ISH), and measurement of MMP levels in the body fluids of cancer patients. Positive correlations have been demonstrated between MMP expression and tumour invasion and metastasis *in vitro* [8, 85–87] as well as in *in vivo* animal models [88–90]. MMPs have been associated with the malignant phenotype in a wide variety of human tissues, including lung, prostate, stomach, colon, breast, ovaries and thyroid, as well as squamous carcinoma of the head and neck [8, 85–87].

Interstitial collagenase, which degrades the triple helical domains of the fibrillar collagens (types I, II, III, VII, VIII and X) into 1/4 amino-terminal and 3/4 carboxy-terminal fragments, is enhanced in a variety of human tumours. Statistically significant correlations have been found between the degree of histological differentiation in human colorectal tumours and the level of proteolytic activity of interstitial collagenase against soluble type I collagen [91]. In colorectal tumour samples, IPS analysis revealed enhanced staining for interstitial collagenase in the stromal cells and collagen fibres adjacent to the malignant nests of tumour cells, whilst normal, benign or malignant epithelium did not stain for the enzyme [92]. Augmented interstitial collagenase transcripts have been observed in 40% of primary pulmonary malignancies, but not in samples from adjacent normal lung tissue [93]. A number of studies using ISH demonstrate elevated transcripts for interstitial collagenase in squamous cell carcinomas of the head and neck that localized to the stromal fibroblasts adjacent to the malignant tumour masses [94–96].

The stromelysins are composed of stromelysins-1, 2 and 3, as well as matrilysin, and proteolyse laminin, fibronectin, proteoglycans, and nonhelical domains of type IV collagen. In addition, matrilysin cleaves urokinase to separate the catalytic and receptor-binding domains, which may have implications for the regulation of the functional activity of this plasminogen activator [97]. Stromelysin-1 and 2 mRNA production correlates with increased local invasiveness of head and neck carcinomas, and transcripts localize to the fibroblasts of the tumour stroma immediately adjacent to areas of basement membrane disruption [96]. ISH studies

a)

TIMP-1	-----MAPFE	PLASGILLLL	WLIA-PSRA***C***	T***C***VPPHPQTA	F***C***NSDLVIRA	44
TIMP-2	MGAAARTLRL	ALGLLLLATL	L---RPADA***C***	S***C***SPVHPQQA	F***C***NADVVIRA	47
TIMP-3	-----MTPWL	GLI-VLLGSW	SLGDWGAEA***C***	T***C***SPSHPQDA	F***C***NSDIVIRA	44
TIMP-1	KFVGTPEVNQ	TTLY------	-QRYEIKMTK	MYKGFQALGD	AADIRFVYTP	87
TIMP-2	KAVSEKEVDS	GNDIYGNPIK	RIQYEIKQIK	MFKGPEK---	--DIEFIYTA	92
TIMP-3	KVVGKKLVKE	G------PFG	TLVYTIKQMK	MYRGFTKM--	-PHVQYIHTE	85
TIMP-1	AMESV***C***GYFH	RSHNRSEEFL	IAGKL–QDGLL	HITT***C***SFVAP	WNSLSLAQRR	137
TIMP-2	PSSAV***C***GVSL	DVGGKKE–YL	IAGKAEGDGKM	HITL***C***DFIVP	WDTLSTTQKK	142
TIMP-3	ASESL***C***GLKL	EV–NKYQ–YL	LTGRVY–DGKM	YTGL***C***NFVER	WDQLTLSQRK	133
TIMP-1	GFTKTYTVG***C***	EE***C***TVFP***C***LS	IP***C***KLQSGTH	***C***LWTDQLLQG	SEKGFQSRHL	187
TIMP-2	SLNHRYQMG***C***	-E***C***KITR***C***PM	IP***C***YISSPDE	***C***LWMDWVTEK	NINGHQAKFF	191
TIMP-3	GLNYRYHLG***C***	-N***C***KIKS***C***YY	LP***C***FVTSKNE	***C***LWTDMLSNF	GYPGYQSKHY	182
TIMP-1	A***C***LPREPGL***C***	TWQSLRSQIA	----------			207
TIMP-2	A***C***IKRSDGS***C***	AWYRGAAPPK	QEFLDIEDP-			220
TIMP-3	A***C***IRQKGGY***C***	SWYRGWAPPD	KSIINATDP-			211

b)

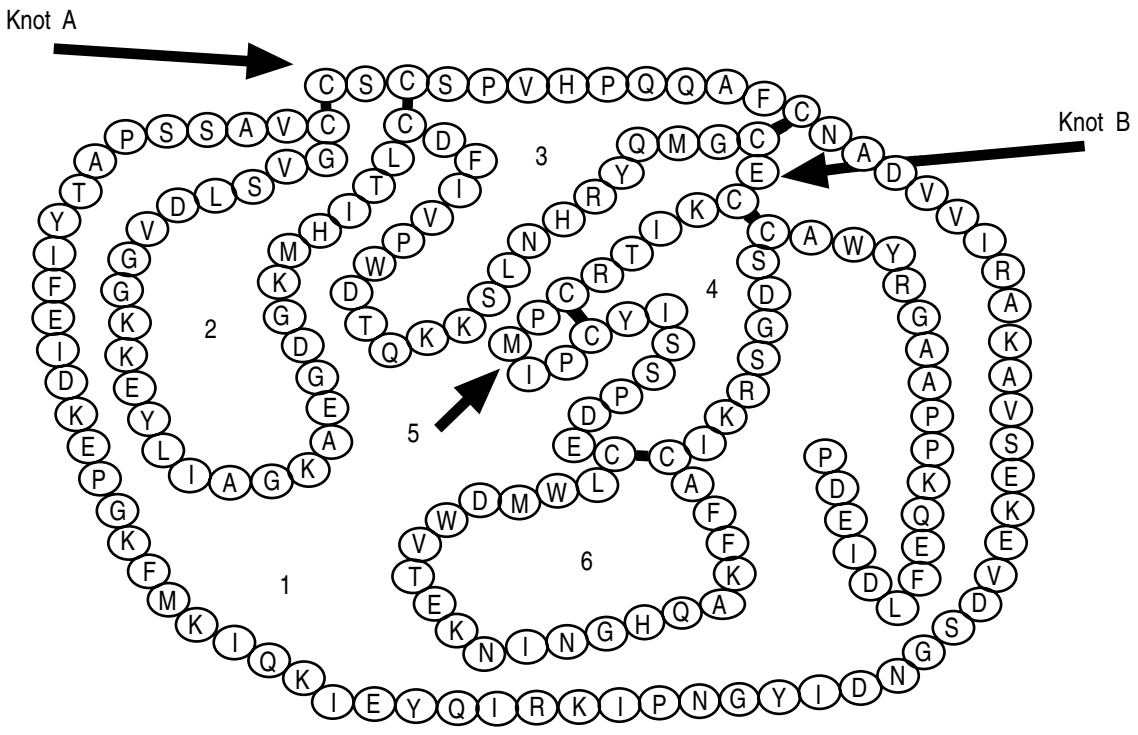

Fig. 2. – a) Alignment of amino acid sequences using single letter amino acid abbreviations for family members of the human tissue inhibitor of metalloproteases. The amino acid sequences of TIMP-1 [44], TIMP-2 [59], and TIMP-3 [49, 52] were extracted from the Swiss-Prot database. Optimal alignment was performed with a protein alignment program contained in Geneworks version 2.2.1 software package from intelligenetics (Mountain View, CA, USA). Residuels which are identical in all three sequences are shaded. The 12 conserved cysteines are denoted by bold italic C's (**C**). b) Disulphide bond pattern of TIMP-2. This figure depicts the predicted six loops numbered 1–6, and two knots (A and B) formed by the characteristic disulphide-bonding pattern of the TIMP family. The amino-terminal domain consits of loops 1, 2 and 3, and represents the domain responsible for binding and inhibiting the catalytic activity of the MMPs. The carboxy-terminal protein domain consits of loops 4, 5 and 6 and represents the domain that associates with the carboxy-terminus of gelatinase A.

demonstrate the matrilysin transcripts, but not stromelysin-1 and 2, are augmented in human gastric and colonic carcinomas [98]. ISH has also been used to localize expression of matrilysin in the epithelial cells of primary prostrate adenocarcinoma and in some foci of epithelial dysplasia, but not in the stroma. Matrilysin transcripts were present in 14 of 18 RNA samples from human prostate adenocarcinoma, but in only 3 of 11 normal prostate biopsy samples [99].

Stromelysin-3 has been studied only at the mRNA level, and its protease activity and substrate specificity has not yet been elucidated. Its message levels correlate with human breast cancer progression [12] and localize to the stromal cells surrounding invasive breast carcinoma. Other studies demonstrate association of stromelysin-3 with the invasive and malignant potential of primary pulmonary carcinomas [93], squamous cell carcinomas of the head and neck [100, 101] and basal cell carcinomas [100].

As was noted previously, there is consistent expression of stromelysins and interstitial collagenase in the stromal fibroblasts adjacent to the malignant epithelium of many human tumours. This suggests that release of soluble factors from invasive tumour cells may be stimulating production of these MMPs by fibroblasts. A collagenase stimulatory factor has been isolated and partially sequenced from a human lung carcinoma cell line [102]. The sequence data indicates that the protein is novel, and has no homology to any growth or motility factors. The factor is released into the tumour cell media and associates with tumour cell membranes. Recent data demonstrate that this factor may co-ordinate synthesis and secretion of several MMPs (notably interstitial collagenase, gelatinase A and stromelysin-1) [103].

The gelatinases degrade denatured collagens (gelatin) in addition to a number of native collagen types that contain helical disruptions. Numerous IPS studies demonstrate expression of gelatinase A in many types of human tumours that is limited to the malignant epithelial cells. Two variants of bronchioalveolar carcinoma, sclerosing and mucinous, exhibit gelatinase A staining, which may contribute to the poor prognoses of these subgroups [104]. Elevated gelatinase transcripts have been identified from primary human pulmonary carcinomas [93], although gelatinase A transcripts were occasionally present in normal uninvolved lung tissue. Gelatinase B was also found in 5 of 9 pulmonary tumours. Recently, ISH has been used to characterize gelatinase B mRNA expression in squamous cell and adenocarcinomas [105]. All 12 squamous cell carcinomas express this enzyme. Gelatinase B mRNA was found both in the tumour cells and in the host stromal cells surrounding the tumour, but not in normal lung fibroblasts. Gelatinase B expression was not found in the adenocarcinomas of the lung or in the stroma surrounding these tumours. Low levels of gelatinase B expression was seen in a variety of normal tissues, including bronchial epithelium, basal cell hyperplasia of bronchial epithelium, alveolar macrophages, and in bronchial mucous glands. Expression of gelatinase B is also evident in squamous cell carcinomas of the skin and colon [106, 107].

Primary breast cancers [12], prostate, and colon carcinomas [87] also contain elevated gelatinase A mRNA. In both the breast and prostate tissues examined there was a gradual increase in the immunohistochemical reactivity for human gelatinase A as the lesions progressed from atypical hyperplasia to carcinoma *in situ*, with frankly invasive carcinoma showing the strongest levels of gelatinase A staining. This evidence strongly supports a specific role for gelatinase A in most human tumours studied. It also suggests that tumour cells may begin to produce progelatinase A very early in their progression. Furthermore, this suggests that whereas production of progelatinase A is necessary, it is not sufficient for attainment of the invasive phenotype. Thus, critical points in the evolution of invasive carcinomas may be the activation of latent enzyme and the down regulation of active enzyme by specific inhibitors. Direct demonstration of the role of MMPs in general, and specifically gelatinase A, in cell invasion comes from studies in which both TIMP-2 and antibodies to gelatinase A were used to neutralize invasion of HT1080 human fibrosarcoma cells across reconstituted basement membranes [108, 109].

TIMPs and cancer

Numerous studies correlate low TIMP expression with enhanced invasive and metastatic properties in a number of murine and human tumour cell lines. Overexpression of TIMP-2 in ras transformed rat embryo fibroblasts results in reduction of *in vivo* growth rate and locally invasive character when the transfected cells producing TIMP-2 are injected subcutaneously, as well as loss of lung colony formation when these cells were injected intravenously in nude mice [110]. TIMP-1 has also been shown to inhibit *in vitro* invasion of human amniotic membranes [111, 112], and *in vivo* metastasis in animal models [112, 113]. Disruption of TIMP-1 by homologous recombination results in increased invasive behaviour in embryonic stem cells. This effect was reversed by the addition of exogenous TIMP-1 [114]. In addition, downregulation of TIMP-1 using an antisense construct transfected into NIH3T3 cells causes enhanced invasion of human amniotic membranes and formation of tumours in athymic mice [57]. Therefore, both TIMP-1 and TIMP-2 may function as natural suppressors of cellular invasion.

Angiogenesis, MMPs and TIMPs

Tumour cell invasion and angiogenesis share a number of functional similarities. Initiation of cellular invasion in both processes requires attachment to a basement membrane, followed by creation of a proteolytic defect in the basement membrane and migration through this defect. After the invading cell crosses this connective tissue barrier, cell proliferation and continued invasive behaviour result in production of either a new vessel lumen or metastatic foci. In addition to sharing these functional similarities, angiogenesis and

tumorigenesis may be mutually stimulating. Formation of new blood vessels permits expansion of tumour foci in three dimensions [5]. Prior to vascularization, tumour foci exist as small, asymptomatic lesions restricted by the limitation of passive oxygen and nutrient diffusion. Following vascularization, the tumour foci undergo rapid local expansion and acquire enhanced metastatic potential that correlates directly with the degree of vascularization of the primary tumour [115]. Thus, tumour invasion and metastasis formation are closely linked to tumour-induced neoangiogenesis.

Evidence for the role of MMPs and TIMPs in angiogenesis come from a number of studies. Nanomolar concentration of TIMP-2 will block the angiogenic response to bFGF, a principal angiogenic cytokine produced by vascularized human tumours, in the chick chorioallantoic membrane assay (Stetler-Stevenson, un-published data). TIMP-1 has also been shown to inhibit endothelial cell invasion of human amniotic membranes *in vitro* [111]. Cartilage-derived inhibitor (CDI), a TIMP-related protein isolated from bovine articular cartilage, blocks endothelial cell proliferation and angiogenesis [116, 117]. In addition, TIMP-1 and TIMP-2 inhibit chick yolk sac vessel morphogenesis in response to polyamines [118].

SCHNAPER *et al*. [119] recently demonstrated the critical nature of the balance of MMPs and TIMPs in an *in vitro* model of angiogenesis. These experiments show that addition of exogenous TIMPs inhibits endothelial cell tube formation on the reconstituted basement membrane matrix. This effect was mimicked by the addition of antibodies which neutralized gelatinase A. Up to a certain level, addition of increasing concentrations of exogenous gelatinase A resulted in enhancement of tube formation that was inhibited by addition of TIMP-2. However, addition of excess activated gelatinase A beyond a critical level resulted in a decrease in tube formation that was reversed by addition of exogenous TIMP-2. These results suggest that the early stages of endothelial tube formation are dependent on a critical balance of active protease, gelatinase A, and inhibitor, TIMP-2. Excess protease activity, although initially stimulatory, becomes inhibitory in higher concentrations, and TIMP-2, can reverse this effect. These results demonstrate the critical nature of the balance between active protease and protease inhibitor, but also demonstrate that the balance can be altered by addition of exogenous protease inhibitors to block both endothelial cell invasion in angiogenesis and tumour cell invasion in metastasis. This suggests that MMP inhibitors, particularly gelatinase-A-specific inhibitors, may have dual potential for clinical prevention of tumour cell dissemination and tumour-associated neovascularization.

Whilst the mechanism for TIMP-mediated inhibition of tumour invasion and angiogenesis appears, at least in part, to be through inhibition of protease activity required for cellular invasion, recent observations suggest that TIMPs affect other distinct groups of biological activities through mechanisms other than MMP inhibition. These include biological activities that are required for angiogenesis and tumour cell invasion. In fact, TIMP-1 was independently identified and cloned as having erythroid-potentiating activity (EPA) [120]. TIMP1/EPA augments red blood cell colony formation by erythroid precursors (CFU-E, BFU-E) and TIMP-2 has been shown to have similar activity [121]. The growth-stimulatory activity in these assays is thought to be due to a direct cellular effect mediated by a cell surface receptor and not through inhibition of metalloproteinase activity, although the precise mechanism is not yet known. Recently, several laboratories have reported growth stimulatory effects of TIMPs on a number of cell lines *in vitro* [83, 22]. The mechanism of these effects and the requirement for metalloprotease inhibitory activity are unknown.

A novel growth-inhibitory activity of TIMP-2, which is unique to this inhibitor and independent of its metalloproteinase inhibitory activity, was recently demonstrated [123]. The ability of TIMP-1 and TIMP-2 to inhibit endothelial cell growth *in vitro* was examined. TIMP-2, but not TIMP-1, specifically inhibited the proliferation of human microvascular endothelial cells stimulated with basic fibroblast growth factor bFGF. Also, a synthetic metalloproteinase inhibitor, BB94, effective at nanomolar concentrations, did not mimic the inhibitory effect of TIMP-2 on endothelial cell proliferation. Thus, the ability of TIMP-2 to block bFGF-stimulated microvascular endothelial cell growth is apparently not due to inhibition of matrix metalloproteinase activity. This is the first demonstration that TIMP-2 has growth-inhibitory properties that are unrelated to protease inhibitory activity. These findings suggest that, in addition to directly blocking tumour cell and endothelial cell invasion, TIMP-2 can also block bFGF- stimulated endothelial cell growth. This further suggests that TIMP-2 may have several activities that could be exploited in the oncology clinic: blocking primary tumour growth through inhibition of bFGF-stimulated angiogenesis as well as prevention of matrix degradation necessary for cellular invasion, thus blocking infiltration of the primary tumour mass by new blood vessels and tumour cell dissemination. Recent findings from ALBINI *et al*. [124] demonstrate that TIMP-2 blocks cellular invasion and angiogenesis induced by conditioned media from Kaposi's sarcoma cell conditioned media. The mechanism and potential clinical utility of this effect are now under study.

Future directions

The emphasis of prior work has been on the role of MMPs in ECM turnover associated with the pathology of tumour invasion and arthritis. In the future, studies should also be directed towards understanding the regulation of these enzymes and their inhibitors in conditions of excess matrix accumulation. This may have important implications for a number of pulmonary diseases, such as idiopathic pulmonary fibrosis, bronchopulmonary dysplasia and emphysema, to name but a few.

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