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The Expression of Tissue Inhibitor of Matrix Metalloproteinase-2 In Activated T Cells: Role in T Cell Growth Modulation

Megan So-Young Lim

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Abstract

The balance between the activities of matrix metalloproteinases (MMPs) and the tissue inhibitors of metalloproteinases (TIMPs) is an important control point during turnover of the extracellular matrix (ECM). Deregulation of this system is associated with many physiologic and pathologic processes including inflammation, embryo implantation, lymphocyte motility, tumor invasion and metastasis. These proteins are expressed in normal and malignant lymphoid cells, and thus they may play a role in their physiology. TIMPs are multifunctional proteins with growth modulatory activities in addition to their metalloproteinase-inhibitory functions. Detailed analysis of MMP and TIMP expression in lymphoid cells demonstrated specific patterns of expression in cells of B and T cell lineage. Moreover, expression of MMP-9 and TIMP-2 was elevated in PHA and IL-2 stimulated peripheral blood T cells.

The role of TIMP-2 on T cell growth and apoptosis was examined. The studies demonstrate that rTIMP-2 increased apoptosis in activated peripheral blood T cells in a dose-dependent manner. This effect was specific to TIMP-2 and was not observed with TIMP-1. Recombinant TIMP-2 also induced apoptosis of neoplastic T cells. The metalloproteinase inhibitory function of TIMP-2 was important in this process as synthetic metalloproteinase inhibitors also enhanced apoptosis. Reduced and alkylated rTIMP-2 did not induce apoptosis while a TIMP-2 neutralizing antibody reduced the level of apoptosis. This effect was not observed with anti-TIMP-1 antibody or an isotype control antibody.

Molecules involved in ligand-mediated pathways of T cell apoptosis are the Fas/Fas ligand and TNF receptor /TNF system. The potential role of TIMP-2 in modulation of cell surface and soluble Fas ligand expression was studied. Analysis of cell surface expression of Fas ligand, and determination of soluble forms of Fas ligand in conditioned media of cells grown in the presence or absence of TIMP-2 and BB-94 support the hypothesis that these molecules are involved in inducing T cell apoptosis. The identity of the metalloproteinase responsible for the cleavage of molecules involved in this process is unknown. These data indicate that MMPs and TIMPs can modulate the growth and function of reactive and neoplastic lymphoid cells suggesting a potential for therapeutic intervention of immune-mediated and neoplastic disorders.

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Table of Contents

List of Tables

List of Figures

Chapter 1. Introduction

Chapter 2. Materials and Methods

Chapter 3. Results

List of Abbreviations

General:

GAPDH	glyceraldehyde 3-phosphate dehydrogenase
MMP	matrix metalloproteinase
TIMP	tissue inhibitor of metalloproteinase
ECM	extracellular matrix
TPA	tetradecanoyl phorbol acetate
TGF-β	transforming growth factor-β
IL	interleukin
TN-α	tumor necrosis factor-α
PCR	polymerase chain reaction
PHA	phytohematglutinin

Units of Measurement:

cpm	counts per minute
kDa	kiloDaltons
kb	kilobase
°C	degrees Celsius
M	molar
mM	millimolar
*u*M	micromolar
g	gram
mg	milligram
*u*g	microgram
ng	nanogram
pg	picogram
L	liter
ml	milliliter
*u*L	microliter
*u*Ci	microcurie
bp	basepair

Chemicals and Solutions:

BCS	bovine calf serum
BSA	bovine serum albumin
DTT	dithiothreitol
EtBr	ethidium bromide
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol bis(B-aminoehtyl ether)-N,N'tetraacetic acid
MOPS	3-(N-morpholino(propanesulfonic acid
FCS	fetal calf serum
PBS	phosphate-buffered saline
PMSF	phenylmethyl-sulfonyl fluoride
SDS	sodium dodecyl sulfate
SSC	standard saline-citrate
TE	TRIS-EDTA buffer
TBS	TRIS-buffered saline
TRIS	tris-(hydroxymethyl)aminomethane
TSFM	total serum-free medium

Nucleic Acids:

cDNA	complementary DNA
mRNA	messenger RNA
RNA	ribonucleic acid
DNA	deoxyribonucleic acid
dNTP	dexoynucleoside 3'-triphosphate
dATP	deoxyadenosine 3'- triphosphate
dCTP	deoxycytidine 3'- triphosphate
dGTP	deoxyguanosine 3'- triphosphate
dTTP	deoxythymidine 3'- triphosphate
ATP	adenosine 3'- triphosphate
CTP	cytidine 3'- triphosphate
GTP	guanosine 3'- triphosphate
UTP	uridine 3'- triphosphate

Chapter 1. Introduction

1.1. Overview of Extracellular Matrix

The extracellular matrix (ECM) is a complex network of proteoglycans, glycoproteins and polysaccharides which envelope and supports cells in multicellular organisms. Previously considered to be an inert structure, it is now believed that the ECM is a highly dynamic system. Not only does the ECM provide physical support and architectural properties, it also acts to regulate aspects of cellular differentiation, metabolism, growth and motility (1-3). Matrix turnover is a highly regulated process requiring coordinated regulation in a spatial and temporal fashion. The balance between proteinases and their inhibitors is an important control point of this process (4). Unbalanced activities of metalloproteinases and their inhibitors are associated with various physiologic and pathologic conditions including inflammation, embryo implantation, tumor invasion and metastasis (Table 1).

1.2 Overview of Matrix Metalloproteinases

1.2.1 Proteases

Proteases responsible for matrix turnover have been identified in all four classes: (I) serine proteases from the plasminogen activator (PA) system which include plasmin, tissue type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA); (ii) cysteine proteinases (cathepsin B and L), (iii) aspartyl proteases, such as cathepsin D and (iv) the matrix metalloproteinases (5). The matrix metalloproteinase (MMP) family of enzymes is well characterized and represents a family of zinc dependent endopeptidases which work at physiological neutral pH that

together can cleave all of the protein components of the ECM. They are thought to be primarily responsible for much of the degradation of the ECM that occurs during physiologic and pathologic events. Inhibition of the enzymes by zinc chelation prevents the bulk of matrix degradation. (Reviewed in (6-8)).

1.2.2 Classification of MMPs

The MMPs have been subgrouped into at least four types based on substrate specificity and sequences: interstitial collagenases, stromelysins, gelatinases and the membrane-type MMPs (MT-MMPs). (See Table 2). Except for stromelysin-3 (STM-3) and the MT- MMPs (9), MMPs are secreted as pro-enzymes and subsequently activated extracellularly by proteolytic cleavage of an amino-terminal domain. For STM-3, intracellular activation by a furin-like proteinase has been demonstrated to occur via a recognition sequence, RXKR (10, 11). This recognition sequence is also found in the deduced amino-acid sequences of the MT-MMPs. However, the exact details of the cellular mechanism for activation via these furin-like proteinases, also known as prohormone convertases, are not well understood.

1.2.3 Structural Features

A number of common structural features are shared among members of the MMP family. Their modular structural characteristics are depicted in Figure 1. Following the amino-terminal signal peptide, all MMPs contain a propeptide domain that conceals the active site of the enzyme. This propeptide domain must be cleaved in

order to activate the enzyme. The catalytic domain contains two histidine residues and a glutamic acid residue that coordinate zinc atom-binding and in the latent state, the pro-enzyme domain contains a fourth coordinating position in which a cysteine residue coordinates the zinc atom. Activation of the enzyme occurs when the zinc-sulfur coordination is disrupted ('cysteine switch') and thus exposing the catalytic site (12-14). This results in a partially active intermediate form of the enzyme that can cleave the propeptide region by autocatalysis, thereby rendering the enzyme fully active. A proline-rich hinge region is then followed by a carboxy-terminal hemopexin-like domain which acts, at least in some MMPs, to confer substrate specificity in conjunction with the catalytic domain (14). This region is also involved in interactions of pro-MMP-2 and pro-MMP-9 with the endogenous inhibitors (tissue inhibitors of metalloproteinases, TIMPs). An important physiological activator of pro-MMPs is plasmin, a serine proteinase that is generated from plasminogen by the action of tissue or urokinase-plasminogen activator (uPA). As mentioned previously, the serine proteinase furin plays a role in activation of MT-MMPs and STM-3 by catalyzing the removal of their propeptides.

1.2.4 Levels of Regulation

To avoid excessive proteolysis and tissue damage a precisely coordinated and temporally regulated system is required. Thus, proteolysis is regulated at several levels. These include; (1) gene expression encompassing both transcriptional and translational regulation, (2) post-translational modification and secretion of latent MMP, (3)

activation of pro-MMPs and, (4) their inhibition by endogenous inhibitors, TIMPs. Activation pathways can cooperate resulting in activation of downstream MMPs such as MMP-9 and Collagenase-1, see Figure 2. As the activation machinery is localized on the surface of the cell, proteolysis is maximal in the immediate pericellular environment, where it can affect cell-cell and cell-ECM interactions.

Table 1. Matrix Metalloproteinase Involvement in Physiological and Pathological Tissue Remodelling Processes.

Physiologic Processes	Pathological Processes
Angiogenesis	Angiogenesis
Blastocyst implantation	Aortic aneurysm
Bone growth plate	Atherosclerosis
Bone remodelling	Cancer invasion
Cervical dilatation	Corneal ulceration
Embryogenesis	Dilated cardiomyopathy
Endometrial cycling	Epidermolysis bullosa
Fetal membrane rupture	Fibrotic lung disease
Hair follicle cycle	Gastric ulcer
Macrophage function	Liver cirrhosis
Mammary development	Osteoarthritis
Neutrophil function	Otosclerosis
Ovulation	Periodontal disease
Salivary gland morphogenesis	Rheumatoid arthritis
Tooth eruption	Tumor metastasis
Uterine involution	Wound/frature

Table 2. The Human Matrix Metalloproteinase Family

Enzyme	MMP	Mass (kDa)	Matrix Substrates
Collagenases:			
Interstitial	MMP-1	54	Collagens I,II,III,VII,VIII
neutrophil	MMP-8	53.4	Collagens I,II,III
collagenase-3	MMP-13	54	Collagens I,II,III
collagenase-4	MMP-18		Xenopus protein
Stromelysins:			
stromelysin-1 transin proteoglycanase	MMP-3	54	Aggrecan;collagens III,IV,V, IX,XI;gelatins;fibronectin; laminin;elastin
stromelysin-2	MMP-10	54	Aggrecan;collagens III,IV,V, IX,XI;gelatins;fibronectins; laminin, elastin
stromelysin-3	MMP-11	55	Alpha-1-anti-trypsin
matrilysin	MMP-7	30	Aggrecan;collagen IV; fibronectin;laminin;gelatins
macrophage elastase	MMP-12	54	Elastin;fibronectin,myelinbasic protein, TNF-a
Gelatinases:			
gelatinase A 72 kDa gelatinase	MMP-2	74	Gelatins, elastin, aggrecan; Collagens IV,V,VII,X,XI
gelatinase B 92 kDa gelatinase	MMP-9	78	Gelatins;elastin;aggrecan; Collagens IV,V
Membrane bound:			
MT1-MMP	MMP-14	66	ProgelatinaseA,collagen, gelatin
MT2-MMP	MMP-15	64	Progelatinase A
MT3-MMP	MMP-16	72	Unknown
MT4-MMP	MMP-17	unknown	Unknown
MT5-MMP	MMP-24		
MT6-MMP			
Other:			
	MMP-19		
Enamelysin	MMP-20		
	MMP-21		
	MMP-22		

Note: MMP-4,-5,-6 and -23 initially thought to belong to the MMP family has been subsequently characterized and excluded.

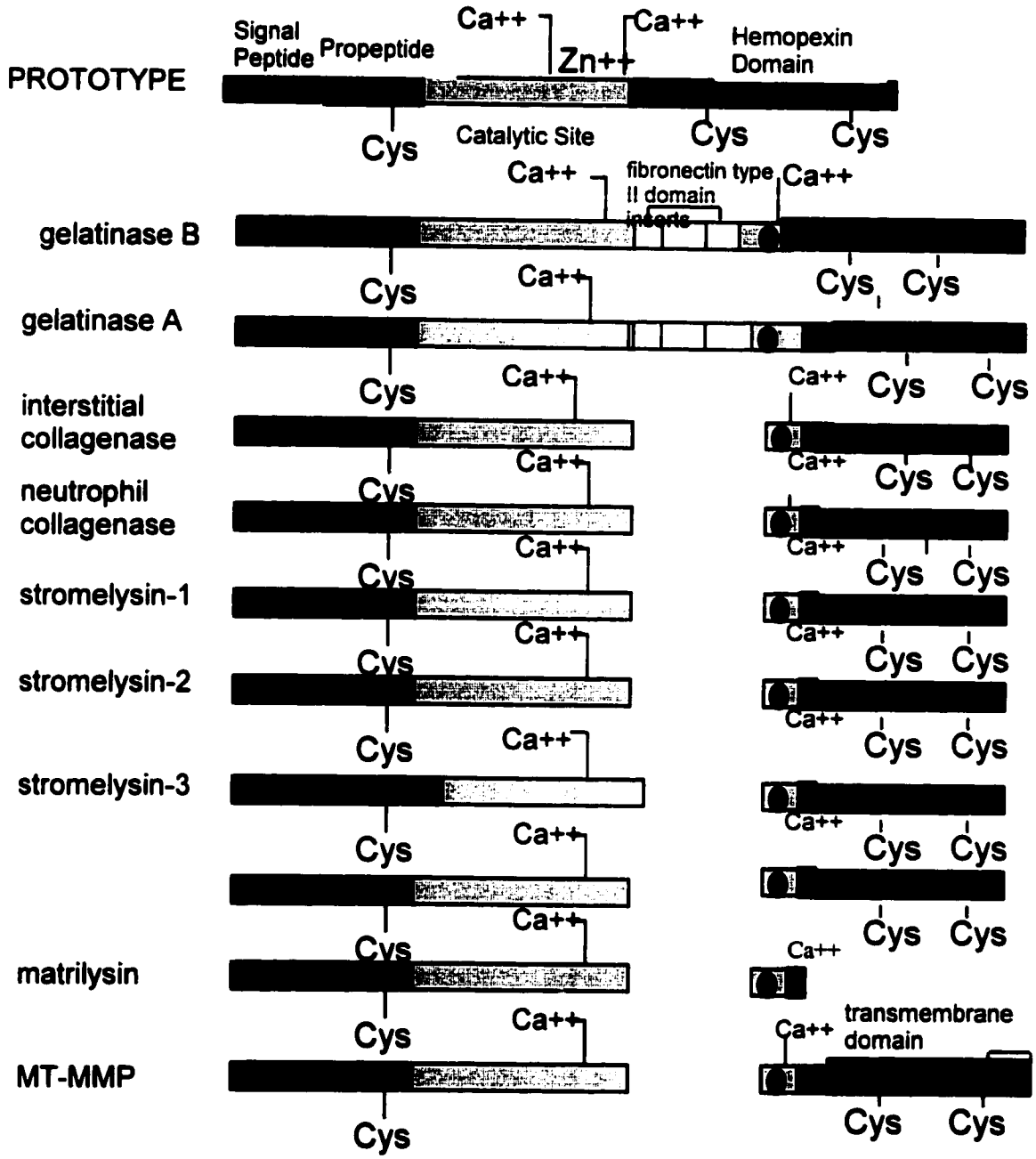


Figure 1. Modular domain structure of the matrix metalloproteinases (MMPs). The MMP family is based on a five-domain modular structure. Three tandem 58 amino acid fibronectin type II repeats distinguish the gelatinases, matrilysin lacks the hemopexin domain, and the transmembrane domain is found only in the membrane-type (MT)-MMPs (3, 5).

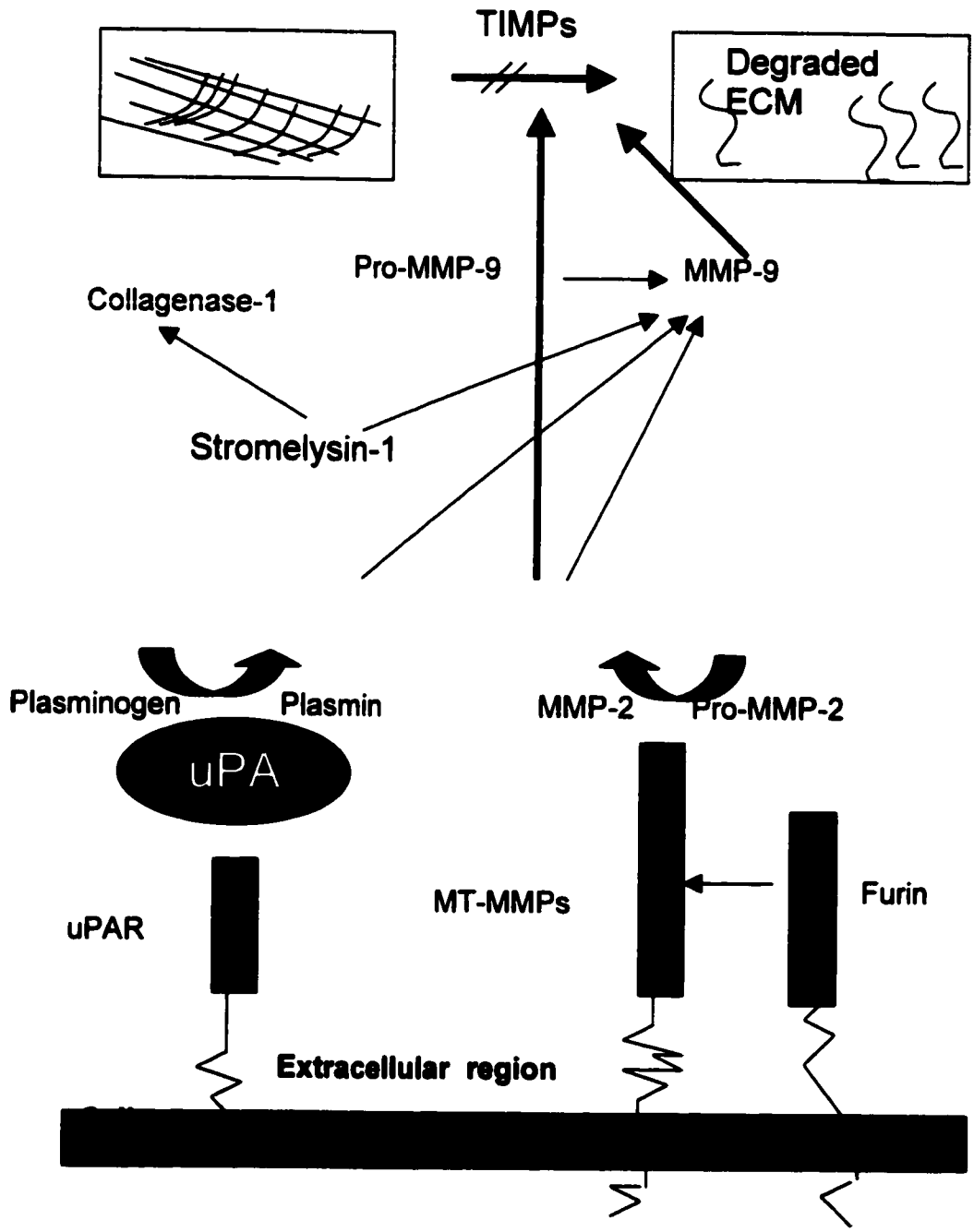


Figure 2. Activation machinery of matrix metalloproteinases. The coordinated activation of several MMPs is initiated by the formation of plasmin. Urokinase plasminogen activator (uPA) which is anchored by its receptor, uPAR catalyzes the activation of plasminogen to plasmin. Plasmin activates a variety of MMPs including stromelysin-1, collagenase-1 and pro-MMP-9. MMP-2 is activated by MT-MMPs which are activated by furin proteinases. The result is an amplification of the activation cascade.

1.3 The Gelatinases

The members of the gelatinase subfamily gelatinase A (72 kDa gelatinase, MMP-2) and gelatinase B (92 kDa gelatinase, MMP-9) are important effectors of cellular invasion. These enzymes can degrade Types IV and V collagen present in basement membrane, as well as elastin, fibronectin and proteoglycan core proteins (5). The gelatinases are structurally distinct from other MMP members by virtue of three fibronectin type II module inserts within the catalytic domain amino-terminal to the zinc binding site (15, 16). These fibronectin modules are thought to confer substrate specificity to the gelatinases, or to play a role in location of the enzyme to the ECM (17). In addition, MMP-9 contains an extended carboxy-terminal collagen V-like domain which may allow it to bind to denatured collagen (gelatin) substrate with high affinity (3).

1.3.1 Regulation of gelatinase expression

Despite similar enzymatic properties, MMP-2 and MMP-9 are controlled by fundamentally different mechanisms. MMP-2 is expressed by a wide variety of cell types in an essentially constitutive fashion (18) and functional enzyme is generated by a very specific cell surface activation mechanism involving another MMP, namely MT-MMP (19). In contrast, proMMP-9 is activated by a variety of proteinases, including stromelysin-1 (20), MMP-2 (21) tissue plasmin and tissue kallikrein (22) with localized inducible expression of the enzyme being of more importance for control than in the case of MMP-2 (23). MMP-9 is expressed by a more limited range of cell types,

including fibroblasts, keratinocytes, polymorphonuclear leukocytes, monocytes/macrophages, and a number of tumor or transformed cells (5). Transcription of MMP-9 can be activated by growth factors, cytokines and tumor promoters (24, 25). The promoter structure of these MMPs provides an explanation for the above observations. The MMP-2 promoter, unlike most of the MMP promoters, does not have a TATA box or the common AP-1 element, critical for the induction of these genes by PMA, growth factors, and cytokines. The MMP-9 promoter is also unique in its requirement for the NFκB and Sp-1 element for induction by inflammatory cytokines such as TNF -α and IL-1 (26-28) while the ETS/PEA transcription factor is involved in mediating TGF-β regulation (25, 29). Cell-cell interactions as causal events in the upregulation of these enzymes have been examined by Lacraz et al., (30). Upon association with PHA-activated human T lymphocytes, the human monocytic line THP-1 produces interstitial collagenase and MMP-9. While MMP-2 appears to be involved in constitutive ECM remodeling, the inducibility of MMP-9 suggests that it normally plays a role in acute ECM remodeling processes.

1.4 The Tissue Inhibitor of Metalloproteinases (TIMPs)

1.4.1 Introduction

Matrix metalloproteinases are specifically inhibited by their natural inhibitors, TIMP-1, TIMP-2, TIMP-3 and TIMP-4 (12, 31-37). For a summary of the characteristics of TIMPs 1-4, see Table 3. (Reviewed in (38, 39). TIMPs are expressed by a variety of cell types and are present in most tissues and body fluids. TIMP-1 and

TIMP-2 form specific complexes with progelatinase B and A, respectively (40), while activated MMP-2 and MMP-9 bind both (41). TIMP-2 has also been shown to inhibit the activation of pro-gelatinase A by MT-1-MMP by forming a stable complex with activated MT-1-MMP (42). MT-MMPs are not inhibited by TIMP-1 (43). TIMP-3, a unique member of this family has high affinity for components of the extracellular matrix, and as a consequence is sequestered there (36, 44). TIMP-3 has been localized as a component of normal basement membranes (35)including Bruch's membrane (45, 46). There is limited information about the inhibitory mechanisms utilized by TIMP-3 (47). TIMPs differ in the extent of glycosylation, in that TIMP-1 and TIMP-3 are glycosylated whereas TIMP-2 and TIMP-4 are not. The apparent molecular weights of TIMP- 1,2,3 and 4 are 28, 24, and 21 kDa, respectively (4, 37).

1.4.2 Structure-Function

The four members of the TIMP family share several structural features (1, 33, 36, 48, 49). TIMPs bind with high affinity in a 1:1 molar ratio to the N-terminus of active and pro-MMPs and inhibit their proteolytic activity. The TIMPs contain 12 conserved cysteine residues that in TIMP-1 have been shown to be paired into six disulfide bridges forming six peptide loops and two knots (50). The TIMPs are approximately 40% identical at the amino acid level and since the 12 cysteine residues are spatially conserved in all four types, presumably have a similar disulfide loop pattern as that mapped for TIMP-1.

a) N-terminus of TIMPs

The whole N-terminal 20 amino acid is highly conserved in all TIMPs and the extreme NH2 terminal Cys is absolutely essential. A 29 amino acid leader sequence is presumably cleaved off to produce the mature protein. The N-terminal domain comprising the first three disulfide-bonded loops, can fold independently of the C-terminal domain and is necessary and sufficient for MMP inhibitory activity. Mutational studies demonstrated that the region from cysteine-3 through cysteine-13 is critical for MMP inhibitory activity (33, 51). Structural work on TIMP-1 and TIMP-2 has been performed by a combination of NMR and X-ray crystallography studies (52, 53). The N-terminal domain is a 5-stranded β-sheet that is folded over to form a barrel, with homology to the oligonucleotide/oligosaccharide-binding (OB) fold found in several baterial enterotoxins and nucleases as well as yeast aspartyl-tRNA synthetase (54). X-ray crystal structures of full-length TIMPs in MMP-3/TIMP-1 and MT1-MMP/TIMP-2 complexes shows the TIMP molecules to be an elongated, continuous wedge that occupies the entire length of the active site cleft of the MMP (52, 53). Six separate polypeptide segments of TIMP-1 make contact with MMP-3, four contributed by the N-terminal domain and two by the C-terminal domain (52). Cys-1 coordinates the Zn atom in the MMP active site through its N-terminal α-amino group and its carbonyl group. The α-amino group is critical as the extension of TIMP-2 by one Ala residue renders the molecule inactive as an MMP inhibitor (55).

b) Structural features important for specificity

Mutational studies of the N-terminal domain of TIMP-1 highlight the importance of the Cys 1/Cys 70 disulfide bond in TIMP-1 that creates a surface ridge involving residues from Cys 1 to Val 4 and Met 66 to Val 70 that contacts the active site (56, 57). Thr 2 interacts with the S1' pocket of the MMP, which is a critical determinant of substrate specificities of the enzymes. Mutation of Thr2 in TIMP-1 to Ala generated a molecule that is 17-fold more effective against MMP-3 than MMP-1(56). In TIMP-2, mutation of the analogous Ser 2 residue to Lys significantly reduced effectiveness against MMP-3, but did not influence action on MMP-2 or MMP-14 (43).

Another major region contributing to the specificity of TIMPs is the loop between the A and B strands in the β-barrel. In TIMP-1 this loop is very short, whereas TIMP-2 and TIMP-4 have longer AB loops. In TIMP-2, this long loop enables it to fold over the edge of the active site cleft in MT1-MMP and extend into a surface pocket caused by a structural feature known as the "MT-loop" that is unique to MT1-3 MMPs. The "MT-loop" is formed by an 8-residue insertion in the MT-MMPs relative to other MMPs (53). Mutation of Tyr 36 in the TIMP-2 AB loop reduces its ability to inhibit MT1-MMP, without affecting actions on other MMPs (43). The inability of TIMP-1 to inhibit MT-MMP may be explained by the short AB loop, however, TIMP-3, which also has a relatively short AB loop, can bind and inhibit MT-MMP as well as TIMP-2. These results suggest that other parts of the molecule are important in MMP:TIMP interactions (52).

c) C-terminus interaction with hemopexin-like domain of MMPs

The interaction of the C-terminal domain of TIMP via the hemopexin-like C-terminal domains of the MMPs is important for the formation of specific complexes with gelatinases (14, 58). These interactions are very strong between TIMP-1 and TIMP-3 with MMP-9 and between TIMP-2, -3 and –4 with MMP-2 (32, 59-61). These interactions are important for several reasons. Firstly, they provide a means by which the docking of TIMP with an active MMP facilitates the interaction of the N-terminus of TIMP with the catalytic site of MMP. Secondly, interaction of TIMPs with latent forms of gelatinases regulate the activation of the enzymes. Thus, pro-MMP-9 which is complexed with TIMP-1 is less susceptible to activation by other MMPs such as MMP-3 and MMP-2 (62). The interaction between TIMP-2/pro-MMP-2 is quite different in that their interaction regulates the activation of pro-MMP-2 by MT1-, 2- and 3-MMPs. In this scenario, the inhibition of active MT-MMP on the cell surface by N-terminal domain interactions with TIMP-2 creates a "receptor" for pro-MMP-2 (59). This would suggest that the relative levels of TIMP-2 and MT-MMPs are critical, as excess TIMP-2 would block MT-MMP function, thereby preventing pro-MMP-2 activation.

Another level of specificity is provided by the highly acidic C-terminal "tail" of TIMP-2 which is a critical factor in the association of TIMP-2 with pro-MMP-2 (14). The corresponding tails of TIMP-3 and TIMP-4 are not as acidic and may not substitute for TIMP-2 to form a pro-MMP-2 receptor (60). These subtle mechanisms of MMP activation and interactions allow for highly specific levels of control in the pericellular microenvironment.

1.4.3 Regulation of TIMP Expression

The transcriptional/post-transcriptional regulation of the TIMP genes seem to be independent from that of MMP regulation. TIMP-1 is responsive to a variety of external stimuli with TIMP-1 mRNA induced by a variety of cytokines and growth factors such as TGF-β, (63), TGF-α, EGF, basic-FGF, retinoic acid, oncostatin M (64), leukemia inhibitory factor (65), IL-1 and IL-6 (66), phorbol esters, and serum (36, 67) whereas TIMP-2 seems to be constitutively expressed in many different tissues and cell lines (68, 69). Exceptions to this have been demonstrated in rat liver cells where TIMP-2 expression was induced in response to lipopolysaccharides and prostaglandin E2 (70) and in injured arteries (71). In addition, TIMP-1 transcription is enhanced by TAX, a protein encoded by HTLV-1 (72). TIMP-3 is induced by EGF, TGF-β and TPA. Dexamethasone treatment, however, selectively induced the expression of TIMP-3 and suppressed the EGF or TPA mediated induction of TIMP-1 (36). TIMP-3 is induced as a delayed early cellular response to hepatocyte growth factor in keratinocytes, kidney and mammary epithelial cells (73). The differential regulation of TIMP gene expression supports the notion that individual TIMP family members may have specific physiologic roles in a cell-specific manner.

Table 3. Properties of Tissue Inhibitors of Metalloproteinases.

	TIMP-1	TIMP-2	TIMP-3	TIMP-4
Size	28k Da	21k Da	24k Da	22k Da
Glycosylation	Yes	No	Yes	No
Localization	Diffusible	Diffusible	ECM	Diffusible
Gene	Xp11.23-11.4	17q2.3-2.5	22q12.1-13.2	3p25
Transcripts	0.9	3.5,1.0	4.5 (2.8, 2.4)	1.2
Expression	Inducible	Constitutive (largely)	Inducible & constitutive	?Constitutive (restricted)
Pro-MMP complex	MMP-9	MMP-2	MMP-2/-9	MMP-2
EPA	Yes	Yes	?	?
Mitogenicity	Yes	Yes	Yes	?
Growth Inhibition	Yes(B16F10)	Yes(endo)	Apoptosis	?

1.5 MMPs and TIMPs in Cancer

Invasive growth of solid tumors and their metastases are dependent on matrix turnover. At either the primary or secondary tumor sites, extracellular matrix degradation is required for tumor cell invasion and spread. Metastatic cells by inference would require sufficient degradative enzymatic capacity to break down the basement membranes. Alternatively, some of the required proteolytic activity may be derived from tumor-associated host tissues, including adjacent stromal tissue and tumor-infiltrating immune cells. There is extensive literature demonstrating the association of MMP family members and tumor progression (74, 75). Several generalizations can be made from these studies. 1) The number of different MMP family members that can be detected tends to increase with progression of the tumor. 2) The relative levels of any individual MMP family members tend to increase with increasing tumor stage and 3) MMPs can be made by either tumor cells themselves or, as a host response to the tumor. A simplified concept initially proposed by the early studies suggested that the major role of MMPs in metastasis was to facilitate the breakdown of physical barriers to metastasis. However, recent evidence suggests that the MMPs and TIMPs may have a more complex role in metastasis and that they make important contributions at other steps in the metastatic process (76-78). Studies in which levels of MMPs and TIMPs are pharmacologically or genetically modulated show that they are key regulators of growth of tumors at both primary and metastatic sites. Thus, MMPs and TIMPs are important in creating and maintaining an environment that supports the initiation and maintenance of growth of primary and

metastatic tumors. *In vivo* and *in vitro* studies indicate that the expression of one or more MMPs increases the metastatic potential of a wide spectrum of tumors including breast cancer, melanoma, and prostate cancer (18, 41, 79-81).

In non-Hodgkin's lymphomas, high levels of MMP-9 mRNAs show a strong inverse relationship with patient survival (82). In most epithelial tumors, MMP-2 has been located both on the surface of tumor cells and in the stroma, but appears to be expressed mainly by stromal cells and subsequently recruited to the tumor cell membrane by a specific MMP-2 receptor (80). Candidates for this receptor are: MT1-MMP and $\alpha V\beta 3$ integrin (83). MMP-9 appears to be expressed mainly by stromal macrophages with only matrilysin (MMP-7) and stromelysin-2 (MMP-10) being truly epithelial cell specific. The above is a simplistic view and it must be remembered that analysis of tissue localization studies can give only a snapshot at one point in time. There are difficulties in interpreting these studies as exact localization of secreted proteins is difficult to ascertain using light microscopy alone.

1.5.1 In Vivo Studies

Evidence implicating MMPs in metastasis and tumor invasion has been obtained from *in vivo* studies. Schultz et al., (84) showed that an intraperitoneal injection of recombinant TIMP-1 reduced lung colonization of intravenously injected B16-F10 melanoma cells. A reduction in TIMP-1 levels by antisense RNA in mouse fibroblasts resulted in formation of metastatic tumors in nude mice (85). Subsequent studies (86-89) using recombinant or transfected TIMP-1 or TIMP-2 in experimental

and spontaneous metastasis assays further suggested that MMPs could play a causal role in metastasis. Transfection of a TIMP-2 expressing plasmid into a transformed rat embryo cell line partially suppressed hematogenous metastasis, while TIMP-1 over-expression reduced the metastatic potential of B16-F10 melanoma cells (85, 90-92). Overexpression of tissue inhibitor of metalloproteinases-2 by retroviral-mediated gene transfer *in vivo* inhibited melanoma tumor growth and invasion (93).

1.5.2 Synthetic Metalloproteinase Inhibitors

Studies with synthetic MMP inhibitors further support a requirement for MMP activity in the establishment of metastatic foci. These low-molecular -weight compounds are unlikely to have complicating activities distinct from inhibition of metalloproteinase activity but the spectrum of MPs inhibited and their biologic functions are not yet fully known. The British Biotech inhibitor batimastat (BB-94) was shown to reduce metastasis of melanoma (94) mammary carcinoma (95) and colorectal tumor cells (96) in experimental metastasis assays and of human colon (97) and breast tumor cells (98) injected orthotopically in nude mice. Other broad-spectrum MMP inhibitors have shown similar results in lung colonization assays (99).

1.5.3 Differential Effect of MMP Family Members in Metastasis

Specific MMP family members have been implicated in playing a role in tumor cell invasion and metastasis and have been studied more extensively than others. Bernhard et al., (100) have demonstrated that MMP-9 expression is strongly associated

with the metastatic ability of rat embryo fibroblasts and that its overexpression results in increased metastatic potential following injection into nude mice (101), while ribozyme inhibition of this enzyme decreases lung colonization (102). Transfection of MMP-2 cDNA in a bladder cancer cell line increased the area of lung metastases (87), and MT1-MMP overexpression enhanced the survival of mouse lung carcinoma cells in the lungs of intravenously injected mice (103). *In vitro* studies measuring invasion through amnion basement membrane, smooth muscle cell-generated basement membrane or reconstituted basement membrane (Matrigel; Collaborative Research, Inc., Waltham, MA) also provide support for the effect of MMP activity in metastasis. An inhibition of *in vitro* invasion has been observed following the addition of recombinant or transfected TIMP-1 or TIMP-2 (84, 90, 104-106). Targeted disruption of the TIMP-1 gene resulted in an increase in *in vitro* invasion (107).

1.5.4 Multifunctional Role of MMPs and TIMPs

Given the significance of MMPs and TIMPs as regulators of not only extracellular matrix turnover, but also cell attachment and motility it is reasonable to propose that they may exert some influence on cell growth and cell signaling. MMPs and TIMPs have functions other than cleaving/clearing of unwanted proteins. There is biochemical and *in vivo* evidence that proteases and metalloprotease inhibitors modulate cellular behavior by altering cell surface-extracellular matrix interactions and regulating the processing of growth factors. In addition, some of these proteases can play a non-proteolytic role through the interaction of non-catalytic domains (EGF-like, disintegrin, hemopexin etc.)

with other proteins, thereby regulating cell migration and adhesion, egg-sperm interactions, angiogenesis, and the cell cycle (108, 109). Evidence supports the role of these proteins in proliferation, migration, invasion, anoikis and apoptosis, likely through complex mechanims that are still under investigation. MMP-2 and urokinase plasminogen activator (uPA) facilitate the sprouting of blood vessels important for maintaining tumor viability. UPA receptor has also been linked with chemotaxis, adhesion and signaling. The uPA-uPAR interaction increases the binding of vitronectin to the cell (110). MMPs cn also modulate the rates of tumorigenesis in mice models. Deletion of MMP-3 in certain genetic background reduced the rates of tumour formation (111). Overexpression of MMP-3 in phenotypically normal mouse mammary epithelial cells induced an altered stromal environment and promoted the phenotypic conversion and malignant transformation of mammary epithelial cells (112) while coexpression of TIMP-1 blocked this process. Overexpression of MMP-3 also modulates cell-cell interaction by cleavage of E-cadherin (79). As loss of E-cadherin function activates the transcription of MMP-7, this may further activate the tissue remodeling process. MMP-9 is a key regulator of developing chondrocyte apoptosis and angiogenesis as mice lacking the gene show failure of vascularization and apoptosis in the skeletal growth plate (113).

Proteases can positively regulate tumor growth in several different ways. This may be accomplished in a direct manner, alternatively, MMPs and TIMPs may positively or negatively regulate the actions of growth factors such as basic fibroblast growth factor (FGF) and transforming growth factor (TGF)-β. They may modulate effectiveness of growth factor receptors by regulating shedding of membrane associated receptors.

Alternatively, they may modulate the local availability of growth factors by controling the degradation or sequestering of binding proteins. The proteolytical cleavage of the membrane-anchored surface growth hormone receptor (GHR) (114) as well as insulin like growth factor binding protein (IGFBP) was blocked by synthetic hydroxamic acid-based inhibitors (114). The mannose 6-phosphate/insulin like growth factor (IGF)-2 receptor which binds to IGF-2 and thereby preventing the cell-proliferative signaling is modulated by the MPs and TIMPs. (114). Overproduction of TIMP-1 suppressed T-antigen induced hepatocellular carcinomas (115), possibly by preventing MMP-mediated degradation of IGF-binding proteins and, thus, reducing the local availability of IGF-2. As IGFs I and II have mitogenic and metabolic effects and participate in the regulation of growth and differentiation of a number of cell types their modulation would play a significant role in tumor progression and metastasis.

1.6 MMPs and TIMPs as Growth Modulators

1.6.1 MMPs as Growth Modulators

Although most data indicate that MMPs and their inhibitors are strongly linked with the process of metastasis and its inhibition, respectively, there are examples of a lack of a consistent effect in some of these assays. No change in *in vitro* invasion was detected in loss-of-function studies in which expression of stromelysin-3 (116) and matrilysin (117) were ablated by use of antisense technology. Other studies have shown that stromelysin-3 gene inactivation by homologous recombination altered the malignant processes. The suppression of stromelysin-3 resulted in a decreased 7,12-

dimethylbenzanthracene-induced tumorigenesis in stromelysin null mice (118). **These results raise the possibility that at least some MMPs may affect steps in metastasis other than extravasation.** In addition, several investigators have studied the metastatic process by intravital videomicroscopy of tumor cells in the microvasculature (119, 120). Results from a series of experiments (121, 122) suggest that the primary functional contribution of MMPs and their inhibitors in metastasis may be at steps after the extravasation stage and may involve modulating the growth of tumor cells (76). Evidence for a role of MMPs in tumor cell establishment and growth comes from studies in which levels of a specific MMP were manipulated. Stromelysin-3 overexpressed in human breast cancer cells or removed by antisense RNA from murine fibroblasts were assayed for subcutaneous tumor development in nude mice. Manipulation of stromelysin-3 levels altered the tumorigenicity of the cells but did not alter the growth of established tumors, their invasion, or their metastatic capability (116). Collagenase expression in the skin of transgenic mice resulted in earlier onset and increased numbers of papillomas arising after chemical initiation and promotion (123). Expression of stromelysin-1 in mammary glands of transgenic mice resulted in the development of aggressive malignant mammary tumors (124). In contrast, chemically initiated mammary tumors were actually reduced in other stromelysin-1 transgenic mice (117). However, this effect was related to an increase in both proliferation and apoptosis in target mammary epithelial cells. Taken together, these results suggest that stromelysin expression can promote tumor maintenance and suggest that metalloproteinases may favor cancer cell survival. Genetic manipulation of

matrilysin levels in human colon tumor cell lines resulted in an effect on the tumorigenicity of the cells following orthotopic injection into the cecum of nude mice, with little detectable effect on invasive or metastatic ability (117). Crosses between matrilysin-deficient mice and mice carrying a germline mutation of the APC gene resulted in a significant reduction in both number and size of intestinal adenomas compared to wild-type control mice (111). In gain-of-function experiments, matrilysin expression in the mammary epithelium of transgenic mice significantly accelerated development of MMTV-neu-induced tumors (125). These results support a role for MMPs in the development and growth of early stage tumors.

1.6.2 TIMPs as Growth Modulators

Recently, the effect of TIMP-1 on the initiation and growth of liver tumors was documented in transgenic mice expressing either sense or antisense TIMP-1 constructs (91). TIMP-1 overexpression inhibited SV40 T-antigen-induced tumor initiation, growth and angiogenesis, while TIMP-1 reduction resulted in more rapid tumor initiation and progression. TIMP-1 transfection in B16-F10 melanoma cells resulted in a decline in primary tumor growth following a subcutaneous injection as well as a reduction in lung colonization following an intravenous injection(88). These results are in contrast to earlier studies reported by Schultz et al.,(84) in which an intraperitoneal injection of recombinant TIMP-1 reduced the number of lung colonies but did not alter the size of lung nodules nor the growth of subcutaneously injected tumors. These apparently contradictory results might be explained by a difference in the experimental

protocol or by effects of tumor versus host expression of TIMP-1, since systemic TIMP-1 was elevated following injection of recombinant protein, while in the transfection studies, tumor cell TIMP-1 levels were specifically altered. This possibility is supported by recent studies of Soloway et al., (126) where co-isogenic cells and genetically manipulated mice varying in expression of TIMP-1 demonstrated that lung colonization is influenced by the TIMP-1 genotype of the tumor but not that of the host. Although systemic TIMP-1 may influence extravasation, the initiation and growth of primary tumor cells can be markedly affected by alterations in tumor TIMP-1 levels.

TIMP-2 also has been demonstrated to reduce tumor cell growth as well as metastasis. Transfection or retroviral introduction of TIMP-2 into transformed rat embryo fibroblasts reduced primary tumor growth as well as hematogenous metastasis (93, 106). TIMP-2 overexpression reduced the growth of metastatic human melanoma cells injected subcutaneously in immunocompromised mice, although it did not prevent metastasis in this study (93). The growth-inhibitory effect of TIMP-2 was shown to require a three-dimensional collagen matrix and was not observed in gelatin-coated dishes; in the presence of matrix, TIMP-2 expressing melanoma cells demonstrated a reduction in growth rate and assumed a differentiated morphology. Thus, it appears that both TIMP-1 and TIMP-2 can have growth inhibitory effects, and this effect can be dependent on the cellular environment and on the tumor cells themselves producing the inhibitor.

a) Erythroid potentiating activity (EPA) of TIMPs

The direct effects of TIMPs on cell growth modulation originate with the observation that TIMP-1 has erythroid potentiating activity (EPA). TIMP-1 stimulates the growth and differentiation of erythroid precursors, erythroleukemia cell lines, K-562 (127, 128) and ELM-I-1-3 (129) *in vitro*, as well as stimulating murine erythropoiesis *in vivo* (130). TIMP-2 also promotes the growth of erythroid precursors (69) as well as acting as an autocrine growth factor for SV40-transformed human fibroblasts (131). A broad spectrum of mitogenic activity has been demonstrated for TIMP-2 in a variety of cell lines including human gingival fibroblasts, Raji Burkitt's lymphoma cells, and a promyelocytic leukemia cell line, HL-60 (132-134). The mitogenic property of TIMP-2 was retained after reductive alkylation, which completely destroyed its function as a MMP inhibitor (133, 134). Related studies by Chesler et al., (135) used TIMP-1 proteins with point mutations in the N-terminal domain which abrogated MMP inhibitory activities. In an *in vitro* erythroid burst-forming unit assay, these authors demonstrated that the growth-stimulatory effects of these N-terminal TIMP-1 point mutants were similar to that of wild type TIMP-1. A C-terminal truncated TIMP-1 lacking complete antiproteolytic activity also stimulated growth in the erythroid burst-forming unit assay. This suggests that the effect of TIMP on cell growth involve functions that are distinct from MMP inhibition (51).

In vivo correlative evidence also exists to support the growth stimulatory potential of TIMP-1. Studies by Kossakowska et al., (82, 136, 137) and Lim/Mansoor et al., (manuscript in preparation) demonstrated a positive correlation between TIMP-1 RNA

expression and clinical aggressiveness as well as histologic grade in non-Hodgkin's lymphomas. HTLV-1 and HTLV-2 infected cell lines have enhanced expression of TIMP-1 mediated by viral TAX protein. This observation led the authors to postulate that TIMP-1 may have lymphoid growth factor activity (72).

More recently, studies by Guedez et al., (138) demonstrated that overexpression of TIMP-1 in a Burkitt's lymphoma cell line JB38, results in resistance to apoptotic stimuli. Other studies indicate that the effect of TIMPs on cell growth is not a uniform one. TIMP-2 (139) and TIMP-3 (140) exhibited growth inhibiting properties, under certain conditions. Potent inhibition of angiogenesis by TIMPs has been demonstrated in cartilagenous (141) and endothelial tissue culture systems (142, 143). *In vitro* studies of human microvascular endothelial cells demonstrated that TIMP-2 but not TIMP-1 is capable of inhibiting the growth of basic-FGF-stimulated cells, which was not mimicked by the addition of metalloproteinase antibodies or a synthetic metalloproteinase inhibitor (49, 142). In addition, growth of Kaposi's sarcoma (KS)-like lesions produced by KS cell-free supernatants and the angiogenic HIV-1 TAT protein were inhibited by TIMP-2 (144, 145). *In vivo* studies by Soloway et al., (126) demonstrated that, although the lack of TIMP-1 expression enhanced lung colonization in two pairs of isogenic cells with wild-type and mutant TIMP-1, in a third pair, lung colonization was reproducibly decreased in the absence of functional TIMP-1 suggesting that in certain cellular contexts TIMP-1 can faciliate tumor growth. Overexpression of TIMP-1 in the gastrointestinal tract also enhanced development of benign gastrointestinal tumors in a line of transgenic mice carrying a germline mutation in the adenomatous polyposis coli (APC) gene (146).

b) Cell-type specificity

As the expression of TIMPs appear to be cell-type specific and activation-state dependent, there is accumulating evidence that the growth modulatory activity of TIMPs may also be cell type-specific, concentration -dependent and regulated by the presence or absence of other growth factors. Nemeth and coworkers (147) have demonstrated that recombinant (r) TIMP-2 is growth stimulatory for a number of different cell lines including human foreskin fibroblasts, lung adenocarcinoma cells, human melanoma cells and the Burkitt lymphoma cell line RAMOS in a concentration-dependent manner. Normal human colon and lung fibroblasts however, showed no response to rTIMP-2. In addition, for certain cell lines TIMP-2 alone was not sufficient for a growth-stimulatory response, requiring the presence of factors such as insulin (147). In the nonadherent promyeloleukemia cell line HL-60, rTIMP-2 inhibited [^{3}H]-thymidine incorporation. In B16-F10 melanoma cells, a highly invasive and metastatic cell line, TIMP-2 overexpression correlated with a reduction in invasion. TIMP-2 overexpressing transfectants displayed limited tumor growth and neoangiogenesis when cells were injected subcutaneously in mice. Interestingly, although necrosis was increased, TIMP-2 overexpressing clones were found to be more resistant to apoptosis than parental and control melanoma cells (139). TIMPs and their role in tumor cell survival have also been demonstrated in corneal epithelial cells. Both recombinant TIMP-1 and TIMP-2 were shown to enhance the spreading of corneal epithelium *in vivo* and proliferation of cultured corneal epithelial cells *in vitro* (148).

A concentration dependence of these effects was observed in that concentrations higher than 200 ng/mL for TIMP-1 and 50 ng/mL for TIMP-2 did not further enhance either the DNA synthesis as measured by BrdU incorporation or the spreading of the epithelium. Whether at higher concentrations a cytotoxic or apoptotic effect was produced was not discussed in this study. Further *in vivo* evidence for the growth inhibitory effect of TIMP-2 is provided by studies reported by Imren et al., (93). Colon cancer cell lines overexpressing TIMP-2 exhibited reduced tumor growth in nude mice. The possibility that this effect may be an indirect result of an anti-angiogenic response cannot be ruled out.

c) Indirect mechanisms of growth modulation

An indirect mechanism of growth modulation by MMPs and TIMPs is illuminated by the ability of certain MPs to function as enzymes that regulate shedding of soluble cytokine receptors such as tumor necrosis factor (TNF) receptors (149, 150) and modulate processing of transmembrane cytokines and growth factors such as (macrophage colony-stimulating factor (M-CSF), transforming growth factor (TGF)-β, stem cell factor (SCF) (151), TNF-α (152, 153), IL-6R (150) and Fas ligand (154, 155). Metalloproteinases are also involved in the shedding of cell adhesion molecules such as L-selectin (156-158) and in the degradation of growth factors binding proteins such as the insulin-like growth factor binding protein (159, 160). The physiologic relevance of this will be discussed in Section 1.10.

d) TIMP-3 in growth modulation

The growth modulating effects of matrix-specific TIMP-3 has also been reported. TIMP-3 is unique among the TIMPs in that it appears to complex specifically with as yet unidentified components in the ECM (36, 44). Yang and Hawkes (161) demonstrated that chicken TIMP-3 can stimulate the proliferation of growth-retarded, non-transformed chicken fibroblasts in serum free conditions, suggesting that mammalian TIMP-3 may have a role in growth modulation. Moreover, an anti-angiogenic activity of TIMP-3 has been demonstrated both *in vivo* and *in vitro* (162). In contrast, others (163) have shown no effect of TIMP-3 overexpression in the growth, tumorigenicity or invasion of mouse epidermal cells. Bian et al., (164) have demonstrated that TIMP-3 overexpressing colon carcinoma line, DLD-1 displayed a serum-dependent growth inhibition in a monolayer culture and a decreased growth potential in nude mice, an effect not mimicked by BB-94. They subsequently demonstrated that TIMP-3 induces cell death by stabilizing the TNF-α receptors present on the surface of human colon carcinoma cells (165). In a similar study, adenovirus-mediated gene delivery of TIMP-3 resulted in inhibition of invasion of melanoma cell lines as well as inducing apoptosis (166). Furthermore, TIMP-3 expression is elevated in mid-G1 in cycling fibroblasts, indicating the possible linkage of TIMP-3 production and cell cycle progression (167).

e) Effect of synthetic MPI on tumor cell growth

The role of MMPs in tumor establishment and growth is further supported by studies using synthetic matrix metalloproteinase inhibitors such as BB-94 (95, 96, 168).

Intraperitoneal administration of BB-94 was shown to block the growth of human ovarian carcinoma xenografts (169) as well as murine melanoma metastasis (94). BB-94 also inhibited human colon tumor growth and spread in an orthotopic model in nude mice and this was associated with increased numbers of necrotic cells (97). In a model evaluating liver invasion of a human colorectal tumor line, invasive growth was inhibited by batimastat treatment (96). Tumors that did form had advanced necrosis, which was interpreted as indicative of a reduction in tumor vascularisation. It has been suggested that in some cases, the effects of MMP inhibitors of tumor growth *in vivo* may be related to their effects on tumor angiogenesis. MMP inhibitors block angiogenesis as assayed in chick and rodent models of neovascularization (143, 170, 171). The mechanism of inhibition of tumor growth may reflect both indirect effects involving suppression of angiogenesis as well as more direct effects on the growth of tumor cells themselves.

f) TIMPs and apoptosis

Given that growth inhibition may be due to decreased cell proliferation or increased cell death via apoptosis and other cell death mechanisms, it may be hypothesized that TIMPs may decrease growth by increasing apoptosis. To date, few studies have reported the induction of apoptosis by a TIMP. TIMP-3 induced cell death by stabilizing the TNF-α receptors present on the surface of DLD-1 colon carcinoma cells (165). Overexpression of TIMP-3 induced apoptosis in melanoma cells while inhibiting

invasion (166, 172). The direct effects of TIMPs on apoptosis and mechanisms involved therein, have not been well studied. There is precedence for proteinases and their inhibitors regulating cellular apoptosis. PAI-2, a serpin (serine proteinase inhibitor) expressed in primitive hematopoietic cells and activated T lymphocytes can inhibit TNF-α-mediated apoptosis (173, 174). This has potential significance as synthetic MPI have been reported to prevent lethal graft vs host disease in mouse models of allogeneic bone marrow transplantation (175). The biologic effect of BB-94 on lymphoid cell growth and function has not been directly studied although in animal models of arthritis, BB-94 was shown to have an immunosuppressive effect (176).

g) Intracellular signals involved in TIMP growth modulation

The growth-modulatory function of TIMPs suggests the presence of a receptor-mediated mechanism. The cell-type specific growth modulatory response may be dependent on the manifestation of cells having an appropriate receptor for the domain of TIMP containing the growth-promoting activity. This has been supported by studies demonstrating specific, saturable binding of TIMP ligands to high affinity cell surface receptors of cells sensitive to TIMP-mediated growth effects (134, 177). There is very little information on signal transduction pathways involved in mediating the growth modulatory effect of TIMPs (70, 178). Recently, it has been demonstrated that TIMP-2 stimulated fibroblast growth via a cAMP-dependent mechanism (179). Others have shown that growth stimulation of human osteosarcoma cell line MG-63 was significantly stimulated after the addition of either TIMP-1 or TIMP-2 alone. Tyrosine kinase

inhibitors such as genistein, erbstatin, and herbimycin A almost completely inhibited the [^{3}H]-Thymidine incorporation stimulated by either of the TIMPs. These inhibition studies suggest a crucial role for tyrosine kinase in the signal transduction of TIMPs. Phosphotyrosine-containing proteins were significantly elevated by the treatment with both TIMPs. TIMP stimulated an increase in mitogen-activated protein (MAP) kinase activity, suggesting that MAP kinase plays a role in TIMP- dependent growth signaling (180).

Others have demonstrated the signalling potential of TIMP-1 using a more direct approach. Secreted TIMP-1-EGFP, a chimeric protein in which the enhanced green fluorescent protein of the jelly fish *Aequorea victoria* was fused to the carboxyl-terminus of TIMP-1 could be visualized binding to the surface of MCF-7 breast carcinoma cells and localized to the nucleus of MCF-7 cells. These findings suggest that TIMP-1 may preferentially bind to and be taken up by malignant breast epithelial cells and that TIMP-1 may play a yet unidentified role in nuclear functions (181). In Burkitt lymphoma cell line JD38, TIMP-1 suppression of apoptosis was associated with induced expression of Bcl-X_L but not BCL-2 as well as decreased NF-κB activity (138).

1.7 The Expression and Role of MMPs and TIMPs in Cells of the Immune System

1.7.1 Inflammation

Metalloproteinases and their inhibitors are believed to play a crucial role in the extracellular matrix degradation that occurs as a consequence of the inflammatory response (182). The recruitment of leukocytes into a site of tissue damage is dependent upon a dynamic and complex series of events, involving endothelial cell activation by inflammatory cytokines, leukocyte activation and the expression of leukocyte cell-derived adhesion molecules. This facilitates leukocyte-endothelial cell adhesion, diapedesis, and leukocyte migration beyond the vascular barrier (183). Transendothelial migration of leukocytes beyond the vascular compartment is dependent on expression of integrin molecules, the movement along leukocyte-specific chemotactic gradients and the secretion of matrix degrading enzymes (184). Migration of monocytes and lymphocytes into extra-lymphoid tissues requires penetration of subendothelial basal lamina. Upon contact with matrix proteins, both macrophages and T cells secrete metalloproteinases. Interactions between activated T cells and monocytes induce secretion of metalloproteinases at the site of inflammation (185).

Due to their potential hazardous effects, metalloproteinases are regulated at different steps during the inflammatory reaction. This control is mainly mediated by cytokines (24, 28, 186) although other inflammatory factors such as prostaglandins can also induce metalloproteinase expression. For example, in monocytes and natural killer

cells, prostaglandin E2 induced metalloproteinases through a cAMP-dependent mechanism (187, 188).

Activation of peripheral blood T cells by stimulation of the T cell receptor (TCR) TCR/CD3 complex leads to several biologic responses which are of great importance in both cell-mediated and humoral immunity. Although a comprehensive and detailed analysis of the expression profiles of MMPs and TIMPs by reactive lymphocytes and other hematopoietic cells has not been reported, studies have shown that activation of T cells by PHA and IL-2 induced the expression of latent and activated forms of MMP-9 (189) as well as stromelysin-2 (190). In addition, cell-cell interactions as causal events in the upregulation of these enzymes have been examined (30). Upon association of PHA-activated human T lymphocytes, the human monocytic line THP-1 produced interstitial collagenase and MMP-9. Whereas isolated T cells expressed MMP-9 alone, after the stimulation of β-1 integrin or vascular cell adhesion molecule (VCAM) by inflammatory mediators (191-193) T cells secreted both MMP-2 and 9. The expression of these gelatinases is differentially regulated in T cells. In contrast to what has been reported in fibroblasts, T cells secreted MMP-9 in a constitutive manner whereas MMP-2 requires induction (194). The transendothelial migration of human blood T cells and cultured T lymphoblastoma cells of the Tsup-1 line stimulated by prostaglandins and leukotrienes was mediated by MMPs -2, -3 and -9 (195). In addition, expression of pro-MMP-9 and TIMP-1 are differentially regulated by inflammatory cytokines and chemokines in distinct T cell subsets (196). Analysis of highly purified leukocyte subpopulations demonstrated that in the absence of

exogenous stimuli, purified CD4 + T lymphocytes produced similar quantities of pro-MMP-9 and elevated levels of TIMP-1 compared with peripheral blood mononuclear cells, while purified CD8 + and CD3 + populations exhibited less MMP-9 and TIMP-1 activity. In comparison, CD56 + (natural killer) cells secreted barely detectable levels of pro-MMP-9 and TIMP-1. The secretion of proMMP-9 by peripheral blood mononuclear cells (PBMC) and purified CD3 +, CD4 + and CD8 + lymphocytes was selectively modulated by chemokines and proinflammatory cytokines. Pro-MMP-9 secretion by CD3 + and CD4 +, but not by CD8 + T cells was augmented in response to TNF-α and IL-1, and down-regulated by interferon (IFN)-γ, while macrophage inflammatory protein (MIP)-1α, and RANTES (regulated upon activation and normally T cell expressed and secreted) chemokines up-regulated the secretion of pro-MMP-9 by all of the lymphocyte subsets. TIMP-2 and TIMP-3 expression was not reported in these studies.

Another mode of regulation of MMP-9 expression has been described in an early "double negative" human T cell line HSB.2 by the EP_3 subtype of prostaglandin E_2 (PGE_2) receptor via a $[Ca^{2+}]$ -dependent increase in transcription. Given that the expression of MMP-9 is regulated by multiple functionally distinct subtypes of PGE_2 receptors (which are the principal determinants of specificity and diversity of the immune effects of PGE_2), this highlights the potential role of MMP-9 in T cell function. In human blood T cells of mixed CD4 + and CD8 + composition, PGE2 stimulated the surface expression and secretion of MMPs -2 and -3, as well as MMP-9 (195).

T lymphocytes interact with macrophages and regulate the expression of MMPs and TIMPs. Macrophages are highly mobile immune modulators that play an important role in antigen processing and presentation to effector cells. Macrophages have been shown to produce several types of metalloproteinases that are induced by specific cell surface determinants present on T cells. Cytokines from both Th1 and Th2 helper cells regulated the expression of MMPs in macrophages (197). IL-1-β and TNF-α are major pro-inflammatory cytokines produced by activated monocytes and Th cells. They control the expression of interstitial collagenase, stromelysin-1 and gelatinase B in a post-transcriptional manner (24). Furthermore, pro-inflammatory cytokines enhanced the expression of metalloproteinases without affecting production of TIMPs (193). Unlike IL-1 and/or TNF-α, Th2 type cytokines such as IL-4 and IFN-gamma inhibited MMPs production even in relatively immature peripheral monocytes, with no effect on TIMP expression (198). IL-10 is secreted by Th2 lymphocytes and is inhibitory to several macrophage functions (199). IL-10 together with IL-6 increased TIMP-1 production. In addition to inducing TIMP-1, IL-10 also inhibited the secretion of MMP-9, an effect not shared by IL-6 (30, 200).

From the above, it is clear that changes in cytokine profiles correlate with the ratio of MMPs to TIMPs observed during the inflammatory response. During the first few hours when pro-inflammatory cytokines are produced there is enhanced expression of MMPs while TIMP expression is low. In the second phase of the inflammatory reaction, IL-6 production is correlated with an increase in TIMP-1 expression. Following secretion of IFN-γ and IL-4, MMP production decreases while TIMP-1

levels remain unchanged. In the final phase, in response to anti-inflammatory cytokine IL-10, maximum expression of TIMP-1 is reached, whereas MMPs levels decrease to basal levels.

1.7.2. Lymphoid Neoplasia

Due to the role of lymphocytes in immune surveillance and their innate migratory capabilities our conceptual framework of the "invasive/metastatic" phenotype used for nonlymphoid malignancies has to be modified in our consideration of the functions of MMPs and TIMPs in the progression of lymphoid neoplasms. Both T and B lymphocytes interact with cellular and structural components of the extracellular matrix. Aggressive lymphoid neoplasms can be observed "invading through the lymph node capsule and into the surrounding soft tissues". Several studies indicate that MMP-9 is a key player in the spread and dissemination of lymphoma cells to peripheral tissues (201). Detailed analysis of MMP and TIMP expression in human malignant lymphoma tissues showed that elevated levels of expression of MMP-9 and TIMP-1 mRNAs are associated with high histologic grade and increased clinical aggressiveness (82, 136, 137, 202). Indeed, MMP-9 expressed in U937 monoblastoid cells correlated with cellular invasion (184). Also, a potential role for MMP-9 in extramedullary tumor formation in leukemias has been proposed (203) by the demonstration of its constitutive expression in a granulocytic sarcoma cell line with high invasive capacity as measured by an in vitro invasion assay. In addition, MMP-9 can function as an elastase (204, 205) and may be involved in vascular invasion occasionally seen in lymphoma specimens (206). As vascular cells

form a dynamic tissue that is able to respond to its environment and/or activating cells through the production of cytokines and cell surface expression of cell adhesion molecules, it is also possible that contact between lymphoma cells and endothelial cells may control the expression of MMPs and TIMPs. Aoudjit et al., (207) have demonstrated that adhesion of mouse T lymphoma cells (164T2) to endothelial cells induced a transient and reciprocal *de novo* expression of MMP-9 mRNA and enzymatic activity by both cell types. Up-regulation of MMP-9 in T lymphoma cells was concomitant to that of TIMP-1, and required direct contact with endothelial cells. Induction of MMP-9 but not of TIMP-1 was blocked by anti-LFA-1 and anti-intercellular adhesion molecule-1 antibodies, indicating that induction of MMP-9 and TIMP-1 in T lymphoma cells required direct, yet distinct, intercellular contact. In contrast, the induction of MMP-9 in endothelial cells by T lymphoma cells did not necessitate direct contact and could be achieved by exposure to IL-1 and TNF-α, or to medium conditioned by cultured T lymphoma cells. Together, these results demonstrate that adhesion of lymphoma cells to endothelial cells participates in the production of MMP-9 in both cell types through bi-directional signaling pathways, and identify intercellular adhesion molecule-1/LFA-1 as a key interaction in the up-regulation of MMP-9 in T lymphoma cells. In some studies, expression of MMP-9 and TIMP-1 are discoordinately regulated, supporting the idea that their induction could be mediated by temporally separated transcriptional activation involving distinct stimuli (137).

Multiple myeloma is a B-cell malignancy characterized by proliferation of malignant plasma cells within the bone marrow and excessive bone resorption. Several

reports have shown that the presence of circulating malignant plasma cells in the peripheral blood of patients could be responsible for tumor progression outside the bone marrow (208). Analysis of primary multiple myeloma cells showed that they constitutively expressed MMP-9 and this production was not regulated by the cytokines and hormones relevant to the biology of this tumor such as IL-6, IL-10, Oncostatin M, TGF-β, TNF-α and IL-1β . (209). Whereas all primary cells expressed functional MMP-9 by zymographic analysis, only 1 of 6 established multiple myeloma cell lines secreted a detectable quantity of the enzyme. Bone marrow stromal cells from patients with multiple myeloma did not express MMP-9 but constitutively expressed MMP-1 and MMP-2. MMP-1 expression was induced by IL-1β, TNF-α, IL-6, and OSM whereas TGF-β had no effect. Dexamethasone, a potent antitumoral agent in multiple myeloma, induced a strong inhibition of MMP-1 production. These studies suggest that the interactions of malignant plasma cells with the bone marrow stroma are important in regulating the expression of metalloproteinases and thereby tumor cell motility and possibly bone resorption. Furthermore, progression of plasma cell tumors which is accompanied by bone marrow neovascularization is paralleled by an increased angiogenic and invasive potential of bone marrow plasma cells which is dependent in part on MMP-2 production (210).

A comprehensive analysis of MMP and TIMP expression in reactive and lymphoid cells of different lineages and differentiation states has not been performed. Given that MMPs and TIMPs may have physiologic roles that are cell-type specific and that they may function in capacities other than matrix degradation, it is important to determine the cell type specific expression and function of these

proteins in lymphoid cell growth modulation. Furthermore, given the role of TIMPs as a growth regulator for certain lymphoid cells (134), it is a plausible hypothesis that MMPs and TIMPs play an important role in immune modulation and lymphoma progression.

1.8 Regulation of T Cell Apoptosis

Programmed cell death (PCD) otherwise known as apoptosis is a cardinal element of homeostatic regulation of the normal immune system. It is a widespread phenomenon that concerns any nucleated cell type and not only lymphocytes (211). It may be defined either morphologically or biochemically. In most cases, apoptosis is accompanied by characteristic ultrastructural alterations (cell shrinkage, cytoplasmic compaction, membrane blebbing, nuclear chromatin condensation) (212) and/or the internucleosomal "ladder-type" fragmentation of nuclear DNA by endogenous endonucleases (213). Whereas apoptosis is thought to require gene expression and protein synthesis, in general, necrosis exemplifies passive cell death caused by exogenous stimuli.

Throughout their lifespan T lymphocytes and their precursors confront the choice of continuing their existence or undergoing apoptosis. In their response to cellular antigens, T cells respond in a cycle of birth and proliferation, followed by death. Memory cells comprise lymphocytes that escape this circuit and persist (214). A plethora of different stimuli cause T cell apoptosis: antigen receptor-mediated

stimulation, cell contact-dependent signals, triggering of cytokine or steroid receptors, as well as many physical and chemical stimuli. It is important to note that no physiological stimuli always provoke the death of T cells. A putative death-inducing stimulus will only trigger apoptosis in a determined context: the differentiation and activation states of the T cell, the presence of other (co)-stimulatory signals, the metabolic environment, the presence of antigen etc. Thus, T cells continuously integrate stimuli arriving via multiple receptors that receive information from antigen-presenting cells, bystander cells, cell matrix proteins, cytokines, hormones and the metabolic microenvironment (215). T cells must then exercise exquisite control of apoptosis in order to minimize the risk of expanding self-reactive clones and of developing leukemias or lymphomas.

1.9 The Role of Fas/Fas Ligand and TNF Receptor/TNFα in T Cell Apoptosis

Stimulation of the T cell receptor (TCR)/CD3 complex with anti-CD3, TCR antibodies or polyclonal activators such as phytohemaglutinin (PHA) leads to several biologic responses which are of central importance in both cell-mediated and humoral immunity. In response to antigen recognition, T cell proliferation is mediated by an autocrine growth pathway, in which the responding T cells secrete their own growth-promoting cytokines. The principal autocrine growth factor for most T cells is IL-2. In addition, T cell activation results in differentiation of native T lymphocytes to effector cells which perform various functions such as secretion of cytokines, and development of antigen-specific memory T cells. The apoptotic pathway is termed "activation-

induced cell death" and plays a role in negative selection in T cell development. Aberrant activation-induced cell death plays a key role in the development and modulation of various pathophysiological processes such as autoimmune disease, immunodeficiency, AIDS and graft versus host disease.

The first signaling event following stimulation of the TCR complex is action of the protein tyrosine kinases Lck, Fyn and ZAP-70, followed by activation of protein kinase C (PKC) and an increase in the cytosolic calcium concentration (216). Further downstream, Raf, Ras and the MAP kinase pathway are activated following PKC activation. Calcium signals, on the other hand, turn on the activity of the phosphatase calcineurin (see Figure 4).

Upon stimulation, lymphocytes upregulate the expression of several members of the TNF and the TNF receptor families. Several of these are involved in apoptosis. The Fas transmembrane protein (also called Apo-1 or CD95) and its ligand, (FasL) in particular are thought to be crucial for TCR-mediated apoptosis in peripheral blood T cells (217, 218) (Figure 3). Fas and FasL play crucial roles in the maintenance of lymphocyte homeostasis (219). FasL, a 40-kDa type II transmembrane protein that is homologous to TNF-α induces apoptosis by binding to its membrane receptor Fas (220). A non-apoptosis inducing version of anti-Fas antibody can inhibit TCR-mediated apoptosis in T cell hybridomas (221). In addition, T-cell hybridomas with defective FasL fail to undergo activation-induced apoptosis (222). The Fas/FasL system is complex in that both the diminution and enhancement of this system may have deleterious effects. Loss of function mutations of Fas and FasL in both humans and

mice result in lymphoproliferative and autoimmune diseases (223, 224). In addition, Fas mutations may be a predisposing factor for development of lymphoid malignancies (225). FasL expressed on activated T cells is partly responsible for tissue damage in fulminant hepatitis (226) and graft vs host disease (227, 228). FasL is constitutively expressed in the testis and eyes, and it has been implicated in the property of immune privilege in these organs (229, 230). It has been reported that the thyrocytes of patients with Hashimoto's thyroiditis express both Fas and FasL, and commit suicide similar to the activation-induced cell death of T cells (231). FasL also functions as a cytotoxic effector molecule of cytotoxic T lymphocyte (CTL) and natural killer (NK) cells and may be responsible for the apoptosis and tissue injury associated with tumors of NK or T cell origin. Soluble (s)FasL is detectable in the serum of patients with certain types of large granular lymphocytic (LGL) leukemias and natural killer (NK) cell lymphomas where as sera from healthy persons do not (232, 233). It has been suggested that sFasL may mediate systemic tissue damage seen in patients with these malignancies. In addition, it has been suggested that the Fas-FasL system plays a role in the loss of CD4+ T cells in AIDS patients (234). The function of Fas expressed on various cells has been investigated using agonistic anti-Fas/Apo-1 mAbs, and the results have indicated that the expression of Fas does not necessarily indicate susceptibility to these agonists (235, 236). Using a fragment of human FasL promoter capable of driving the expression of GFP to allow identification and analysis of FasL activity in Jurkat cells, Su et al., (237) have suggested that distinct populations of T cells with different

susceptibility to apoptosis exist and that this occurs according to different FasL production.

1.10 (a disintegrin and metalloproteinase) ADAMs

The development and study of synthetic metalloproteinases inhibitors led to the recognition that they also targeted non-MMPs. The cleavage of TNF-α, a potent pro-inflammatory cytokine produced mainly by activated monocytes and macrophages was inhibited by the MMP inhibitor GI-129471, resulting in the block of TNF-α secretion (152, 153). TNF-α is synthesized as a precursor protein and processed to a mature 17-kDa soluble form by cleavage of an Ala-Val bond. The soluble form is implicated in cachexia associated with fulminant hepatitis.

Recently, the novel disintegrin metalloprotease (TNF alpha-converting enzyme, TACE) thought to mediate the cleavage of TNF-α was identified (238) (239). It is a membrane-anchored proteinase that is a member of the adamalysin metalloproteinases that possess both a disintegrin and metalloproteinase domains (240, 241). These soluble ADAMs may have proteolytic activity, but their activity may be modified as soluble TACE is much less active than the membrane-bound enzyme (238, 242).

The ADAMs also have Cys-rich, EGF-like regions, followed by transmembrane and cytoplasmic domains in most. To date 28 ADAMS have been identified but the substrates and binding partners for only a few are known. These proteins are related to snake venom metalloproteinases (243). ADAMs are included in the wider family of metalloproteinases, the metzincins which also contains the matrix metalloproteinases

and astacins (244). Biologic roles of the mammalian ADAMs involve such diverse functions as fertilization, cell adhesion and fusion, ectodomain shedding of cell surface receptors or adhesion molecules, and proteolytic activation of cytokines and growth factors. One family member is ADAM-10 or kuzbanian which was originally implicated in myelin degradation (245) but was recently shown to be potentially capable of processing proTNF-α to its soluble form (246). It is also involved in cleavage of the Notch ligand Delta and thus in cell fate determination (247).

1.11 Cleavage of Cell Surface Molecules by Metalloproteinases

Shedding of cell surface receptors plays a pivotal role in the biology of receptor-ligand interactions. In general, soluble cytokine receptors are generated either by proteolytic cleavage of a membrane bound receptor or by alternative mRNA splicing (248). In some cases, the soluble and transmembrane forms do not have identical biological activities (249). Moreover, shedding can reduce the cell- cell juxtacrine stimulation and allows soluble counterparts of transmembrane growth factors to act on distant targets. The soluble counterparts have been demonstrated to retain their ligand binding capacity and thus can compete for cytokines and modulate their bioavailability, either inhibiting or enhancing their action depending upon the microenvironment (250, 251).

Metalloproteinases and their inhibitors have been implicated in the generation of soluble forms of cell adhesion molecules, cytokines and/or their receptors as well as in the proteolytic cleavage of growth factors (158, 252). For instance, cleavage of a

Val368-Met369 bond in the fibroblast growth factor receptor (FGFR) by MMP-2 releases a soluble intact ectodomain (253).

Studies of the TNF receptor have also implicated the cleavage of the two forms of TNF membrane-bound receptors, p55 and p75, by metalloproteinases. Williams et al., (254) demonstrated that the cleavage of p55 TNF receptor was blocked with a synthetic inhibitor of MMPs, BB-2275 which was accompanied by a reduction of TNF and soluble p75 receptor levels as well as an inhibition of MMP activity. The metalloproteinase inhibitor, TNF-α protease inhibitor (TAPI) together with synthetic MP inhibitors have been shown to modulate the shedding of not only p60 TNFR, but also the processing of IL-6R in COS-7 cells (150).

Metalloproteinases have been implicated not only in the processing of mature or soluble forms of membrane proteins, but as described in a recent report, can inactivate or degrade IL-1-β (255). Adhesion molecules important for regulating lymphocyte migration and chemotaxis are also targets of metalloproteinase cleavage. Metalloproteinases have been implicated in the proteolytic cleavage of the integrin L-selectin (157). This metalloproteinase effect has important implications in leukocyte trafficking, since L-selectin controls attachment to endothelium. The fact that sequences around the cleavage sites of these proteins are quite dissimilar suggests the existence of either a novel family of Zn-endopeptidases that selectively cleave individual molecules, or a unique enzyme with a broad substrate specificity recognizing a secondary structure rather than a conserved amino acid sequence (150). Whether natural inhibitors of metalloproteinases can regulate the cleavage and shedding of

growth factors and cytokine receptors has not been well established although induction of apoptosis via stabilization of cell surface TNF-α receptor on colon cancer cells have been attributed to TIMP-3 (165).

Other members of the TNF-α family are also thought to be cleaved by MMPs. The TNF-homologous portion of membrane FasL is thought to be cleaved into a 26-kDa soluble form by a metalloprotease (154, 233). It is not known whether FasL cleavage is also performed by TACE. Treatment with hydroxamic acid inhibitors of MMPs specifically induced accumulation of membrane-bound FasL (p40) of cells transfected with human FasL cDNA or in activated T cells (154, 155, 233). **It is important to stress that the identity of the enzyme involved in FasL shedding is unknown at this time.** It may involve a member of the ADAMs family or the MMP family. This effect of metalloproteinases in FasL regulation has important clinical implications for hematological disorders characterized by high serum soluble FasL such as leukemias of natural (NK) or T cell-type large granular lymphocytes (LGL) or NK-lymphomas (232, 256). These observations suggest that metalloproteinases have an important function in regulating cytokine and growth factor responses and thus modulation of the immune response.

Summary

Detailed studies reporting the expression and role of MMPs and TIMPs in reactive and neoplastic disorders of the immune system are limited. Although monocytes which are related to fibroblasts have been studied extensively, the expression of MMPs and TIMPs

in specific lymphoid cell lineages have not been systematically analyzed. In addition individual MMPs and TIMP family members may have specific and pleiotropic physiological roles. In this study, we have analyzed the expression profiles of a subset of MMPs and TIMPs in both reactive and neoplastic lymphoid cells. The expression of these proteins during T cell activation and the role of TIMP-2 in T lymphocyte growth modulation is the focus of this thesis.

1.12 The objectives of this thesis are:

1. Analysis of expression of MMPs and TIMPs in cells of the hematolymphoid system
2. Expression of MMPs and TIMPs during T cell activation
3. Determine the role of TIMP-2 in T cell growth modulation
4. Characterization of the enhancement of T cell apoptosis by recombinant TIMP-2
5. Determine the mechanism of TIMP-2 induction of T cell apoptosis

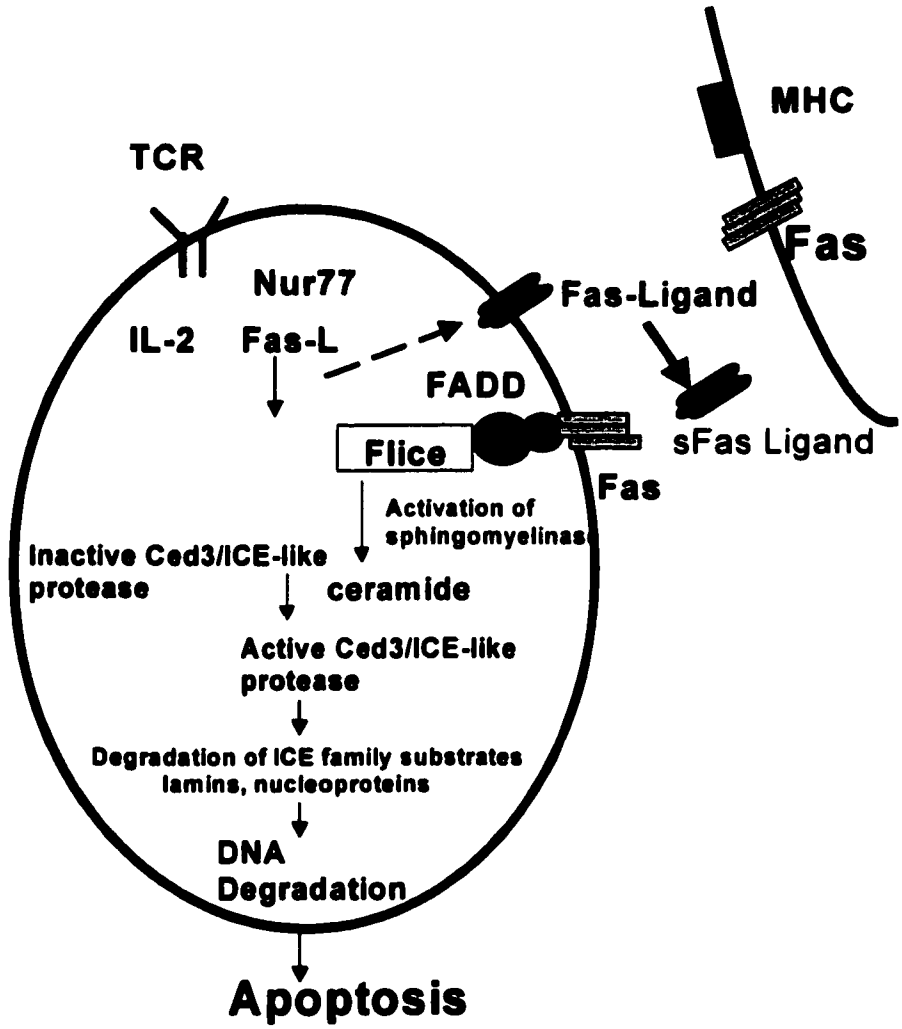


Figure 3. Triggering of Fas death pathway by antigen stimulation of the T-cell receptor.

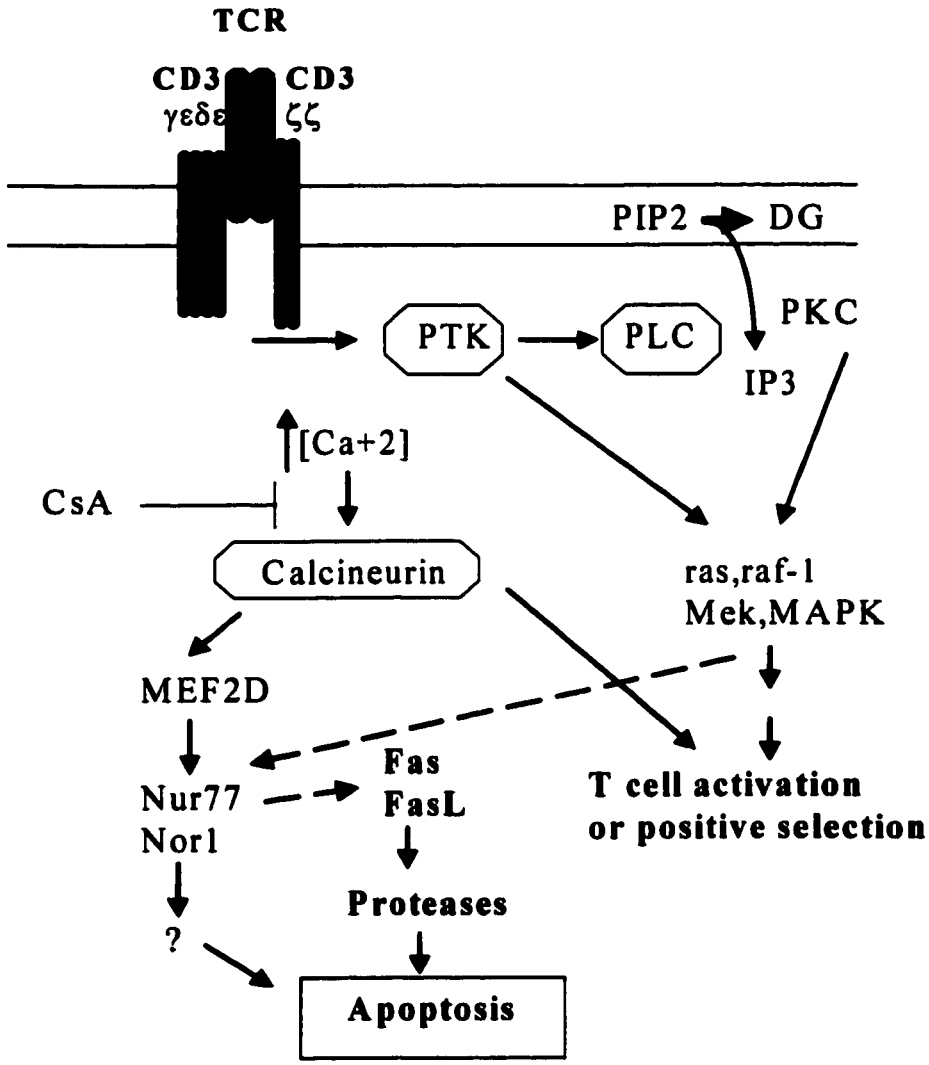


Figure 4. Antigen stimulation of T cell receptor initiates intracellular signal transduction leading to apoptosis.

Chapter 2. Materials and Methods

2.1 Cell Lines and Culture

All cell lines were maintained to 1-2 X 10^6 cells/mL at 37°C and 5% CO_2 at 95% air in RPMI 1640 with 10% FCS (without serum as indicated), 100 mg/mL penicillin G, 100 mg/mL streptomycin sulfate, and L-glutamine or in AIM V media (GIBCO BRL, Gaithersburg, MD) unless otherwise specified. Cell lines used include those developed from Burkitt's lymphoma, follicle center cell neoplasms, and T cell malignancies. The Burkitt's lymphoma cell lines, Jijoye, AG876, DW6, and PA682 were kindly provided by Dr. Ian Magrath (Chief, Lymphoma Biology Section, Pediatric Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD). The follicle center cell lymphoma-derived lines, SUDHL 6 and SUDHL 4, contain the t(14;18) translocation characteristic of these neoplasms. The cell lines derived from T cell neoplasms include Jurkat, Molt-4, and Peer-1. K562, a cell line derived from a patient with chronic myelogenous leukemia (CML) in blast crisis, is a poorly differentiated multipotential line that can differentiate into progenitors of erythrocytic, granulocytic, and monocytic series (obtained from American Type Culture Collection, Rockville, MD).

Cell suspensions were prepared from hyperplastic tonsillar tissue obtained from patients less than 15 years of age. Unprocessed tonsillar tissue contained 62% B cells and 19% T cells with the remainder of the population being comprised primarily of monocytic lineage cells. Tonsillar B cells were isolated as described (257). Tonsillar B cells were stimulated with *Staphylococcus aureus* Cowan strain 1 and phorbol 12-myristate 13-acetate (PMA) with cell samples collected at 0, 4, and 24 hours of

stimulation. Peripheral blood and tonsillar B cells were isolated as described (258). Briefly, components of tonsillar lymphocytes or peripheral blood were separated by density centrifugation of buffy coat using Ficoll-Hypaque (Amersham Pharmacia Biotechnology Inc., Piscataway, NJ). Mononuclear cells were harvested and resuspended in RPMI containing 10% FCS. Monocytes were allowed to adhere to plastic tissue culture plates by incubating at 37°C for 2 hours. The nonadherent population composed mostly of lymphocytes was then passed through a nylon wool (Polysciences Inc., Warrington, PA) column for 45 minutes at 37°C at 5% CO_2 to collect B cells. B cells were further purified by using magnetic beads conjugated to anti-CD19 (MACS, Miltenyi Biotec Inc, Auburn, CA) (259) and sorted using FACStar (Beckton Dickinson).

2.2 Isolation of Peripheral Blood T cells

T cells were separated from peripheral blood mononuclear cells as described (260). Briefly, components of peripheral blood were separated by density centrifugation of buffy coat using Ficoll-Hypaque (Amersham Pharmacia Biotechnology Inc., Piscataway, NJ). Mononuclear cells were harvested and resuspended in RPMI containing 10% FCS. Monocytes were allowed to adhere to plastic tissue culture plates by incubating at 37°C for 2 hours. The nonadherent population composed mostly of lymphocytes was then passed through a nylon wool (Polysciences Inc., Warrington, PA) column for 45 minutes at 37°C at 5% CO_2 to remove B cells. In some experiments, T cells were obtained by counterflow elutriation

(Dr. Larry Wahl, National Institute of Dental Research, National Institutes of Health, Bethesda, MD). Two color immunophenotypic analysis using FITC-conjugated anti-CD3, PE-conjugated anti-CD14, and PE-conjugated anti-CD19 by flow cytometry showed greater than 90% T cells, less than 1% B cells and natural killer cells comprising the remaining cell population. No monocytes were detected. Purfied T cells were maintained in complete media and used within 24 hours of isolation.

2.3 Stimulation of Cells for Time Course Experiments

Cells were grown in AIM-V growth media or RPMI 1640 with or without FCS at concentrations ranging from 1-2 X 10^6 /mL unless otherwise specified. Reagents used for T cell stimulation were as follows, PHA (1 ug/mL) (Sigma, St. Louis, MO) and IL-2 (25 ng/mL) (Sigma, St. Louis, MO). Cells and conditioned media were collected at specified time points and analyzed appropriately.

2.4 Collection of Protein Samples from Cell Culture

Conditioned media analyzed for gelatinase and TIMP activity were collected by centrifugation at 1000 *g* for 8 minutes (Sorval centrifuge model RT6000, Dupont, Wilmington, DE) at 4°C to remove cells and then stored at -20°C.

2.4.1 Preparation of Cell Lysate for Western Blot Analysis

Cells were obtained and pelleted at 15,000g for 4 minutes at 4°C, resuspended in 1 mL of ice-cold RIPA buffer containing 150 nM NaCl, 1% NP-40, 0.15% SDS, 50

mM Tris-HCl (pH 8.0), 0.001% paraphenylmethylsulphonylfluoride (PMSF), aprotinin .23U/mL and leupeptin 10 uM (Sigma, St. Louis, MO), kept on ice for 30 minutes. The protein extract was centrifuged for 15 minutes at 4°C at 20,000g, and supernant collected and stored at –80°C until use.

2.5 Protein Concentration Determination

Protein concentrations of conditioned media and cell lysates were determined by the BCA protein assay microtitre plate protocol (Pierce Chemical Company, Rockford, IL). Samples were diluted in a volume of 100 uL of water, then 200uL of BCA protein assay reagent were added and samples were mixed thoroughly. After allowing to equilibrate for 30 minutes at 37°C, absorbance of samples were read at 595 nm. Protein concentrations were determined from a BSA standard curve prepared at the same time as the samples. When necessary, 2 mL of conditioned media was concentrated in Centricon-10 microconcentrators (Amicon, Beverly, MA). Cell extracts and conditioned media for western blot analysis were resuspended in 4X reducing gel-loading buffer (200 mM tris-HCl pH 6.8, 4% SDS, 0.1% bromophenol blue, 40% glycerol, 5% β-mercaptoethanol), heated at 95°C for 5 minutes and then stored at -70°C.

2.6 Northern Blot Analysis

Total cellular RNA from cell lines was isolated by using RNAzol (Cinna/Biotecx Laboratories International, Inc. Friendswood,TX). Approximately 5-10 X 10^6 cells were harvested and washed 2 X with PBS before lysing with 1 mL of

RNAzol solution (Cinna/Biotecx Laboratories Internation, Inc, Friendswood, TX) and 0.2mL of chloroform. After centrifugation at 12,000 g for 15 minutes at 4°C, the aqueous phase was transfered to a fresh eppendorf tube. RNA was precipitated with equal volume of isopropanol and stored at 4°C for 10 minutes. RNA pellet was acquired by centrifugation at 12,000 g for 10 minutes at 4°C. RNA pellet was washed twice with 75% ethanol, dried, dissolved in sterile water, and concentrations estimated by absorbance at 260nm. The absorbance at 260/A280 ratio, an estimate of purity was routinely greater than 1.8. A total of 7.5 to 10 ug of RNA was electrophoresed on a 1% wt/vol agarose-formaldehyde gels before transfer onto nylon filters (Gene Screen Plus, Dupont, NEN products, Boston, MA). The RNA was UV cross-linked to the filter and hybridized using standard conditions. Blots were hybridized with TIMP-1, TIMP-2 and GAPDH probes prepared as previously described (261). These DNA probes were labeled with a [^{32}P] dCTP using a random primer labeling kit (Bethesda Research Laboratories, Gaithersburg, MD). Filters were autoradiographed at -80°C.

2.7 Reverse transcriptase (RT)-polymerase chain reaction (PCR)

Complementary DNA was prepared from 2 ug of RNA and the Superscript Preamplification system for first-strand cDNA synthesis (GIBCO-BRL, Gaithersburg, MD). One tenth of the cDNA was used for each polymerase chain reaction. A total of 2.5 U of Taq polymerase (GIBCO-BRL) and 0.8 uM of each primer were used. The primer pairs used are listed in Table 4. The cDNA was denatured for 5 minutes at 94°C and then 20-32 cycles of PCR were performed as follows: 1 minute denaturation at

94°C, 1 minute annealing at 55°C, and 1 minute extension at 72°C with the exception of the last cycle in which extension was for 5 minutes. Products were analyzed by electrophoresis in TBE polyacrylamide gels (Novex, San Diego, CA) followed by ethidium bromide staining.

2.8 Zymogram Analysis

Gelatinolytic activity was assayed by electrophoresis in polyacrylamide gels containing sodium dodecyl sulfate (SDS) and gelatin as described by Heussen and Dowdle (262). Gelatin zymography is an exquisitely sensitive technique that is capable of detecting 10 pg of gelatinase enzyme (263). Briefly, aliquots of conditioned media were suspended in sample buffer (50 mM Tris-HCL, 2% SDS, 0.1% bromophenol blue, 10% glycerol, pH 6.8) without boiling or thiol denaturation and applied to 10% (wt/vol) acylamide gels containing 1 mg/mL of gelatin (Novex, San Diego, CA) followed by electrophoresis at 20 mA/gel. Gels were then incubated in 2.5% (vol/vol) Triton X-100 for 60 minutes to remove SDS followed by overnight incubation in developing buffer (50 mM Tris-HCL, 0.2 M NaCl, 5 mM CaCl2, 0.02% (wt/vol) Brij-35, adjusted to pH 7.6). Gels were stained for 3 hours in 30% methanol, 10% glacial acetic acid, 0.5% Coomassie Blue G-250 (Bio-Rad, Richmond, CA), destained for 1.5 to 2 hours in 30% methanol, 10% glacial acetic acid, and dried overnight. Gels were dried and scanned using an AGFA flatbed scanner. (263)

2.9 Reverse Zymogram Analysis

Metalloproteinase inhibitory activity was assayed by electrophoresis in polyacrylamide gels containing gelatin as matrix metalloproteinase substrate and HT1080 fibroblast culture-conditioned media as a source of matrix metalloproteinase as previously described (264). Briefly, 12% or 15% polyacrylamide gels were prepared with Tris-HCl containing 0.1% SDS, 2.5 mg/mL gelatin, and 10% (vol) HT1080 fibroblast culture-conditioned media. Aliquots of conditioned media (15uL) were suspended in sample buffer (50mM Tris-HCl, 2% SDS, 0.1% bromophenol blue, 10% glycerol, pH 6.8) and applied to gels followed by electrophoresis at 20 mA/gel. Gels were then incubated in 2.5% (vol/vol) Triton X-100 for 60 minutes to remove SDS followed by overnight incubation in developing buffer (50 mM, Tris-HCl, 0.2 M NaCl, 5 mM $CaCl_2$, 0.02% (wt/vol) Brij-35, adjusted to pH 7.6). This restores the matrix metalloproteinase activity resulting in gelatin degradation. Gels were stained for 3 hours in 30% methanol, 10% glacial acetic acid, 0.5% Coomassie Blue G-250 (Bio-Rad, Richmond, CA), destained for 1.5 to 2 hours in 30% methanol, 10% glacial acetic acid, and dried overnight. TIMPs were visualized as bands of nondegraded gelatin staining positive with Coomassie Blue. Figure 5 illustrates the substrate-based gel electrophoresis method used to detect gelatinases and TIMPs. Gels were dried and scanned using AGFA flatbed scanner.

2.9.1 Quantitation of MMP and TIMP activity

The bands were quantified using an Arcus scanner (Agfa-Gevaert) equipped with a transparency option interfaced to a Macintosh computer as previously described (265). Gels were scanned using Adobe Photoshop (Adobe Systems Inc., Mountain View, CA) in gray-scale mode at 600dpi using the autodensity feature. The scanner records the density for each pixel on a scale from 0(clear) to 255 (opaque). The image was digitally inverted for zymogram analysis so that the integrations of bands would be reported as positive values. For reverse zymogram, western blot and northern blot analysis native images were processed for density quantification. Images saved to disk in TIFF format were analyzed using NIH Image 1.41, a public domain program. Within this program, the integrated density of a particular band is calculated by summing the pixel values within a selected area and subtracting from this total the modal density. This assumes that the background pixel intensity is relatively even and can be approximated by selecting the most common pixel. Values of integrated density are reported in volume units of pixel intensity x mm2. Each gel was scanned three times and the average value of the integrated density for a particular band was used in further calculations. The standard deviation of the mean value was generally only 1 to 2 % of the mean.

2.10 Western Blot Analysis

Five to 10 ug of total protein was electrophoresed on 10% or 12% SDS-polyacrylamide gels using a BioRad minigel apparatus, and electroblotted onto nitrocellulose membrane (Novex, CA) using a BioRad Trans-Blot electroblotter (Bio-

Rad, Richmond, CA). Samples were dissolved in gel loading buffer containing 5% *B*-mercaptoethanol and heated at 95°C for 5 minutes before loading. Efficiency of protein transfer was assessed by Coomassie Blue staining of gels post transfer. Membranes were blocked with 5% non-fat dry milk in 20nM Tris-buffered saline pH 7.5, 1% Tween-20 (TBS-T), washed in TBS-T, then incubated with primary antibody in TBS-T for 1 hour at 37°C. After washing 4 times with TBS-T, the membranes were incubated in a 1:5000 dilution of goat-anti-mouse-horseradish peroxidase-conjugated secondary antibody (Pierce, Rockford, IL), for 30 minutes at 37°C. Following washing in TBS-T, the blots were developed by using an enhanced chemiluminescence detection system for HRP-labeled secondary antibody (Amersham, Arlington Heights, IL) and exposed on Kodak BMR film, according to the manufacturer's directions.

2.11 Staining of Cell Surface Antigens

Peripheral blood T cells and neoplastic lymphoma cell lines were washed twice with cold PBS and preincubated with 1% human albumin to prevent binding of the antibodies to Fc receptors. For direct staining of antigens, the cells were incubated with 10 to 20 uL of FITC-conjuated or PE-conjugated monoclonal mouse anti-human antibodies or mouse IgG (Pharmingen, San Diego, CA) as isotype control for 30 min at 4°C in the dark. Antibodies and respective manufacturers are listed in Table 5. Indirect staining of cell surface antigens was carried out by incubation of washed cells with primary antibody (volumes used were as indicated by manufacturer) for 30-45 min at 4°C in the dark after which the cells were washed 2 X with PBS. A goat anti-mouse IgG-FITC-conjugated antibody (Caltag Lab, San. Fransisco, CA) or a goat anti-mouse

IgG-PE-conjugated secondary antibody (Caltag Lab, San. Fransisco, CA) was then added (20 uL) and incubated at 4°C in the dark for an additional 30 minutes. After removing excess unbound secondary antibody with PBS the cells were resuspended in 0.1 % paraformaldehyde before analysis. The labeled cells were analyzed by FACScan (Becton Dickinson, San Jose, CA) using either Lysis or CellQuest software to determine percentage of positive cells. For each sample, 10,000-15,000 events were collected in list mode, and cell debris and doublets were excluded from the data acquisition using a doublet discriminator (Becton Dicksinson).

Table 5. Antibodies used for cell surface antigen staining and functional assays.

Cluster Designation	Clone	Type	Label	Source
CD4	Leu 3a	Mouse a-human Monoclonal	FITC/PerCP	Pharmingen, San Diego, CA
CD8	Leu2a	Mouse a-human Monoclonal	FITC/PerCP	Pharmingen, San Diego, CA
CD20	L26	Mouse a-human Monoclonal	PE	Beckton Dickinson, CA
CD3		Mouse a-human Monoclonal	FITC	Beckton Dickinson, CA
CD14	LeuM 3	Mouse a-human Monoclonal	PE	DAKO Corp. Carpinteria, CA
IgG1		Isotype control	unlabeled	Beckton Dickinson, CA
CD95 Fas Inhibits apoptosis	ZB4	IgG1	unlabeled	Immunotech, Marseille, France
CD95 Fas	UB2	IgG1 Mouse a-human Monoclonal	FITC	Immunotech, Marseille, France
CD95L Fas L	NOK-1	IgG1 Mouse a-human Monoclonal	unlabeled	Immunotech, Marseille, France
CD95L	Clone 33	IgG1 for western blot	unlabeled	Transduction Lab, Lexington, KY
TIMP-1		Rabbit IgG polyclonal	unlabeled	Dr.W.G.Stetler-Stevenson
TIMP-2		Rabbit IgG polyclonal	unlabeled	Dr.W.G.Stetler-Stevenson
MMP-9		Rabbit IgG polyclonal	unlabeled	Dr.W.G.Stetler-Stevenson

FITC: fluorescein isothiocyanate
PE: phycoerythin
PerCP: peridinin chlorophyll protein

2.12 Determination and Quantitation of T cell Apoptosis

Two assay conditions were used to detect T cell apoptosis. Fetal bovine and calf serum contains gelatinases as well as TIMPs including TIMP-1 and TIMP-2. To prevent contamination of reaction conditions with TIMPs and gelatinases, T cells were activated with PHA and IL-2 in the presence of serum after which the cells were washed 2X with PBS. The cells were then resuspended and cultured in RPMI without serum. The effect of TIMP-2 on T cell apoptosis was analyzed at specified time points. The other assay protocol tested the effect of TIMP-2 and other reagents on T cells cultured in RMPI 1640 containing 3% FCS during the activation period. Apoptosis was determined either by Annexin V-FITC and propidium iodide staining (Section 2.12.4) or by trypan blue dye exclusion using the following calculation:

$$\frac{\text{Total \# of blue cells}}{\text{Total \# of cells}} \times 100$$

Percentages of apoptotic cells were measured in triplicate under the light microscope. Cells counted as apoptotic included cells with characteristic nuclear chromatin condensation and fragmentation as previously described (266). Percentages of apoptotic cells counted under the light microscope were confirmed by flow cytometry analysis after incubation with Annexin V-FITC and propidium iodide. These methods have been used to quantitate apoptosis in peripheral blood T cells (267). Mean and standard deviation values were obtained by using the Microsoft Excel (Redmond, WA) program. P values were obtained using the Student's t-test.

2.12.1 Reagents Used for Studying Effect of TIMP-2 on T Cell Apoptosis

Recombinant TIMP-2, TIMP-1, reduced and alkylated TIMP-2 were prepared and purified as previously described (179).

Affinity-purified polyclonal rabbit anti-TIMP-2 antibody (isotype IgG) and polyclonal rabbit anti-TIMP-1 antibody (isotype IgG) were used for TIMP neutralizing experiments. A mouse IgG was used as isotype control.

Synthetic metalloproteinase inhibitors were obtained from the following companies; Batimastat (BB-94) a hydroxamic acid analog (4-{hydroxyamino}-2R-isobutyl-3S-{thiophen-2-ylthiomethyl}-succinyl]-L-phenylalanine-N-methyalmide) and Galardin (GM-6001) (N-[2R-2{hydroxamido-carbonymethyl}-4methylpentanoil]-L-tryptophan methylamide) (British Biotech, UK), KB-8301 (Pharmingen, San Diego, CA)

Fas blocking antibody; clone ZB4 (Immunotech, Miami, Florida) was used at 1 ug/mL. Fas Ligand antibody used for cell surface staining (purified mouse anti-human Fas ligand monoclonal antibody; Cat # 65321A, clone NOK-1, mouse IgG1) was purchased from Pharmingen, San Diego, CA.

Fas Ligand antibody used for western blot analysis purified mouse anti-human Fas ligand monoclonal antibody; Cat #F37720, clone 33, isotype IgG1, 37kDa) was purchased from Transduction Laboratories, Lexington, KY and was used at 2.5 ug/mL.

2.12.2 Cell Viability Assay

Cells were incubated in the presence or absence of rTIMP-2 or other specified reagent for variable time periods. Absolute number of viable cells were determined by multiplying the number of cells excluding trypan blue (0.4% in 0.9% saline) by the volume of culture media. Percentage of viability was obtained by dividing number of cells excluding trypan blue by the total number of cells counted

2.12.3 DNA Fragmentation Assay by Agarose Gel Electrophoresis

To identify DNA fragmentation after exposure to TIMP-2 or BB-94, treated cells were incubated for 18hours at 50° C in lysis buffer (100mM NaCl, 10 mM Tris/HCl, pH 8, 25 mM EDTA, 0.5% SDS, 0.1 mg/ml proteinase K). Samples were extracted twice with phenol/chloroform (1:1) treated with 20 ug/ml RNAse A, and precipitated with 7.5 M ammonium acetate and 2 volumes of 100% ethanol. DNA was recovered by centrifugation and pellets were washed with 70% ethanol, air dried, and resuspended in TE buffer (10 mM Tris/HCl pH 8.0, 1mM EDTA). DNA samples were separated on 1.8 % agarose gels at 60V for 2 hours and stained with 0.25 ug/mL ethidium bromide. Bands were visualized by UV fluorescence and photographed.

2.12.4 Annexin V-FITC Staining and Flow Cytometric Analysis

Detection of phosphatidylserine exposure on cell membranes was performed using Annexin V-fluorescein isothiocyanate (FITC) which was purchased from Nexins Research B.V. (Maastricht, The Netherlands or R &D Systems, Abingdon, UK). Cells

(5 X 10^5 cells/mL) were treated with rTIMP-2 or BB-94 as indicated above. Cells were harvested, washed in ice-cold PBS and resuspended in 200 uL of annexin binding buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$ and 2 mM $CaCl_2$). The cells were stained with Annexin V-FITC for 10 minutes on ice in the dark, according to the manufacturer's instructions, and analyzed by a FACScan flow cytometer. Annexin V "bright" cells were measured on a flow cytometer by gating on FSC/SSC settings for control untreated cells. Peripheral blood T cells treated with 3% formaldehyde and incubated on ice for 30 minutes were used as positive control for Annexin V positive cells.

2.13 Quantitation of soluble Fas Ligand by ELISA

To quantitate the level of soluble Fas Ligand in the conditioned media of cells treated with TIMP-2 and BB-94, a sFas Ligand ELISA Kit (Cat# 5255 Medical & Biological Laboratories Co., LTD, Nagoya, Japan) was used following manufacturer's instructions.

Table 4. Oligonucleotide Primer Pairs For RT-PCR.

GENE	5'PRIMER	3'PRIMER
hTIMP-1 (641bp) (-9-632)	ATAGTCGACATGGCCCCCTTT--GAGCCCCTG (29nt)	GGAATTCCTCAGGCTATC--TGGGACCGCAGGGA (32nt)
hTIMP-2 (470bp) (377-873)	GGCGTTTTGCAATGCA--GATGTAG (23nt)	CACAGGAGCCGTCACT--TCTCTTG (23nt)
hTIMP-3 (457bp) (323-780)	CTTCTGCAACTCCGAC--ATCGTG (22nt)	TGCCGGATGCAGGCGT--AGTGTTT (23nt)
hMT1-MMP (548bp) (1156-1704)	GCCCATTGGCCAGTTCT--GGCCGG (23nt)	CCTCGTCCACCTCAAT--GATGATC (23nt)
GAPDH (306bp)	CGGAGTCAACGGATTT--GGTCG (21nt)	AGCCTTCTCCATGGT--GGTGAAGAC (28nt)

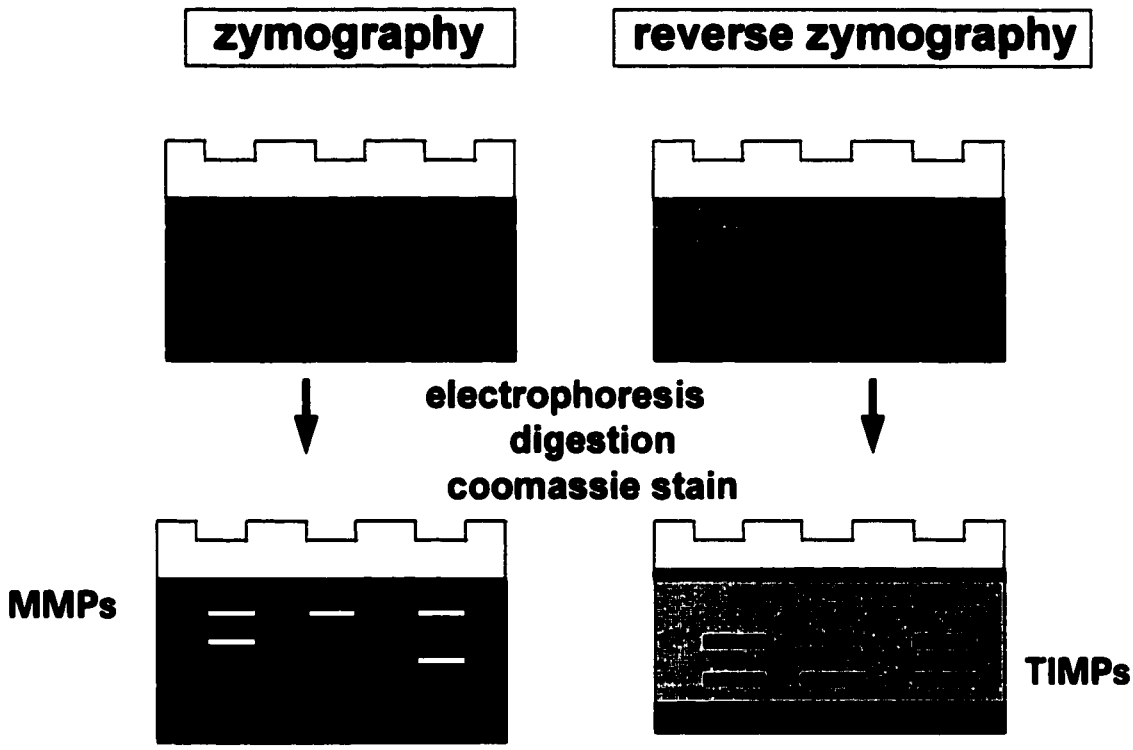


Figure 5. Gelatin substrate-based gel electrophoresis used to detect gelatinases and tissue inhibitor of metalloproteinase.

Chapter 3. Results

3.1 Expression of matrix metalloproteinases and tissue inhibitors of metalloproteinases in cells of the hematolymphoid system

Lymphoid neoplasms differ fundamentally from solid tumor neoplasms. Due to their role in immunosurveillance, lymphocytes are normally found throughout the organs and connective tissues of the body, and as such, have a normally invasive phenotype. Recent evidence suggests that matrix metalloproteinase activity may be involved in the transmigration of lymphocytes from the vascular compartment (194, 268). Aggressive lymphoid neoplasms can be observed "invading" through the lymph node capsule and, in some cases, extensively involving the surrounding soft tissue. In addition, invasive behavior has been documented in neoplastic lymphoid cell lines using the membrane invasive culture system. This invasive behavior directly correlated with the expression of MMP-2 and associated metastatic behavior in severe combined immunodeficiency (SCID) mice was observed in cell lines expressing high levels of MMP-2 (201). Studies by Kossakowska et al., (136) demonstrated TIMP-1, but not TIMP-2 expression, in non-Hodgkin's lymphomas. TIMP-1 expression was localized to the stromal and endothelial cells by *in situ* hybridization. However, low level expression of TIMP-1 by the tumor cells could not be excluded. The expression of TIMP-1 did not correlate with MMP-2 or interstitial collagenase expression (MMP-9 expression was not studied in this report). Paradoxically, increased TIMP-1 expression was observed in high-grade non-Hodgkin's lymphomas and in advanced stage disease, leading the investigators to postulate that TIMP-1 may have lymphoid growth factor activity (136). Further studies by this group demonstrated an association between

survival and MMP-9, but not TIMP-1 expression in high-grade, large cell immunoblastic lymphomas (82). Uchijima et al., (72) observed that human T-cell leukemia virus type 1 (HTLV-1) and HTLV-2-infected cell lines express elevated levels of TIMP-1. They have proposed that TIMP-1 may be an autocrine growth factor produced by the infected cells and may actually modulate the clinical course in patients with adult T-cell leukemia. These studies suggest that gelatinases and TIMPs may be of importance in lymphomas.

We have studied the expression of MMP-2, MMP-9 as well as TIMP-1 and TIMP-2 and TIMP-3 in reactive lymphoid cells and a series of cell lines derived from neoplasms of B-cell and T-cell lineage. These studies using cell lines and isolated lymphoid cells were performed to elucidate the possible involvement of matrix metalloproteinases and TIMPs in lymphoid neoplasms without the confounding influence of stromal and endothelial cells present in lymphoma tissue specimens.

3.1.1 Expression of MMPs in Lymphoid Cells

In cells of lymphoid origin, expression of MMP-9 (gelatinase B) was seen at variable levels while expression of MMP-2 was not observed. MMP-9 expression was seen in reactive and malignant B lymphoid cells (Figure 6), as well as unstimulated resting and activated T lymphocytes (Figure 9). Transcripts for MMP-2 and MMP-9 were not detected for any of the neoplastic cell lines (data not shown). Zymography, a more sensitive method to detect gelatinase activity, failed to demonstrate MMP-2 in any cell lines studied. A 92-kDa gelatinase activity consistent with MMP-9 was observed

(Figure 6) in the Burkitt cell lines Jijoye and PA682, but was absent from the follicle center cell lines (SUDHL-4 and SUDHL-6) and the T-cell lines (Molt-4, Jurkat, and Peer 1). Conflicting reports have been published regarding the expression of MMP-2 by lymphoid cells. Studies from two laboratories using highly purified T cells reported the absence of MMP-2 expression whereas its expression was observed in another study that did not eliminate B cells from their preparation. In pure cell suspension cultures as was used in these studies, MMP-2 expression was not seen in any of the reactive or malignant lymphoid cells studied. MMP-2 expression has been observed in some lymphoblastoid cell lines (201) which suggests that they may be a consequence of *in vitro* culture conditions. In contrast to B lymphoma cell lines, MMP-9 was not expressed in any of the neoplastic T cell lines. Analysis of lymphoid cells for the expression of matrilysin and stromelysins 1,2, 3, as well as other members of the MMP family have not been reported although we have detected stromelysin-3 in activated peripheral blood T cells by ELISA technique (data not shown). RT-PCR demonstrated absence of MT1-MMP expression in any of the reactive or neoplastic lymphoid cells (Figure 7).

3.1.2 Expression of TIMPs by Lymphoid Cells

TIMP-1 transcripts (0.9 kb) were detected in all four Burkitt cell lines (Jijoye, AG876, DW6, and PA682) and in the multipotential neoplastic K562 line, but were not detected in the low-grade follicular center cell lymphoma cell lines (SUDHL-4 and SUDHL-6) or neoplastic T-cell lines (Figure 8A). This is further supported by the demonstration that secreted and functional TIMP-1 is found in the conditioned media

from these cell lines detected by reverse zymography (data not shown). A semiquantitative assessment of the levels of protein expression correlated well with the northern blot data.

TIMP-2 transcripts (1.0 and 3.5 kb) were detected at high levels in the neoplastic T-cell lines (Figure 8B). Secreted and functional TIMP-2 was detected in activated (Figure 112) and neoplastic T cells by reverse zymography (Figure 9). We observed restricted expression of TIMP-1 and TIMP-2 in B and T lymphoid cells (Table 6). TIMP-1 is not expressed by neoplastic T cell lines, low grade B-cell lymphoma cell lines or isolated peripheral blood B cells. It was however, expressed in stimulated tonsillar B-cells and in a subset of high grade Burkitt cell lines. TIMP-2 expression was restricted to cells of T cell lineage with high levels being observed in neoplastic T cell lines and lower levels in hyperplastic tonsils. TIMP-3 was not detected in the conditioned media of any of the hematolymphoid cells by reverse zymography. No transcripts for TIMP-3 were detected by the sensitive RT-PCR assay (Figure 10). These results are consistent with previous reports showing that TIMP-3 is specifically localized in the extracellular matrix. The expression of TIMP-3 in lymphoid organs may be restricted to stromal cells such as endothelial cells and fibroblasts.

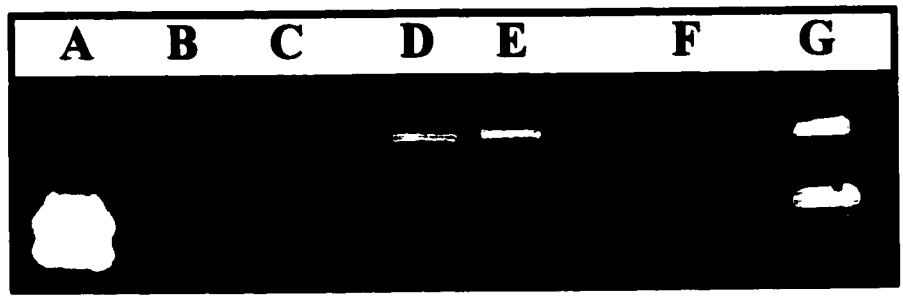


Figure 6. Gelatin zymography of reactive and neoplastic lymphoid cells.

Cell suspensions of SUDHL-6, Jijoye, Molt-4, PA682 and reactive tonsillar lymphocytes were grown in AIM-V media at concentrations of 0.5-1 X 10^6 cells/mL. A total of 16 uL of conditioned media was electrophoresed on 10% SDS polyacrylamide gels containing gelatin. SDS was removed following electrophoresis and gels incubated to restore gelatinase activity. Gels were scanned by an AGFA Arcus flatbed scanner. Lane A, MMP-2; lane B, SUDHL-6; lane C, Molt-4; lane D, Jijoye; lane E, PA682; lane F, tonsil; lane G, HT1080-conditioned media (positive control for MMP-2 and MMP-9). (from Stetler-Stevenson et al., (261))

Figure 7. Reverse transcriptase-polymerase chain reaction analysis shows absence of MT1-MMP mRNA transcript in cells of the hematolymphoid system.

RT-PCR was performed as described in chapter 2. PCR amplification of MT1-MMP cDNA was performed for 32 cycles. β-actin was used as a control for RNA integrity. A2058, a malignant melanoma cell line was used as a positive control for MT1-MMP. Lanes 1,2 (PHA/IL-2 stimulated peripheral blood T cells; RNA control with no cDNA and cDNA, respectively); Lanes 3,4 (MOLT-4; RNA control and cDNA respectively); Lanes 5,6 (DeFoe RNA control and cDNA respectively); Lanes 7,8 (KK562; RNA control and cDNA respectively); A2058 (+Control). Mwt marker (100 bp ladder). Size of deduced amplification product of MT1-MMP is 773 bp; B-Actin is 125 bp. The identity of the weak band at deduced size of approximately 1 kbp is unknown.

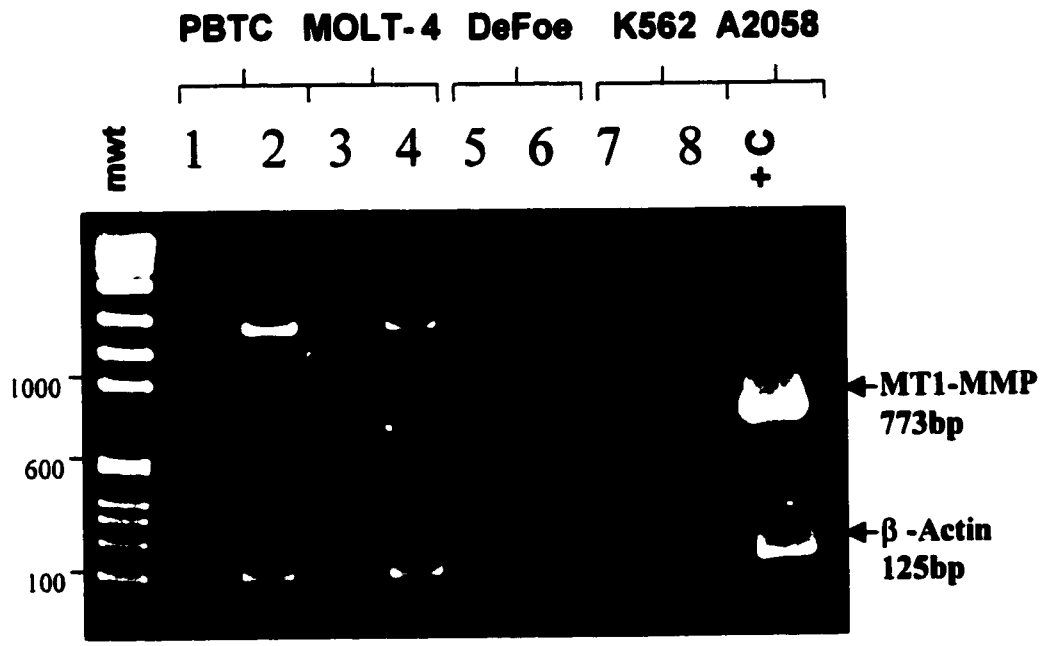

PBTC
MOLT-4
DeFoe
K562
A2058
mwt
1
2
3
4
5
6
7
8
C+
1000
600
100
MT1-MMP
773bp
β -Actin
125bp

Figure 8A and B. Northern blot analysis of TIMP-1and TIMP-2 in reactive and neoplastic hematolymphoid cells.

SUDHL-6, SUDHL-4, K562, PA682, AG876, DW6, Peer 1, Jurkat, Molt-4, Tonsillar lymphocytes, and peripheral blood T cells were harvested for RNA extraction following the protocol described in Chapter 2. A total of 7.5 ug of total RNA was electrophoresed on 1% wt/vol agarose-formaldehyde gel, transfered onto nylon filters, and hybridized with TIMP-1 (0.9 kb) (**A**), TIMP-2 (2 transcripts 3.5 kb, 1.1 kb) (**B**) and GAPDH probes. The filters were autoradiographed at -80°C. Radiographs were scanned by an AGFA Arcus flatbed scanner (from Stetler-Stevenson et al., (261)).

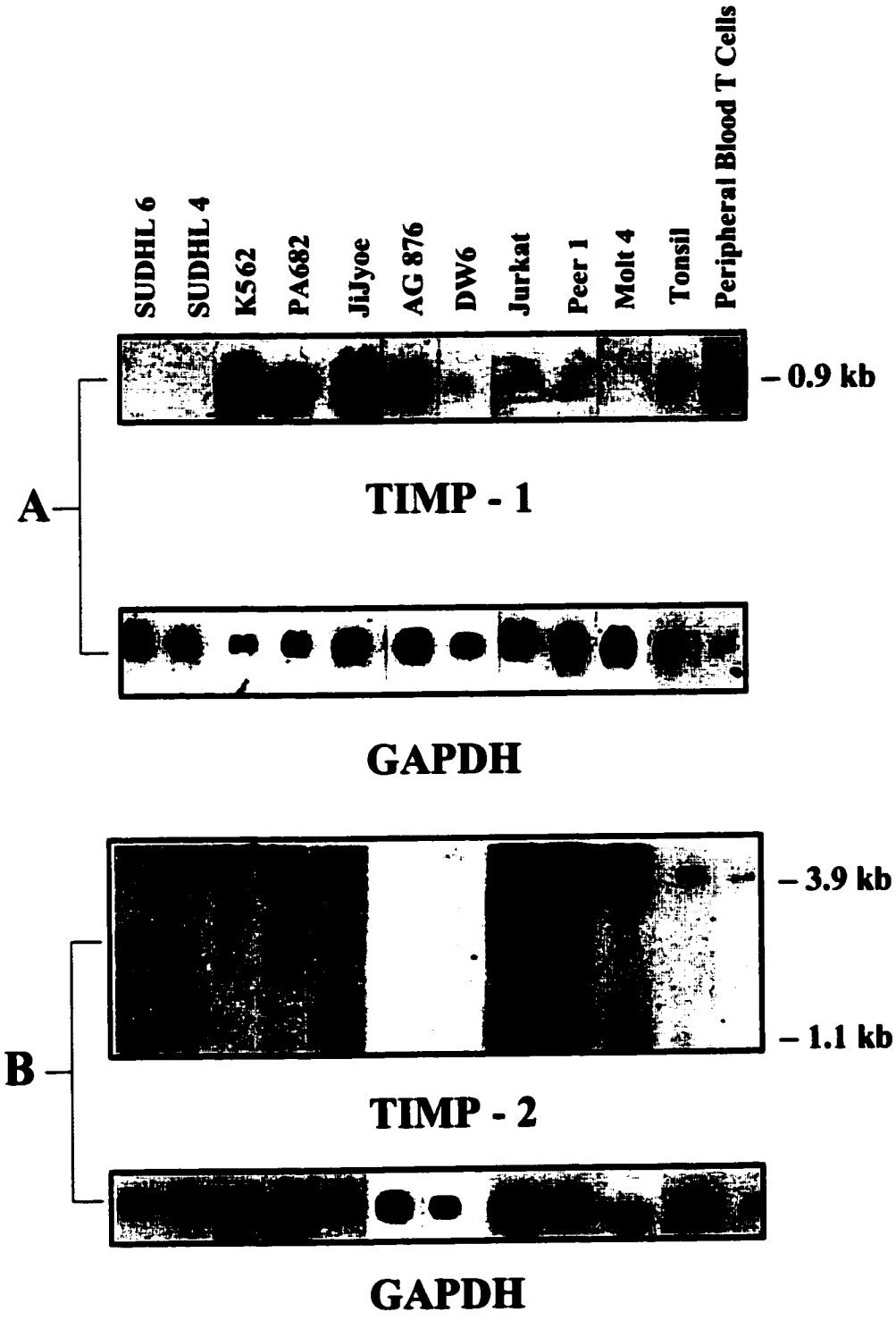

SUDHL 6
SUDHL 4
K562
PA682
JiJyoe
AG 876
DW6
Jurkat
Peer 1
Molt 4
Tonsil
Peripheral Blood T Cells
– 0.9 kb
A
TIMP - 1
GAPDH
– 3.9 kb
– 1.1 kb
B
TIMP - 2
GAPDH

Figure 9. Reverse zymography of neoplastic T cells.

Neoplastic T cell lines express TIMP-2 but not TIMP-1. MOLT-4, Jurkat, Tsup lymphoblastic lymphoma cell lines were cultured in AIM-V media at 0.5-1 X 10^6 cells/mL. A total of 20 uL of conditioned media was electrophoresed on 15% SDS polyacrylamide gels containing gelatin and HT1080 fibroblast culture conditioned media. SDS was removed following electrophoresis and gels incubated to restore gelatinase activity. Gels were scanned by an AGFA Arcus flatbed scanner. A dominant band with an apparent molecular weight of 60 kDa most likely represents albumin which is a component of the AIM-V serum free media.

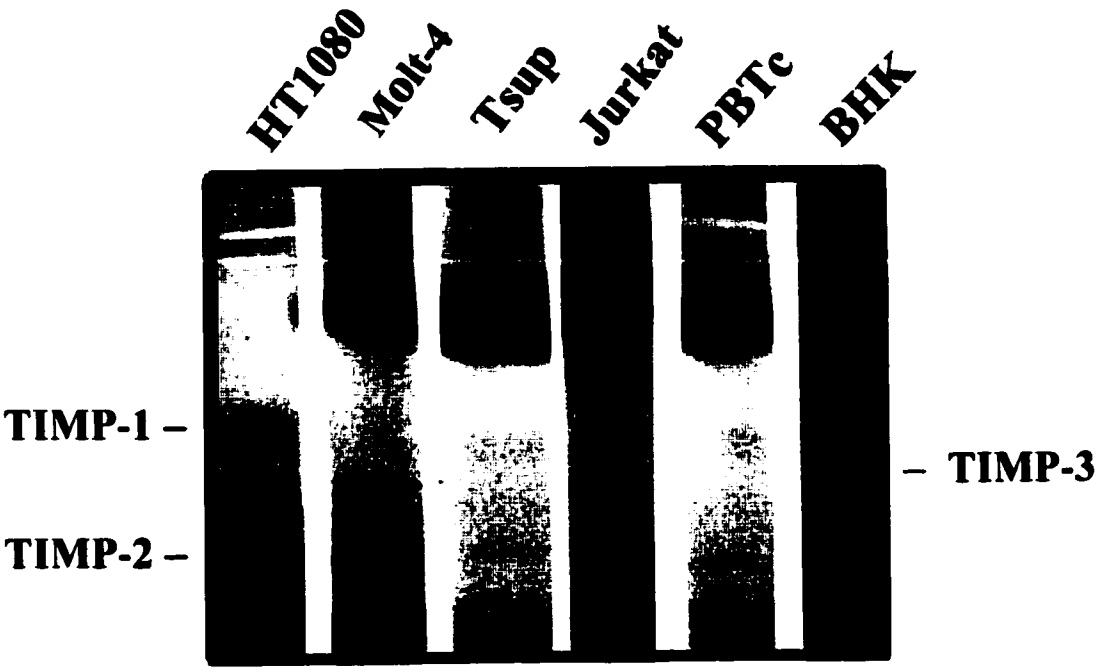

HT1080
Molt-4
Tsup
Jurkat
PBTc
BHK
TIMP-1 –
TIMP-2 –
– TIMP-3

Figure 10. Reverse transcriptase-polymerase chain reaction analysis shows absence of TIMP-3 mRNA transcript in cells of the hematolymphoid system.

RT-PCR was performed as described in chapter 2. PCR amplification of TIMP-3 cDNA was performed for 32 cycles. β-actin was used as a control for RNA integrity. A2058, a malignant melanoma cell line was used as a positive control. Mwt marker (100 bp ladder).

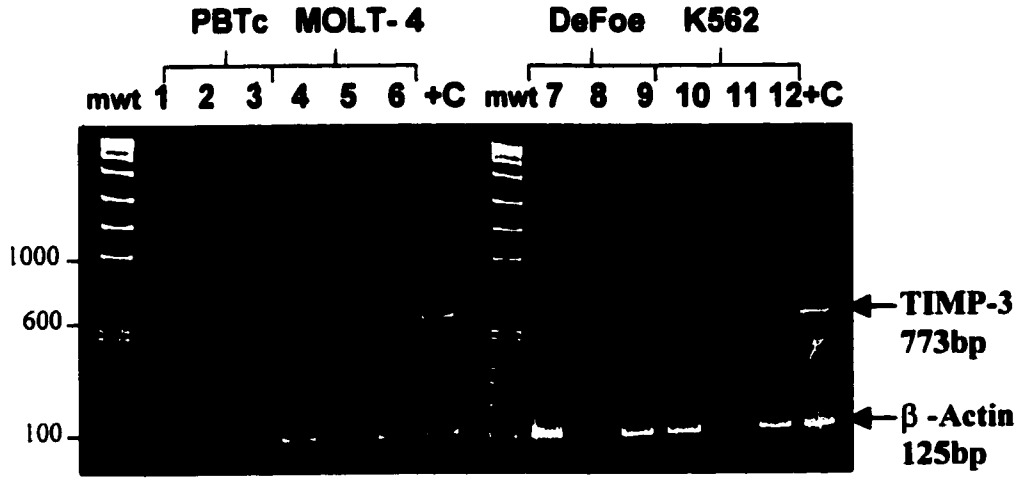
PBTcells cDNA with B-actin primer pairs alone Lane 1, TIMP-3 primer pairs alone Lane 2, β-actin + TIMP-3 primer pairs Lane 3;

MOLT-4 cDNA with β-actin primer pairs alone Lane 4, TIMP-3 primer pairs alone Lane 5, β-actin + TIMP-3 primer pairs Lane 6;

DeFoe cDNA with β-actin primer pairs alone Lane 7, TIMP-3 primer pairs alone Lane 8, β-actin + TIMP-3 primer pair Lane 9;

KK562 cDNA with β-actin primer pairs alone Lane 10, TIMP-3 primer pairs alone Lane 11, β-actin + TIMP-3 primer pairs Lanes 12;

A2058 (+Control). Size of deduced amplification product of TIMP-3 (635 bp); β-Actin (125 bp).


PBTc
MOLT-4
DeFoe
K562
mwt 1 2 3 4 5 6 +C
mwt 7 8 9 10 11 12+C
1000
600
100
TIMP-3
773bp
β -Actin
125bp

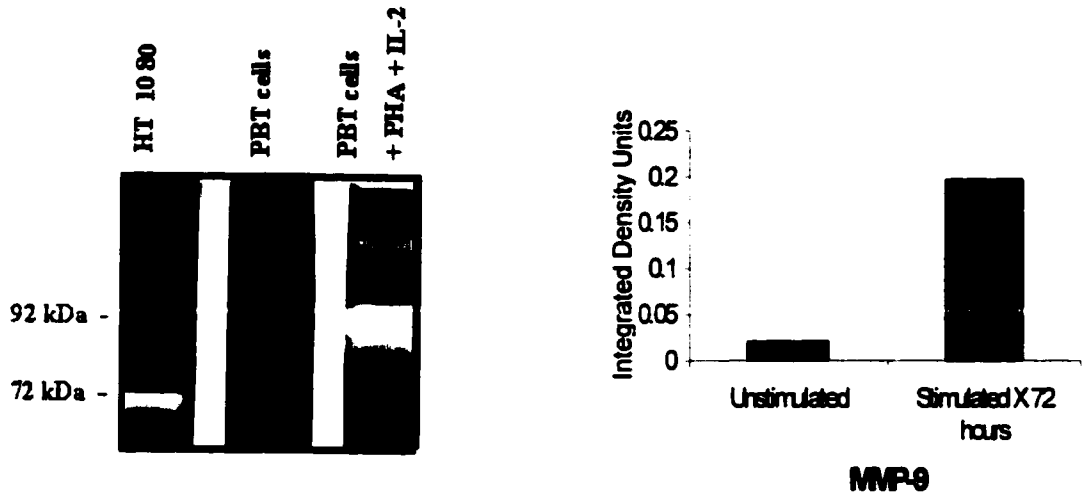
3.2 Induction of MMP-9 and TIMP-2 protein expression in activated peripheral blood T cells.

Having detected a differential pattern of TIMP-1 and TIMP-2 expression in neoplastic B- and T cell lines, respectively, we studied the expression of these protease inhibitors and associated MMPs in non-neoplastic T-cell populations. A 92-kDa gelatinase activity consistent with MMP-9 was observed in the tonsillar cell suspension by gelatin zymography (Figure 6).

Activation of peripheral blood T cells by PHA and IL-2 induced the expression of TIMP-2 protein, and latent and activated forms of MMP-9. A greater than 10-fold increase in MMP-9 protein was seen after 72 hours of T cell activation (Figure 11A and B). Peripheral blood T cells cultured without mitogens demonstrated a low basal level of TIMP-2 activity, as assessed by reverse zymography (Figure 12A). However, on stimulation with IL-2 plus PHA, active TIMP-2 could be detected in the media within 24 hours. A 16-fold increase in TIMP-2 protein expression was seen over a 72 hour time period (Figure 12B). The expression of TIMP-1 however, did not change significantly during the period of T cell activation.

Although TIMPs are essentially interchangeable in their capabilities as inhibitors of MMPs, they are distinguished by the formation of specific complexes with different pro-MMPs. Conventional models derived from analysis of adherent cell lines involve MMP-2/TIMP-2 and MMP-9/TIMP-1 complexes as playing a critical role in activation of specific latent MMPs (16, 40).

The discordant expression of TIMPs and MMPs in lymphoid cells is puzzling and suggests that; 1) MMPs and TIMPs may have cell-specific physiologic roles, and 2) TIMPs may be functioning in capacities aside from their metalloproteinase inhibitory function. Furthermore, the restricted expression of TIMP-1 in B cells and TIMP-2 in T cells indicates that they may have lineage-specific functions. Some of the data presented in this section have been published in manuscript form (261).



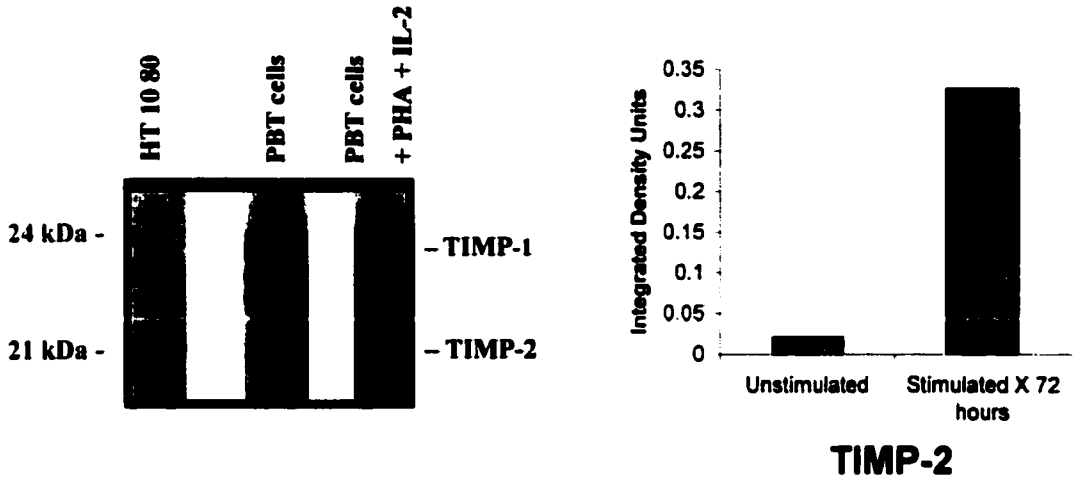
A. B.

Figure 11. Gelatin zymography demonstrates induction of MMP-9 in activated peripheral blood T cells.

Peripheral blood T cells were purified as outlined in Chapter 2. Peripheral blood T cells were cultured in AIM-V growth media and stimulated with PHA (1.5 ug/mL) and IL-2 (50 u/mL) for 24 to 72hours. Conditioned media normalized for protein concentration were electrophoresed on 10% SDS polyacrylamide gels containing gelatin. SDS was removed following electrophoresis and gels incubated to restore gelatinase activity. Gels were scanned by an AGFA Arcus flatbed scanner.

A. Zymogram Lane A; HT 1080 cell-conditioned media (positive control for both TIMP-1 and TIMP-2); Lane B, unstimulated peripheral blood T cell-conditioned media; Lane C, IL-2/PHA-stimulated peripheral blood T cell-conditioned media.

B. Integrated densitometric analysis.



A. B.

Figure 12. Reverse zymography demonstrates induction of TIMP-2 expression in activated peripheral blood T cells.

Peripheral blood T cells were purified as outlined in Chapter 2. Peripheral blood T cells were cultured in AIM-V growth media and stimulated with PHA (1.5 ug/mL) and IL-2 (50 u/mL) for 24-72 hours. Conditioned media normalized for equal protein concentration were electrophoresed on 15% SDS polyacrylamide gels containing gelatin and HT1080 fibroblast culture conditioned media. SDS was removed following electrophoresis and gels incubated to restore gelatinase activity. Gels were scanned by an AGFA Arcus flatbed scanner.

A. Reverse zymogram Lane A; HT 1080 cell-conditioned media (positive control for both TIMP-1 and TIMP-2); lane B, unstimulated peripheral blood T cell-conditioned media; lane C, IL-2/PHA-stimulated peripheral blood T cell-conditioned media.

B. Integrated densitometric analysis.

Table 6. Expression of Matrix Metalloproteinases and TIMPs in Lymphoid Cells

	Gene or Protein Analysis	MMP-3 ELISA	MMP-2 NB	MMP-2 Z	MMP-9 NB	MMP-9 Z	MT-MMP RT-PCR	TIMP-1 NB	TIMP-1 RT	TIMP-1 RZ	TIMP-2 NB	TIMP-2 RT	TIMP-2 RZ	TIMP-3 RT	TIMP-3 RZ
	Cells														
B	SUDHL4	+	-	-	-	-/+	-	-	-	-	-	-	-	-	-
B	SUDHL6	-	-	-	-	-/+	-	-	-	-	-	-	-	-	-
B	RAJI	ND	-	-	-	+	-	+	ND	+	-	-	-	-	-
B	DeFoe	ND	-	-	-	+	-	+	ND	+	-	-	-	-	-
B	PA	-	-	-	-	+	-	+	ND	+	-	-	-	-	-
B	AG	-	-	-	-	+	-	+	ND	+	-	-	-	-	-
B/T	Tonsils	ND	-	-	-	-	-	+	ND	+	+	ND	ND	-	-
T	Molt4	-	-	-	-	-	-	-	-	-	+	+	+	-	-
T	Jurkat	-	-	-	-	-	-	-	-	-	+	+	+	-	-
T	Tsup	-	-	-	-	-	-	-	-	-	+	+	+	-	-
T	SUDHL1	ND	ND	-	ND	-	-	-	-	-	+	+	+	-	-
T	PBTc	-	ND	-	ND	+	-	+	+	+	+	+	+	-	-
T	PBTc*	+	ND	-	ND	+	-	+	+	+	+	+	+	-	-

NB: Northern blot analysis
RT: Reverse Transcriptase PCR
Z: Zymogram
RZ: Reverse Zymogram
PBTc*: Activated peripheral blood T cells
ND Not determined

3.3 Functional Significance of TIMP-2 in T Cell Biology

3.3.1 Regulation of T Cell Growth by TIMP-2

T cell homeostasis is maintained by a complex process of proliferation and apoptosis. In their response to cellular antigens, T cells respond in a cycle of birth and propagation followed by death. A number of different stimuli resulting in antigen receptor-mediated stimulation, cell contact-dependent signals, triggering of cytokine or steroid receptors result in secretion of growth modulatory cytokines. Mature T cells proliferate in response to T cell growth factors, such as IL-2 and IL-4, and undergo apoptosis upon T cell receptor (TCR) religation (269, 270). T cells are resistant to TCR-mediated death during the G1 phase of the cell cycle but become susceptible to TCR-induced apoptosis upon entry into S-phase (267).

The discordant expression of TIMPs and MMPs in lymphoid cells suggests that MMPs and TIMPs may have cell-specific physiologic roles. Furthermore, the restricted expression of TIMP-1 in B cells and TIMP-2 in T cells indicates that they may have lineage-specific functions. The growth-modulatory activities of TIMP-1 and TIMP-2 have not distinguished among different lymphoid cell lineages or states of differentiation. Growth modulatory roles of TIMPs have been reported for a variety of cell types. From these studies it is clear that the responses are cell-type specific as well as being concentration dependent (69, 113, 128, 129, 134, 147, 165, 179, 271). As illustrated in Section 3.2, Figure 12B, significant TIMP-2 protein induction was seen during peripheral blood T cell activation. The next set of experiments sought to determine the physiologic significance of TIMP-2 in T cell growth modulation.

3.3.2 Effect of rTIMP-2 on T Cell Viability

To determine the growth modulatory function of TIMP-2 in T cells, we evaluated the effect of TIMP-2 on T cell growth. Using Jurkat T lymphoblastic lymphoma cells we initially sought to determine whether rTIMP-2 had an effect on cell proliferation. Jurkat T cells were activated with PHA and IL-2, washed and resuspended in fresh media containing 40 nM rTIMP-2. After exposure for 48 hours, cell viability was determined by trypan blue dye exclusion. The percent of cells in S phase as determined by flow cytometric analysis of cell cycle parameters was not affected by rTIMP-2 (data not shown.). In contrast, the number of viable cells as measured by trypan blue dye exclusion was significantly decreased after rTIMP-2 exposure and many cells exhibited changes consistent with cell death.

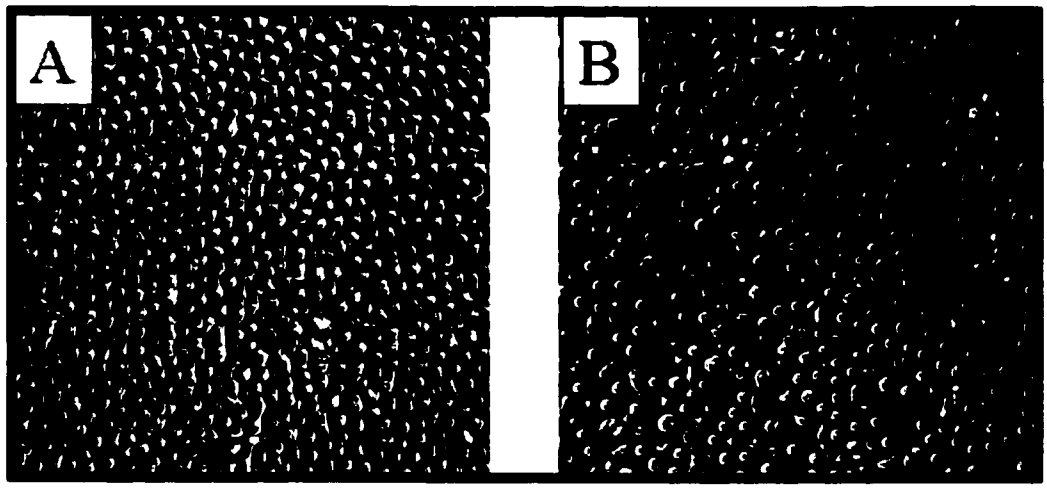
Inhibition of cell proliferation could be the result of the induction of apoptosis, cell cycle growth arrest and/or the inhibition of growth. Thus, in the next set of experiments, we examined the effect of TIMP-2 on peripheral blood T cell apoptosis using four independent methods. To test the effect of TIMP-2 on a physiologically relevant system, the subsequent experiments were performed using peripheral blood T cells. (Figure 12B Section 3.2)

1. Trypan blue dye exclusion
2. Cell morphology
3. Annexin V staining
4. DNA degradation

Figure 13 illustrates the morphology of activated peripheral blood T cells that were exposed to 40 nM rTIMP-2 for 48 hours (Figure 13B). Compared to control cells, the number of viable cells was markedly decreased and many of the remaining cells demonstrated changes characteristic of apoptosis. The cells were shrunken in size and blebbing of plasma membrane was frequently observed. Fragmentation of cellular components and nuclear chromatin was also evident.

Figure 13. Recombinant TIMP-2 induces apoptosis in activated human peripheral blood T cells.

Peripheral blood T cells were purified as outlined in Chapter 2. Peripheral blood T cells were cultured in RPMI growth media with 3% FCS and stimulated with PHA (1.5 ug/mL) and IL-2 (25 u/mL) for 24 hours. Cells were then washed twice with PBS and resuspended in fresh media at concentration of 2-4 X 10^6 cells/mL with and without varying concentrations of recombinant TIMP-2 protein (0-60 nM). Photomicrographs of peripheral blood T cells. Panel **A)** untreated T cells activated with PHA and IL-2; Panel **B)** PHA and IL-2 treated T cells exposed to rTIMP-2 (40 nM) for 48 hours. This experiment was performed at least 5 times with different peripheral blood T cell preparations from 5 different individuals.


A
B

3.3.3 Annexin V Staining Demonstrates Induction of Apoptosis by TIMP-2

Early stages of apoptosis are evident at the cell surface. One of the membrane alterations is the translocation of phosphatidylserine (PS) from the inner side of the plasma membrane to the outer layer, by which PS becomes exposed at the external surface of the cell. Annexin V is a Ca^{2+} dependent phospholipid-binding protein with high affinity for PS. Hence, this protein can be used as a sensitive probe for PS exposure upon the cell membrane. The measurement of Annexin V binding to the cell surface in conjunction with a dye exclusion test is a good measure of apoptosis (272, 273). Annexin V binding was assessed using bivariate flow cytometry: cell staining was evaluated with fluorescein isothiocyanate (FITC)-labelled Annexin V (green fluorescence), simultaneously with dye exclusion of propidium iodide (PI) (negative for red fluorescence). This test discriminates intact cells (FITC-/PI-), apoptotic cells (FITC+/PI-) and necrotic cells (FITC+/PI+).

Figure 14 shows a representative flow cytometric dot plot of peripheral blood T cells stained with Annexin V-FITC and propidium iodide with or without TIMP-2 exposure. Using this method, recombinant TIMP-2 exposure at 40 nM resulted in 35.7 ± 1.5 % T cell apoptosis compared to 15.5 ± 1.0% in control untreated cells. Unstimulated T cells (ie not treated with PHA and IL-2) demonstrated a basal level of apoptosis (6.0 ± 0.4%) with increasing concentrations of rTIMP-2; 6.2 ± 0.4 % at 5 nM, 6.7 ±1.1% at 20 nM and 7.7 ± 1.0% at 40 nM (data from Figure 16).

Several experiments were performed to determine the correlation between % of apoptotic cells by Annexin V-FITC staining and the % apoptotic cells determined by

trypan blue dye exclusion using the following calculation (total number of blue cells)/(total number of cells) X 100. Excellent correlation was observed from two experiments and in most of the subsequent experiments, apoptosis was determined using trypan blue dye exclusion.

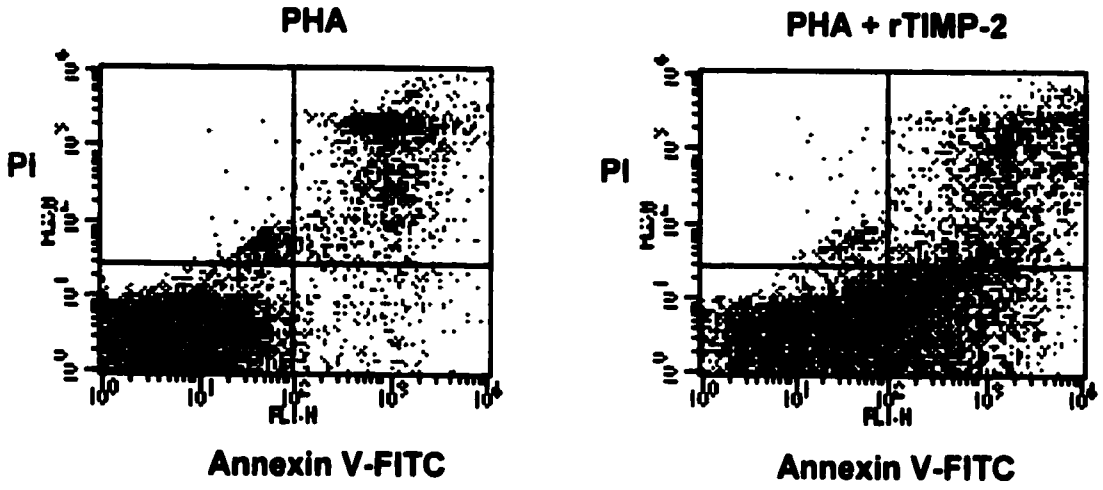


Figure 14. Annexin V-FITC staining of T cells treated with rTIMP-2.

Peripheral blood T cells were cultured in RPMI growth media with 3% FCS and stimulated with PHA (1.5 ug/mL) and IL-2 (50 u/mL) for 24 hours. Cells were then washed twice with PBS and resuspended in fresh media at concentration of 2-4 X 10^6 cells/mL and treated with recombinant TIMP-2 protein (40 nM) for 36 hours. Apoptosis was quantified by bivariate flow cytometry using Annexin V staining of externalized phosphatidylserines and simultaneous staining with propidium iodide. Apoptotic cells stain as Annexin V-FITC+/PI-, while viable cells stain FITC-/PI-. Necrotic cells are represented in FITC+/PI+ quadrant. The experiment was performed with 5 different samples of peripheral blood T cells from 5 different individuals with each reaction condition carried out in triplicate. The data from one representative experiment are shown.

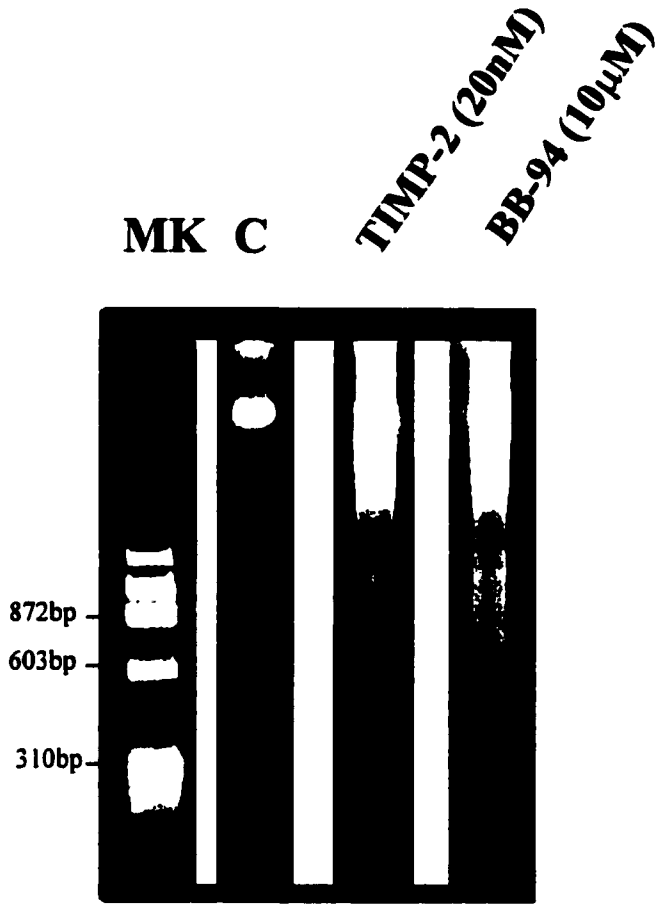
3.3.4 Induction of DNA Fragmentation by TIMP-2

Fragmentation of cellular DNA at the internucleosomal linker regions has been observed in cells undergoing apoptosis. This cleavage produces ladders of DNA fragments that are the sizes of integer multiples of a nucleosome length (180-200 bp) (56, 274). Because of their characteristic patterns revealed by agarose gel electrophoresis, these nucleosomal DNA ladders are widely used as biochemical markers of apoptosis.

Apoptosis was observed in cells treated with 20 nM rTIMP-2 and 10uM BB-94, a synthetic MMP inhibitor as illustrated by the DNA ladder shown in Figure 15. These data indicate that the metalloproteinase inhibitory properties of TIMP-2 is involved in the induction of apoptosis. Control cells that were activated with PHA and IL-2 demonstrated minimal amounts of DNA fragmentation with maintenance of high molecular weight DNA. Although the percent cells demonstrating apoptosis determined by Annexin V-FITC staining was greater in BB-94 treated cells than for rTIMP-2 this quantitative difference could not be demonstrated by DNA fragmentation analysis using agarose gel electrophoresis. This assay is not felt to be a quantitative assay of apoptosis as DNA degradation is a terminal event in the cell death process.

Figure 15. Recombinant TIMP-2 induces DNA fragmentation in peripheral blood T cells.

Peripheral blood T cells were purified as outlined in Chapter 2. Peripheral blood T cells were cultured in RPMI growth media with 3% FCS and stimulated with PHA (1.5 ug/mL) and IL-2 (50 u/mL) for 24 hours. Cells were then washed twice with PBS and resuspended in fresh media at concentration of 2-4 X 10^6 cells/mL with recombinant TIMP-2 protein (20 nM). 1 X 10^6 cells were harvested after 48 hours and used for DNA extraction following the protocol outlined in Chapter 2. Ten ug of DNA was subject to electrophoresis on 1.8 % agarose gel at 60 V for 2 hours. The gel was stained with ethidium bromide before photography. Untreated controls (Lane 1) do not show DNA ladder formation typical of cells in apoptosis, while rTIMP-2 and BB-94 (synthetic metalloproteinase inhibitor) treated T cells (Lane 2 and 3, respectively) demonstrate DNA fragmentation. Lane 1: mwt marker is ϕX174RF DNA/Hae III fragment. The experiment was performed twice with two different peripheral blood T cell preparations from 2 different individuals. A representative experiment is shown.


MK
C
TIMP-2 (20nM)
BB-94 (10μM)
872bp
603bp
310bp

3.4 Dose Dependent Effect of Recombinant TIMP-2

Dose-response analysis demonstrated that there was 26.1 ± 0.4% T-cell apoptosis at 5 nM rTIMP-2 ($P < 0.005$ vs control cells), 35.7 ± 1.5% at 20 nM ($P < 0.005$ vs control cells), and 38 ± 0.5% at 40 nM ($P < 0.005$ vs controls cells) (Figure 16) and that the effect appears to be reaching a plateau by 40 nM TIMP-2. Unstimulated T cells exhibited similar low levels of apoptosis (5.0 ± 5%; Figure 16) for the TIMP-2 treated and untreated cells. These data suggest that the TIMP-2 protein may be saturating a protein or receptor molecule involved in inducing apoptosis. Receptors for TIMPs have not been identified to date although cell surface binding of TIMP-2 and pro-MMP-2/TIMP-2 complexes has been demonstrated in certain cell lines (177). Alternatively, only a subset of T lymphocytes may respond by undergoing apoptosis. Indeed, activation of T cells elicits the expression of apoptosis inducing molecules such as Fas and Fas ligand in only a subset of T cells (267).

3.5 Time Course of TIMP-2 Effect

The effect of exogenous TIMP-2 on T cell apoptosis was not an immediate event as illustrated in Figure 17. A time course analysis of the effect of rTIMP-2 showed that induction of apoptosis was observed initially at 24 hours (25-30%) and peaked at 36-48 hours (35-45%). After 52 hours all cells including cells under control conditions exhibited equal level of apoptosis (>45%). This suggests that the effect of rTIMP-2 on T cell apoptosis is transient and indicates the presence of T cells that may be transiently susceptible to apoptosis. Furthermore, at later time points apoptosis may

be attributed to the depletion of nutrients in the conditioned media. Specific differentiation or activation-associated antigens or intracellular signaling molecules which are expressed during this phase of T-cell activation may contribute to this process. Activated T cells in S phase have decreased levels of FLIP (FLICE-like inhibitory protein), an inhibitor of the Fas signaling pathway while those in G1 phase contain high levels of FLIP protein (267). These finding suggest that activated T cells in S phase of the cell cycle are more susceptible to Fas-mediated apoptosis. Alternatively, cell-cell interactions which is a function of cell concentration may also be a factor.

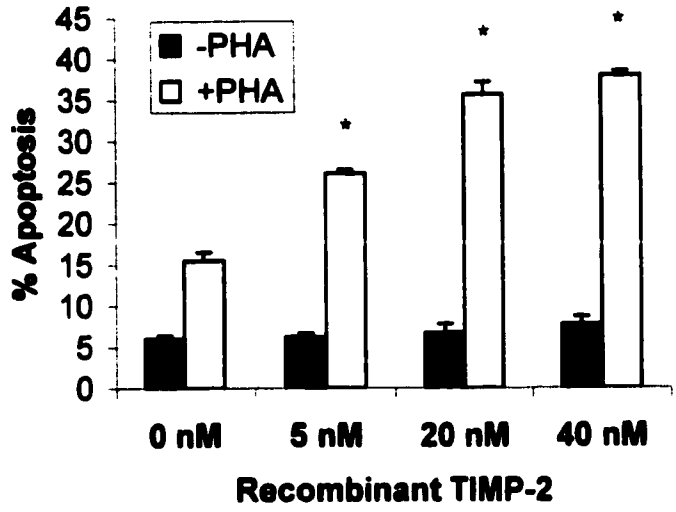


Figure 16. Effect of recombinant TIMP-2 is dose-dependent up to 40nM.

Peripheral blood T cells were cultured in RPMI growth media with 3% FCS and stimulated with PHA (1.5 ug/mL) and IL-2 (50 u/mL) for 24 hours. Cells were then washed twice with PBS and resuspended in fresh media at concentration of 2-4 X 10^6 cells/mL with increasing concentrations of recombinant TIMP-2 protein. At 36 hours, the percentage of apoptotic cells was determined by trypan blue dye exclusion. The experiment was performed 3 times with 3 different T cell preparations. Each reaction was set up in triplicate. Values are expressed as mean of triplicate. Error bars represent standard errors of mean. The data are from a representative experiment. *Indicates statistical significance ($P < 0.005$) vs untreated control.

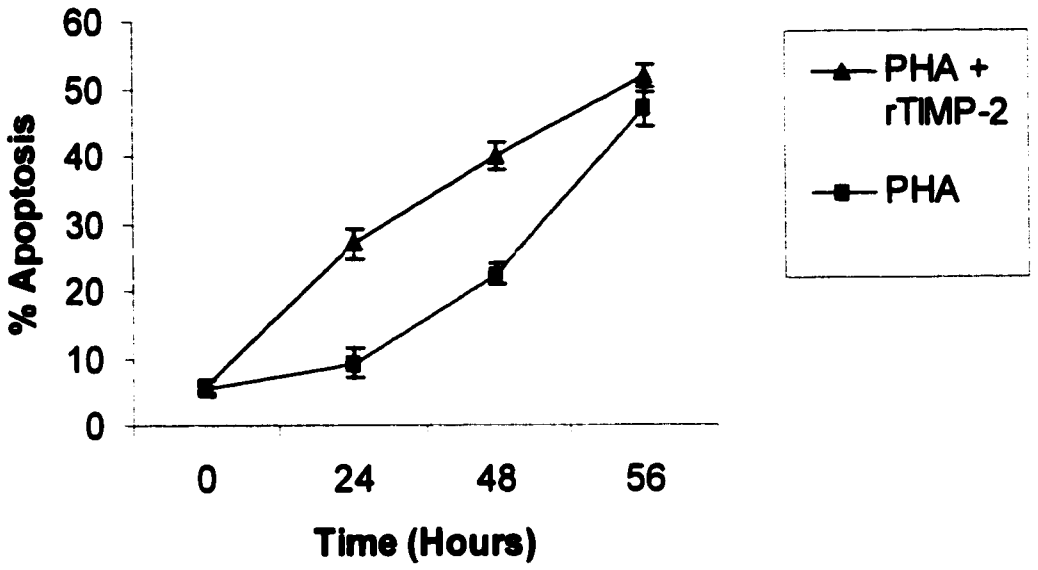


Figure 17. Time course analysis of T cell apoptosis after rTIMP-2 treatment. Peripheral blood T cells were cultured in RPMI growth media with 3% FCS and stimulated with PHA (1.5 ug/mL) and IL-2 (50 u/mL) for 24 hours. Cells were then washed twice with PBS and resuspended in fresh media at concentration of 2-4 X 10^6 cells/mL with recombinant TIMP-2 protein (40 nM). At various time points, apoptosis was quantitifed by Annexin V staining of externalized phosphatidylserines by flow cytometry. The experiment was performed 2 times with 2 different T cell preparations. Each reaction was set up in triplicate. The data from a representative experiment is shown. Values are expressed as mean of triplicates. Error bars represent standard errors of mean.

3.6 T Cell Apoptosis is not Induced by Recombinant TIMP-1

Four members of the TIMP family have been cloned and characterized (Table 3). They share similar structure-function properties and are approximately 40% identical at the amino acid level. The expression patterns and inhibitory specificities of the TIMPs differ, however suggesting that although all proteins inhibit the MMPs, they have some functional differences that may be useful in characterizing the cell type specificity of their physiologic role. TIMP-1 does not block MT-MMPs 1-3 (MMP14-16) as does TIMP-2,-3 or-4 (275, 276). To determine if the effect on apoptosis was mediated by the metalloproteinase inhibitory activity of TIMPs peripheral blood T cells were exposed to rTIMP-1. As shown in Figure 18, TIMP-1 had no effect T cell apoptosis at concentrations that demonstrated maximum effect with TIMP-2 (40 nM). This was demonstrated with two different lots of TIMP-1, one from a commercial source and another from the laboratory of Dr. W. G. Stetler-Stevenson. Recombinant TIMP-1 and TIMP-2 preparations were shown to be biologically active MMPIs in a fluorescent MMP-2 enzyme assay (Dr. Anita Yu/Dr. W.G. Stetler-Stevenson). The lack of effect of TIMP-1 in inducing T cell apoptosis may be due to TIMP-1's inability to inhibit the relevant enzyme(s) involved, alternatively TIMP-1 may be functionally less specific than TIMP-2 in this setting.

Preliminary data suggested moreover that TIMP-1 treated T cells showed increaed cell viability when compared to control cells. Although this was not further pursued it is an enticing observation as TIMP-1 has been shown to have a suppressive

effect on Burkitt lymphoma cell apoptosis (138) and may be involved in the stimulation of proliferation of normal and malignant B cells.

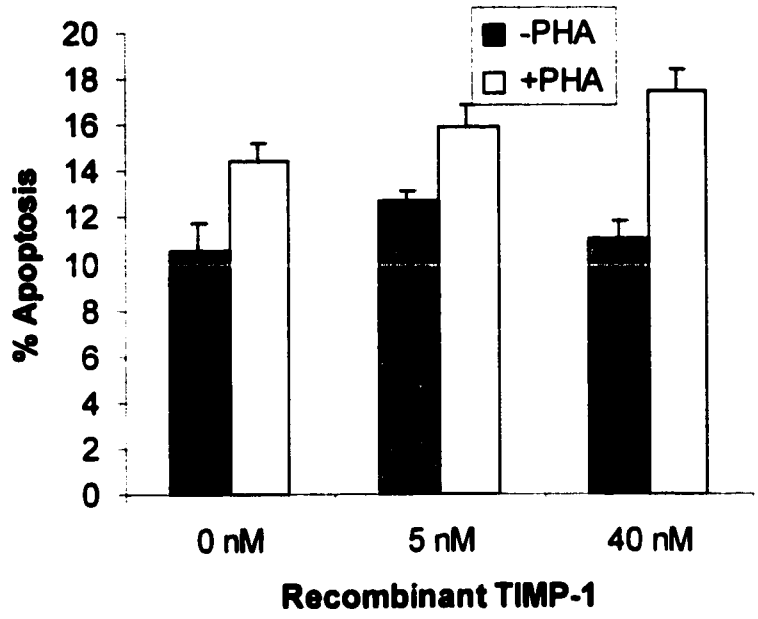


Figure 18. Recombinant TIMP-1 has no effect on T cell activation-induced apoptosis.

Peripheral blood T cells were cultured in RPMI growth media with 3% FCS and stimulated with PHA (1.5 ug/mL) and IL-2 (50 u/mL) for 24 hours. Cells were then washed twice with PBS and resuspended in fresh media at concentration of 2-4 X 10^6 cells/mL with recombinant TIMP-2 protein (40 nM) or recombinant TIMP-1 (0-40 nM). At various time points, cells were analyzed for morphologic changes by transmission microscopy. Apoptosis was determined by trypan blue dye exclusion. The % apoptosis at different rTIMP-1 concentrations were not statistically significant. The experiment was performed twice with two different peripheral blood T cell preparations. Each reaction condition was set up in duplicate. Data from a representative experiment are shown.

3.7 Abrogation of TIMP-2 Mediated Apoptosis by A Neutralizing TIMP-2 Antibody

The next experiment was designed to investigate whether antibody neutralization could abrogate the apoptosis enhancing effect of endogenous or basal TIMP-2 expressed during the process of T cell activation. Neutralization of endogenous secreted TIMP-2 with polyclonal anti-TIMP-2 antibody during activation of peripheral blood T cells decreased the numbers of apoptotic cells (38.6 ± 1.4%), a 35% reduction compared to cells incubated with isotype control (57.5 ± 2.0%) or anti-TIMP-1 antibody (59.6 ± 3.5%) ($P < 0.005$ vs isotype IgG treated cells) (Figure 19). Cells treated with antibody displayed a general increase in apoptosis compared to untreated T cells. The significance of this observation is uncertain, but it suggests that the immunoglobulin component of the antibody may be altering the kinetics of T cell activation. Transmission microscopic analysis of cells demonstrated that the decrease of apoptotic cells was associated with increased numbers of cells showing blast morphology.

Cells incubated with anti-TIMP-1 antibody showed a similar percentage of apoptotic cells at 48 hours compared to mouse isotype IgG treated cells. It is acknowledged that the use of a rabbit rather than a mouse isotype IgG antibody would have been more appropriate in this experiment. Anti-TIMP-1 antibody treatment did not inhibit endogenous TIMP-2 mediated apoptosis as was seen with anti-TIMP-2 treated cells. These data suggest that TIMP-1 may have a different function in T cell homeostasis. This is consistent with previous observations tht rTIMP-1 was not able to

induce T cell apoptosis (Section 3.6 Figure 18). TIMP-1 suppressed apoptosis induced by a variety of stimuli including cold shock and Fas activation in B cells (138) while BB-94 and TIMP-2 did not. These observations highlight the cell type specificity of TIMP function in lymphocyte homeostasis. Whether these activities are mediated by a metalloproteinase inhibitor function or by an as yet undescribed function is unclear from these experiments. In order to explore the requirement for MP inhibition in TIMP-2 mediated apoptosis, in subsequent experiments the effect of synthetic metalloproteinase inhibitors such as BB-94 and GM-6001 and inactivated forms of rTIMP-2 was studied (Section 3.8, 3.9 and 3.10).

Neither anti-TIMP-2 nor anti-TIMP-1 antibody had an effect on unactivated T cells (data not shown) suggesting that molecules that are upregulated during activation are required for TIMP-2 mediated apoptosis. The role of cell receptors for TIMP-1 and TIMP-2 may help elucidate the components of this complex process.

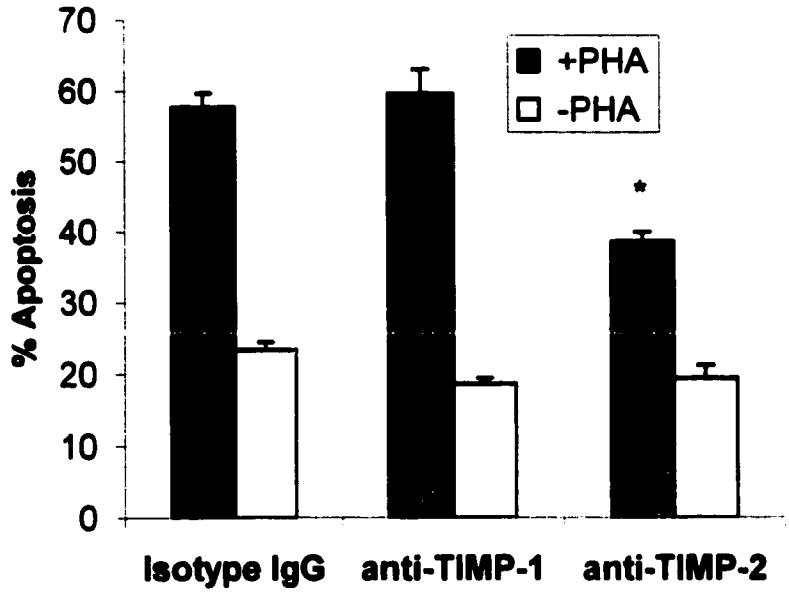


Figure 19. Anti-TIMP-2 antibody abrogates TIMP-2 induction of apoptosis.

Peripheral blood T cells were cultured in RPMI growth media with 3% FCS and stimulated with PHA (1.5 ug/mL) and IL-2 (50 u/mL) at concentration of 2-4 X 10^6 cells/mL for 48 hours with and without affinity purified polyclonal rabbit anti-TIMP-2 antibody (isotype IgG1) at 1-2 ug/mL, polyclonal rabbit anti-TIMP-1 antibody (isotype IgG1) at 1-2 ug/mL. A mouse isotype control antibody was used as negative control. At various time points, cells were analyzed for morphologic changes by transmission microscopy. Apoptosis was determined by trypan blue dye exclusion. The experiment was performed twice with two different peripheral blood T cell preparations. Each reaction condition was set up in duplicate. Representative data obtained at 36 hours are shown. * Indicates statistical significance ($P < 0.005$) vs isotype IgG control cells.

3.8 Decreased Apoptosis with Reductive Alkylation of TIMP-2

Chemical reduction and alkylation completely abrogates TIMP's ability to inhibit MMP activity (134). To determine whether the metalloproteinase-inhibitory activity is involved in the pro-apoptotic effect of rTIMP-2 in T cells, we tested the effect of reduced and alkylated TIMP-2 (obtained from Drs. Anita Yu) on T cell viability and apoptosis. Compared to control peripheral blood T cells treated with functional rTIMP-2 those treated with reduced and alkylated rTIMP-2 (40 nM) demonstrated reduced ability to induce apoptosis (37.3 ± 2.5%) vs (11.7 ± 1.5%) ($P < 0.005$ vs wild type TIMP-2 treated cells) representing a 69% reduction (Figure 20). These results support the hypothesis that the metalloproteinase inhibitory activity of TIMP-2 is required for its effect on T cell apoptosis. Together with data from Section 3.6 this experiment suggests that the metalloproteinase inhibitory (MPI) function is required only in the setting of TIMP-2 in inducing T cell apoptosis. The non-MPI domain of TIMP-1 may define the specificity of this process, alternatively, the putative receptor for TIMP-1 in B cells may not be present or functional in this setting.

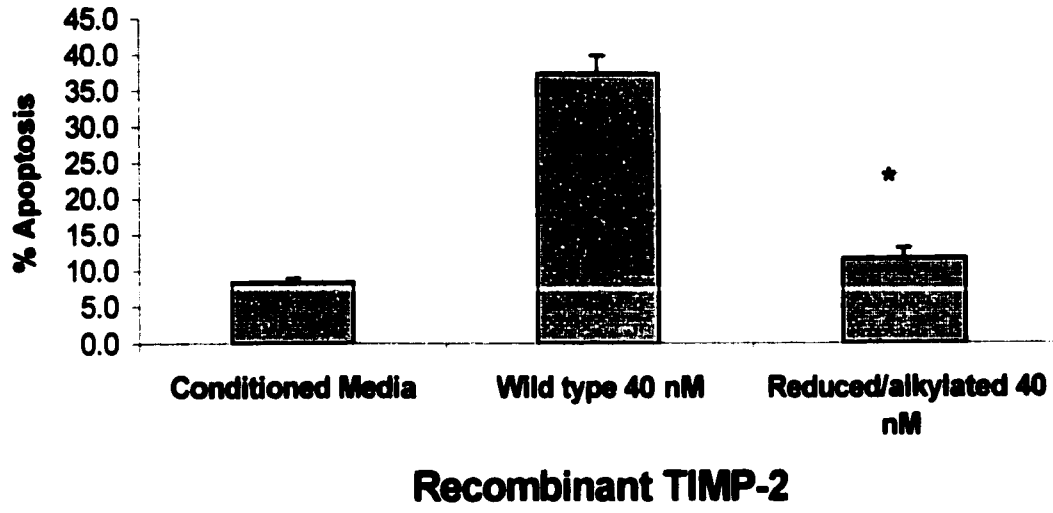


Figure 20. Reductive alkylation of TIMP-2 reduced its ability to induce apoptosis. Peripheral blood T cells were cultured in RPMI growth media with 3% FCS and stimulated with PHA (1.5 ug/mL) and IL-2 (50 u/mL) for 24 hours. Cells were then washed twice with PBS and resuspended in fresh media at concentration of 2-4 X 10^6 cells/mL with recombinant TIMP-2 protein (40 nM) or varying concentrations of reduced and alkylated rTIMP-2. At 36 hours, apoptosis was determined by trypan blue dye exclusion. The experiment was performed 2 times with 2 different T cell preparations. Each reaction was set up in triplicate. The data from a representative experiment is shown. Values are expressed as mean of triplicates. Error bars represent standard errors of mean. * Indicates statistical significance ($P < 0.005$) vs wild type TIMP-2 treated cells.

3.9 Recombinant TIMP-2 Induction of Apoptosis in Neoplastic T cells

To determine whether the effect of rTIMP-2 was specific for reactive T cells, a panel of T cell lymphoma cell lines were exposed to varying concentrations of exogenous TIMP-2 for varying time periods. Cell numbers were determined by the trypan blue dye exclusion method. Two different high-grade lymphoblastic lymphoma cell lines Jurkat and Tsup were susceptible to apoptosis by rTIMP-2. Figure 21 shows data for Jurkat T cells. At 20 nM rTIMP-2, 37.2 ± 1.6% of cells demonstrated apoptosis compared to 10.1 ± 0.3% for 0 nM rTIMP-2. Similar to what was observed with reactive peripheral blood T cells, the effect was seen with preactivated cells and not with unstimulated cells (10.1 ± 0.3% apoptosis at 0 nM; 12.7 ± 0.5% at 5 nM, and 13.1 ± 1.1% at 20 nM). Activation of Jurkat T cells with polyclonal activators such as PHA and IL-2 result in coexpression of Fas and FasL on the cell surface (237). Although B lymphoma cell lines were not tested in this set of experiments, Guedez et al., (138) have reported that rTIMP-2 induced apoptosis in Burkitt (B-cell) lymphoma cell lines. Whether TIMP-2 has a proapoptotic effect on nonlymphoid cancer cells is debatable at this time. *In vitro* data suggest that TIMP-2 inhibits growth of basic-FGF stimulated endothelial cells (142) however whether TIMP-2 has a direct role in inducing apoptosis has not been demonstrated. The growth modulatory function of TIMP-2 may be cell-type specific as overexpression of the protein has been shown to inhibit apoptosis while increasing necrosis in melanoma cells (139).

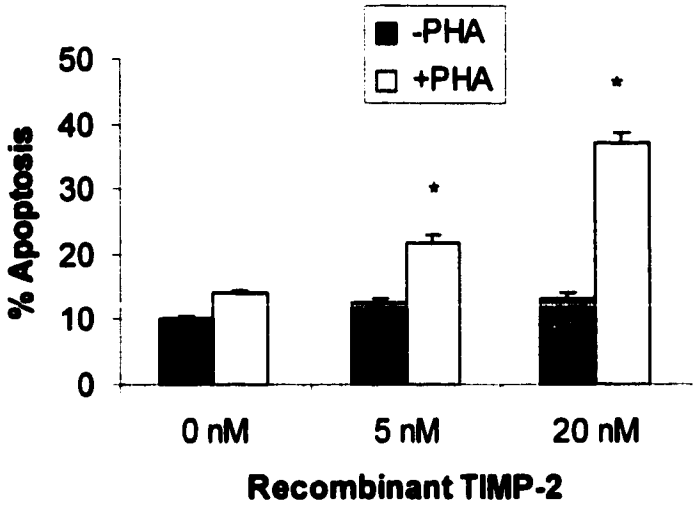


Figure 21. Recombinant TIMP-2 induces apoptosis in human T lymphoblastic lymphoma cells.

Jurkat T lymphoblastic lymphoma cells were cultured in RPMI growth media with 10% FCS and stimulated with PHA (1.5 ug/mL) and IL-2 (50 u/mL) for 24 hours. Cells were then washed twice with PBS and resuspended in fresh media at concentration of 2-4 X 10^6 cells/mL with varying concentrations of recombinant TIMP-2 protein. Apoptosis was determined by trypan blue dye exclusion at 36 hours. The experiment was performed 3 times with each reaction condition performed in triplicate. Data from a representative experiment are shown. Error bars represent standard deviation of mean of triplicate. *Indicates statistical significance ($P < 0.05$) vs control cells.

3.10 Effect of Synthetic Metalloproteinase Inhibitors on T Cell Apoptosis

To further determine whether the MPI activity of TIMP-2 is important for induction of T cell apoptosis, a panel of synthetic MPIs were used. BB-94 (a hydroxamate-based metalloproteinase inhibitor), GM-6001 and KB-8301 are low molecular-weight broad-spectrum synthetic MPIs shown to inhibit not only tumor spread and metastasis (96, 97) but also tumor growth of human ovarian cancer xenografts (169). Their direct effects on cell apoptosis have not been addressed, although the biologic effect of BB-94 on lymphoid growth and function have been implicated in studies where BB-94 was shown to have an immunosuppressive effect in animal models of arthritis (176). Both BB-94 and GM-6001 resulted in induction of apoptosis of preactivated peripheral blood T cells as well as T lymphoblastic lymphoma cell lines, Jurkat and Tsup (Figure 22 shows data for peripheral blood T cells). BB-94 caused a concentration-dependent induction of T cell apoptosis (data not shown). Under these conditions, induction of apoptosis was seen at 5 uM with maximal effect achieved with 10 uM BB-94 (35.9 ± 1.3% apoptosis vs 15.3 ± 0.9% for DMSO treated solvent control cells) ($P < 0.005$ vs DMSO control cells). Further increases in the concentration of BB-94 up to a maximum of 100 uM had no additional effect. GM-6001 demonstrated less potent effect on apoptosis with 24.5 ± 1.7% cells demonstrating apoptosis at 10 uM concentration. ($P < 0.005$ vs DMSO control cells).

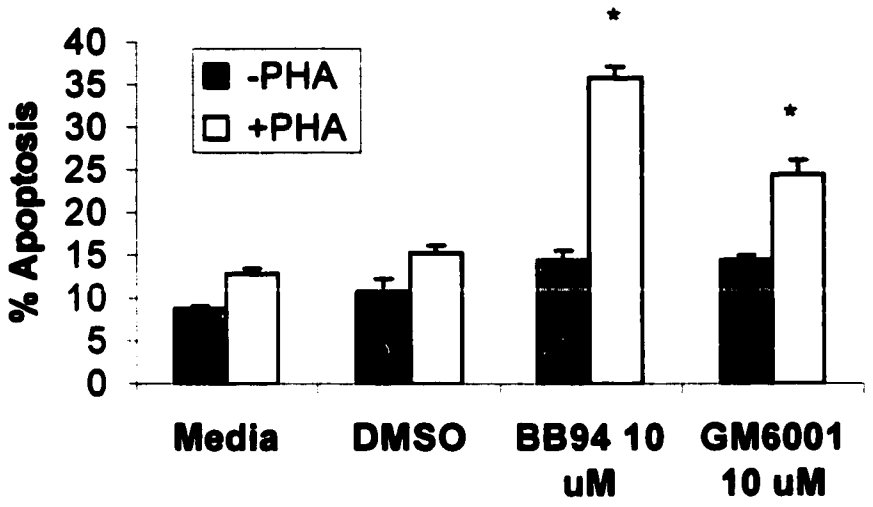


Figure 22. Synthetic metalloproteinase inhibitors BB-94 and GM-6001 induce apoptosis in activated T cells.

Peripheral blood T cells and T lymphoblastic cell lines Jurkat and Tsup were cultured in RPMI growth media with 3% FCS and stimulated with PHA (1.5 ug/mL) and IL-2 (50 u/mL) for 24 hours. Cells were then washed twice with PBS and resuspended in fresh media at concentration of 2-4 X 10^6 cells/mL with varying concentrations of BB-94 (10 uM) and GM-6001 (10 uM) At 36 hours, percent apoptosis was determined by trypan blue dye exclusion. The experiment was performed 4 times with 4 different T cell preparations. Each reaction was set up in triplicate. The data from a representative experiment is shown. Values are expressed as mean of triplicates. Error bars represent standard errors of mean. * Indicates statistical significance ($P < 0.005$ vs DMSO control cells).

3.11 Mechanism of TIMP-2 and Synthetic Metalloproteinase Inhibitor Mediated Induction of T Cell Apoptosis

The results obtained thus far suggest that rTIMP-2 and synthetic MPIs induce apoptosis in human T lymphocytes. These results, however, do not provide any evidence with respect to the mechanism through which they induce apoptosis. Data using neutralizing TIMP-2 antibody, reduced and alkylated TIMP-2 and N-terminal domain mutant peptide suggest that the metalloproteinase inhibitory activity of TIMP-2 is important in the process. **To elucidate the potential mechanisms involved, we investigated the hypothesis that cleavage of a cell surface molecule involved in T cell apoptosis may be modulated by TIMP-2 and BB-94.** Metalloproteinase inhibitors have been implicated in the processing and cleavage of several cell surface receptors and molecules. Metalloproteinase inhibitor KB-8301 is a hydroxamic acid inhibitor of the MMPs that has been shown to block the release of Fas ligand (154). Fas ligand is initially expressed as 40 kDa membrane bound protein (mFas L) on activated T cells and proteolytically processed to a 26 kDa soluble form (sFas L). TNF-α, TNF-receptor, IL-1β, L-selectin have also been shown to be targets of metalloproteinases (153-155, 157, 233, 238, 253, 254). See Section 1. 11.

TNF-α and Fas ligand are important mediators of T cell apoptosis (215, 219, 222, 269, 277). TNF-α and Fas ligand impart differential susceptibility to activation-induced apoptosis. TNF-α is more important for T cells in lymph nodes and spleen whereas Fas ligand is important in peripheral blood T cells (278). We therefore

hypothesized that TIMP-2 and synthetic MPIs inhibit the cleavage of Fas ligand and thereby enhance T cell apoptosis.

Firstly, to determine the effect of TIMP-2 and synthetic MPI on cell surface Fas ligand expression, activated T cells in the presence or absence of TIMP-2 and synthetic MPI were analyzed by flow cytometry. Secondly, levels of sFas ligand in conditioned media of activated T cells treated with TIMP-2 or synthetic MPIs were measured by western blot analysis and ELISA.

3.11.1 Effect of TIMP-2 and Synthetic MPI on Cell Surface Fas Ligand Expression

As shown in Figure 23, treatment of peripheral blood T cells with 10 uM BB-94 and 10 uM KB-8301 resulted in a significant increase in the percentage of activated T cells with cell surface expression of Fas ligand (44.3 ± 4.7 % ($P<0.05$), 32.7 ± 3.7%, ($P < 0.05$), respectively) compared to PHA and IL-2 activated cells (7.8 ± 0.8%). No significant increase in cell surface Fas ligand expression was seen with 40 nM rTIMP-2 (14.2 ± 2.3%) ($P >0.05$). Importantly, the concentrations of BB-94 (10 uM) and KB-8301 (20 uM) required to demonstrate increased cell surface Fas ligand expression were significantly higher than that used for rTIMP-2. This was also true for the apoptosis experiments (Figure 22). Notably, these are the concentrations required to demonstrate increased cell surface Fas ligand expression in previous reports suggesting metalloproteinase-mediated Fas ligand cleavage (155). As the data from dose response analysis demonstrated, TIMP-2 at these concentrations had saturated the effect on T

cell apoptosis. Thus it can be interpreted that BB-94 acts to inhibit a broad spectrum of metalloproteinases other than that which is inhibitable by TIMP-2. BB-94 and KB-8301 are MPIs with broad substrate specificities. It is possible that they inhibit a number of different metalloproteinases that may be responsible for the cleavage of Fas ligand. Additionally, as the concentrations of TIMP-2 used in this preliminary experiment were significantly lower than those used for synthetic MPIs and those used in the literature (1.5 uM for TIMP-3 inhibition of sIL-6R release from myeloma cells, (279), optimum concentrations may not have been used. Another explanation for these observations is that the enzyme responsible for Fas ligand cleavage may be a non-matrix-type metalloproteinase. Furthermore, TIMP-2 may be inhibiting the shedding of other molecules such as TNFR expressed on a subset of T cells thereby inducing apoptosis in this population.

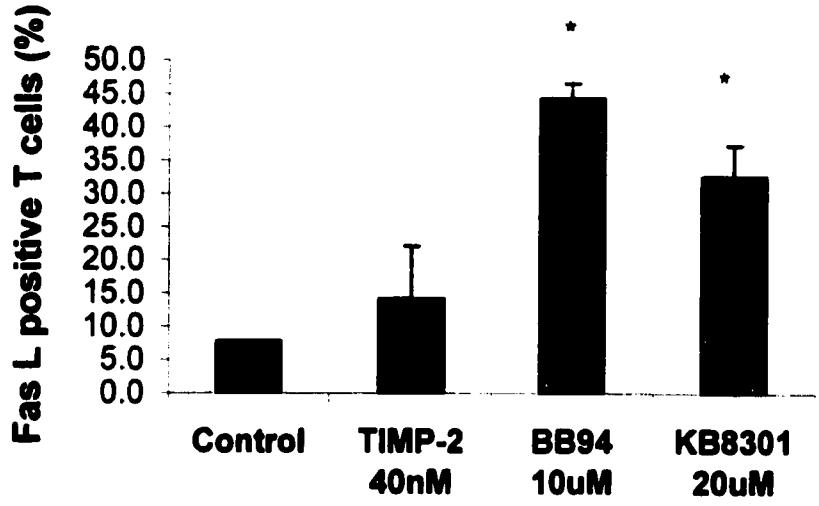


Figure 23. Flow cytometric analysis of cell surface Fas ligand expression in activated T cells exposed to TIMP-2 and synthetic MPI.

Peripheral blood T cells were cultured in RPMI growth media with 3% FCS and stimulated with PHA (1.5 ug/mL) and IL-2 (50 u/mL) for 24 hours. Cells were then washed twice with PBS and resuspended in fresh media at concentration of 2-4 X 10^6 cells/mL with recombinant TIMP-2 protein (40nM), BB-94 (10uM), or KB-8301 (20 uM). Cells were harvested and stained for cell surface Fas ligand. The experiment was performed 2 times with 2 different T cell preparations. Each reaction was set up in duplicate. The data from a representative experiment is shown. * Indicates statistical significance ($P < 0.05$) vs control cells.

3.11.2 Effect of TIMP-2 and Synthetic MPI on Level of Soluble Fas Ligand

KB-8301 when added to cell culture systems increased the levels of cell surface Fas ligand by blocking Fas ligand cleavage (154, 280) with concomitant decrease in soluble Fas ligand in the conditioned media.

We investigated the presence of soluble Fas ligand in the conditioned media of cells treated with or without rTIMP-2 and BB-94 by immunoblotting as well as by ELISA. Western blot analysis using an antibody against sFas ligand (monoclonal, clone 33, isotype IgG1, 37 kDa; Transduction Laboratories) identified a 37-40 kDa band corresponding to the sFas ligand in the conditioned media (Figure 24A). Densitometric analysis showed no differences in the amount of sFas ligand detectable in the conditioned media of treated and untreated T cells.

Analysis of conditioned media by a commercially available ELISA kit (Medical and Biological Laboratories Co., LTD, Nagoya, Japan) however, showed decreased amount of sFas ligand in conditioned media of peripheral blood T cells treated with rTIMP-2 and BB-94 (Figure 24B). After stimulation of T cells with PHA and IL-2 there was a higher level of sFas ligand in the conditioned media from 0.23± 0.02 ng/mL for unstimulated cells than in stimulated cells (0.78 ± 0.03 ng/mL). BB-94 treatment resulted in a significant decrease in the level of soluble Fas ligand (0.25 ± 0.04 ng/mL, a 68% reduction compared to control stimulated cells). Recombinant TIMP-2 treatment had a less significant effect with an 18% reduction in sFas ligand levels (0.64 ± 0.04 ng/mL) compared to control stimulated cells. There was no effect of rTIMP-2 or BB-94 in unstimulated T cells (data not shown) further supporting the hypothesis that T

cell activation-induced Fas ligand shedding is modulated by rTIMP-2 and BB-94.

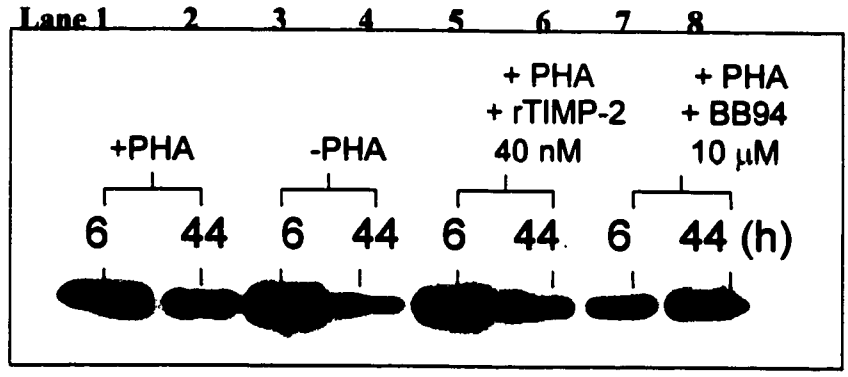


Figure 24A. Western blot analysis of soluble Fas Ligand in the conditioned media of reactive T cells treated TIMP-2 and BB-94.

Peripheral blood T cells were cultured in RPMI growth media with 3% FCS and stimulated with PHA (1.5 ug/mL) and IL-2 (50 u/mL) at concentration of 2-4 X 10^6 cells/mL with recombinant TIMP-2 protein (40nM) or BB-94 (10uM). At 6 and 44 hours, conditioned media was collected and 10ug equivalent was analyzed for soluble Fas ligand by western blot technique and ELISA. Lane 1. Activated PBT cells at 6 hours; Lane 2, Activated PBT cells at 44 hours; Lane 3, Unstimulated T cells at 6 hours; Lane 4, Unstimulated PBT cells at 44 hours; Lane 5, Activated PBT cells treated with TIMP-2 at 6 hours; Lane 6, Activated PBT cells treated with TIMP-2 at 44 hours; Lane 7, Activated PBT cells treated with BB-94 at 6 hours; Lane 8, Activated PBT cells treated with BB-94 at 44 hours. The experiment was performed 2 times with 2 different T cell preparations. The data from a representative experiment is shown.

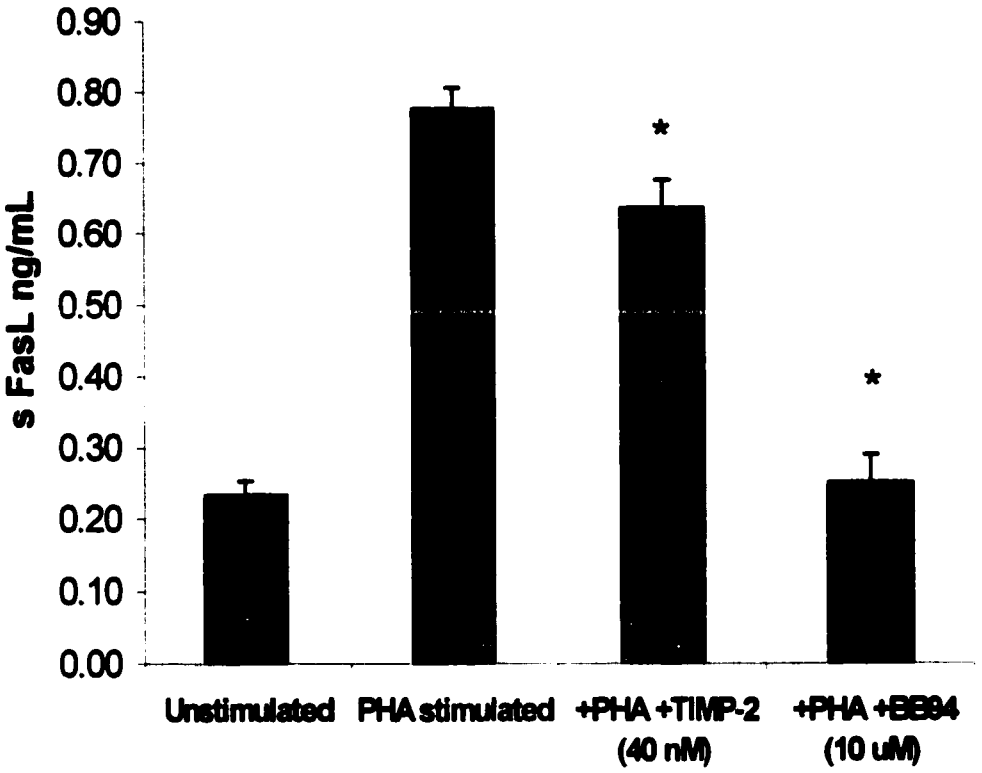


Figure 24B. ELISA of soluble Fas Ligand in conditioned media of reactive T cells treated with rTIMP-2 and BB-94.

Concentration of soluble Fas ligand in ng/mL was determined by ELISA purchased from Medical and Biological Laboratories Co., LTD, Nagoya, Japan. The experiment was performed 2 times with 2 different peripheral blood T cell preparations. Each reaction was set up in triplicate. Values are expressed as mean of triplicates. Error bars represent standard errors of mean. * Indicates statistical significance ($P < 0.05$) vs PHA stimulate control cells.

3.11.3 Effect of Neutralizing Fas Antibody (clone ZB4) on Soluble Fas Ligand Expression and T Cell Apoptosis

To further determine whether the Fas/FasL mediated apoptosis pathway is involved in the MPI mediated apoptosis, T cells were treated with rTIMP-2 and BB-94 in the presence of the anti-Fas antibody ZB4. This antibody binds to cell surface Fas receptor and inhibits apoptosis. Its binding would also prevent sFas ligand from binding to the Fas receptor on the cell surface and allow its accumulation in the conditioned media. As shown in Figures 25 and 26, anti-Fas ZB4 antibody abrogated apoptosis induced by rTIMP-2 (7.3 % +/- 0.6 % apoptosis vs 32.0% +/- 3.6%) demonstrating the neutralizing ability of the antibody. These observations demonstrate that Fas/FasL pathway is involved in the T cell apoptosis induced by rTIMP-2. Interestingly, in contrast to its effect on TIMP-2 mediated apoptosis, ZB4 did not abrogate BB-94-mediated apoptosis (26.7 ± 2.9% vs 33.3 ± 1.5%). One interpretation of these findins are that BB-94 may be inhibiting the cleavage of molecules involved in T cell apoptosis other than Fas ligand whereas TIMP-2 mediated apoptosis may be more dependent on the Fas/FasL pathway.

The abrogation of TIMP-2 mediated T cell apoptosis by ZB4 occurred concomitantly with increased levels of sFas ligand in the conditioned media as measured by ELISA. Conditioned media of T cells incubated with rTIMP-2 without ZB4 had 0.8 ± 0.1 ng/mL of sFas ligand, whereas conditioned media of cells incubated with rTIMP-2 and ZB4 had 4.7 fold higher levels of sFas ligand (3.8 ± 0.2 ng/mL)($P < 0.005$ vs PHA stimulate and TIMP-2 treated cells) (Figure 27). Similarly, cells

incubated with BB-94 in the presence of ZB4 contained a higher amount of sFas ligand in the conditioned media (1.3 ± 0.1 ng/mL) ($P<0.05$) than those incubated with BB-94 alone (0.4 ± 0.1 ng/mL). There are several explanations for these results. ZB4 antibody may be blocking the Fas-Fas ligand interaction such that sFas ligand will accumulate in the conditioned media. Alternatively, ZB4 may be stabilizing the sFas ligand molecule thereby allowing its detection in the conditioned media.

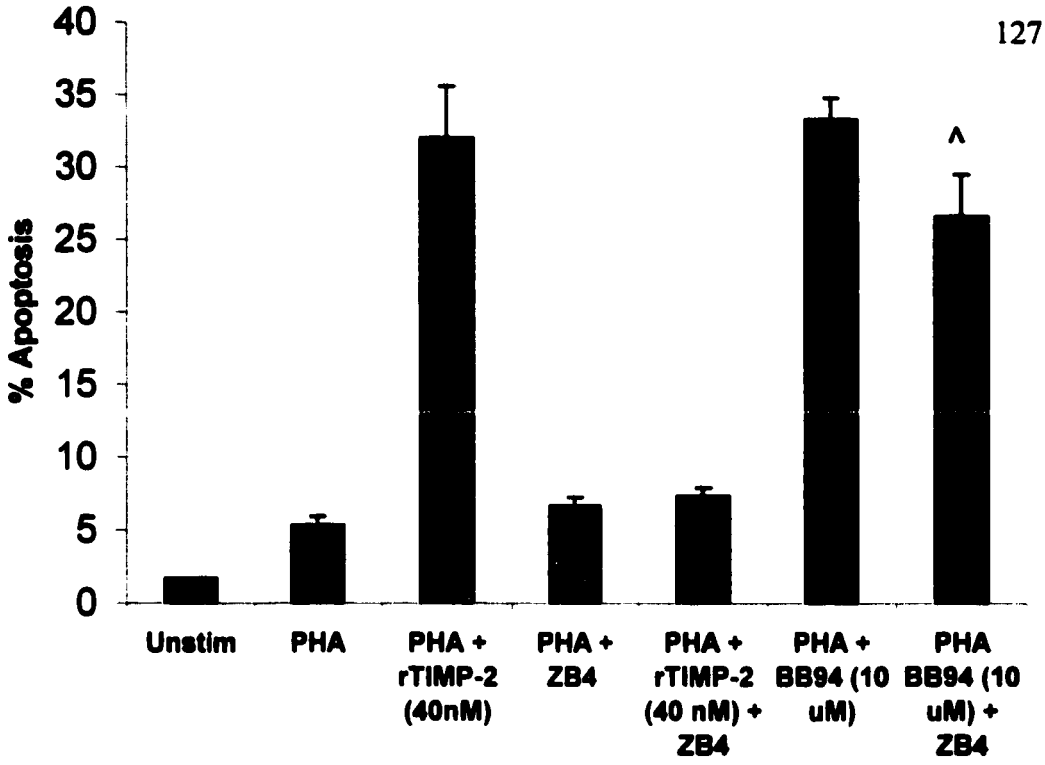
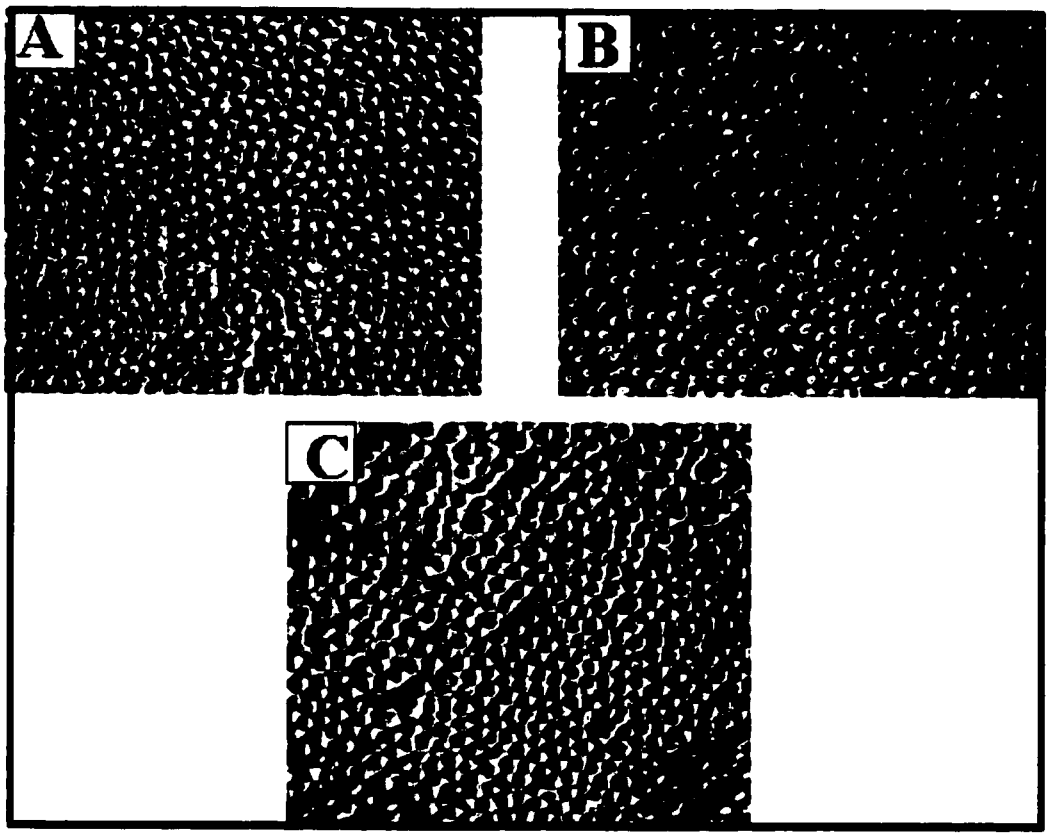


Figure 25. Effect of neutralizing Fas antibody ZB4 on TIMP-2 and BB-94 induction of apoptosis.

Peripheral blood T cells were cultured in RPMI growth media with 3% FCS and preincubated with the neutralizing Fas antibody, clone ZB4 (istoype IgG1)(1ug/mL) for 2 hours with and without recombinant TIMP-2 protein (40 nM) or BB-94 (10 uM). Cells were then stimulated with PHA (1.5 ug/mL) and IL-2 (50 u/mL) for 36 hours. Cell viability and morphology was analyzed at 36 hours. The experiment was performed 2 times with 2 different T cell preparations. Each reaction was set up in triplicate. Percent apoptosis was determined by trypan dye exclusion. Values are expressed as mean of triplicates. Error bars represent standard errors of mean. ^ Indicates statistical significance ($P < 0.01$) vs PHA and BB94 treated cells.

Figure 26. Photomicrograph of T cells: Effect of neutralizing Fas antibody ZB4 on TIMP-2 induction of apoptosis.

Peripheral blood T cells were cultured in RPMI growth media with 3% FCS and preincubated with the neutralizing Fas antibody, clone ZB4 (istoype IgG1)(1ug/mL) for 2 hours with and without recombinant TIMP-2 protein (40 nM). Cells were then stimulated with PHA (1.5 ug/mL) and IL-2 (50 u/mL) 36 hours. Cell viability and morphology was analyzed at 36 hours. The experiment was performed 2 times with 2 different T cell preparations. **Panel A)** Peripheral blood T cells stimulated with PHA and IL-2, **Panel B)** PHA and IL-2 stimulated T cells treated with TIMP-2, **Panel C)** PHA and IL-2 stimulated T cells treated with TIMP-2 in the presence of ZB4.


A
B
C

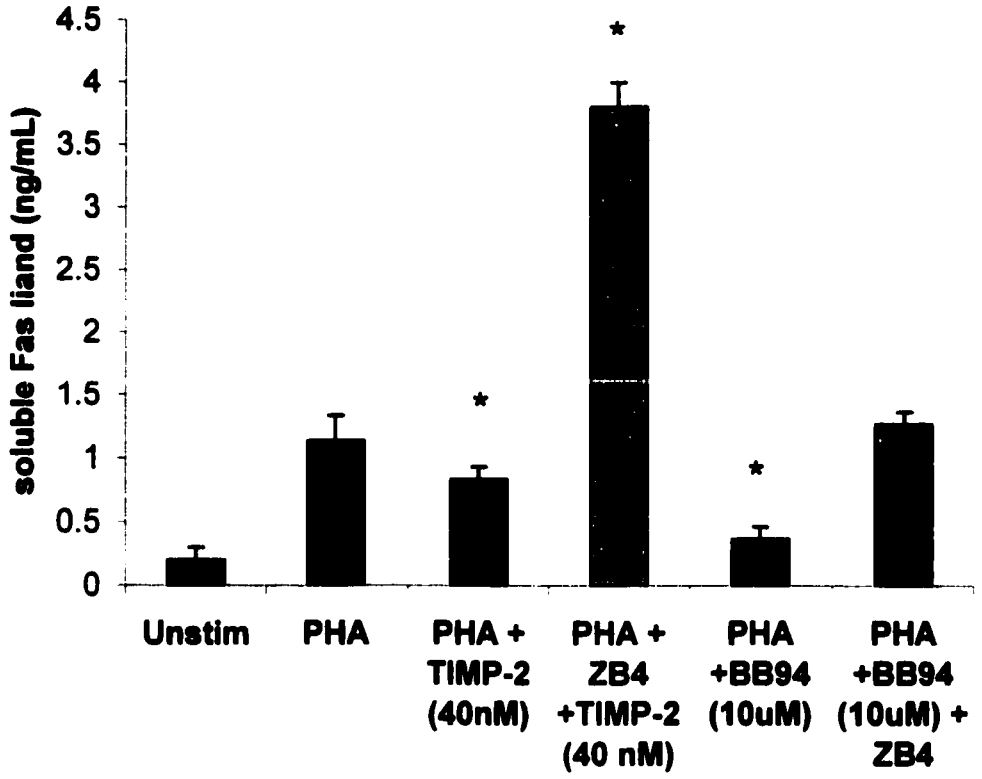


Figure 27. Effect of neutralizing Fas antibody (ZB4) on level of soluble Fas ligand during TIMP-2 and BB-94 mediated induction of apoptosis.

Peripheral blood T cells were cultured in RPMI growth media with 3% FCS and preincubated with the neutralizing Fas antibody, clone ZB4 (istoype IgG1)(1ug/mL) for 2 hours with and without recombinant TIMP-2 protein (40nM) or BB-94 (10uM). Cells were then stimulated with PHA (1.5 ug/mL) and IL-2 (50 u/mL) for 36 hours. Conditioned media normalized for 10ug of protein was analyzed for soluble Fas ligand by ELISA. The experiment was performed 2 times with 2 different T cell preparations. Each reaction was set up in triplicate. Values are expressed as mean of triplicates. Error bars represent standard errors of mean. The data from a representative experiment is shown. * Indicates statistical significance ($P < 0.05$) vs PHA treated control cells.

3.12 Identification of Metalloproteinase Involved in Fas Ligand Cleavage

Metalloproteinases have now been shown to be responsible for a range of membrane-associated protein processing events, termed shedding or cleavage. These include shedding of cytokines such as TNF-α, (149, 152), TNF-R (150, 254), IL-1β (281) and adhesion molecules including L-selectin (156, 282). The identities of the metalloproteinases responsible for most of these shedding events are unknown. The enzyme which processes membrane-associated pro-TNF-α to the mature soluble form has been identified as a member of the novel ADAM (for a disintegrin and a metalloproteinase) family of Zn^{2+}-dependent endopeptidases known as TACE (ADAM-17) (239, 283). Members of the ADAMs family are strong candidates for this activity.

To determine whether MMP-9, a matrix metalloproteinse which is coexpressed and upregulated during T cell activation (Figure 11) is involved in the cleavage of Fas ligand, we incubated activated T cells with a neutralizing anti-MMP-9 antibody under similar conditions used for previous experiments. No effect on T cell apoptosis was demonstrated in this preliminary experiment. Although we do not know whether TACE is expressed by lymphoid cells, it is a potential candidate in the processing of Fas ligand. Recombinant TACE is inhibited by peptide hydroxamate inhibitors including BB-94 as well as TIMP-3 but not TIMP-1, -2, and –4. TIMP-2 and TIMP-4 were weak inhibitors at IC_{25} of 130 nM and 100 nM, respectively. Mouse TIMP-3 can inhibit the shedding of TNF α and IL-6 (284). This however is controversial as Lombard et al., (285) has shown that TIMP-2 inhibits the cleavage of TNFα-receptor with an IC_{50} in the 286-462 nM range.

Chapter 4. Discussion

4.1 Expression of MMP and TIMPs in Cells of the Hematolymphoid System

4.1.1 Expression of MMPs

The involvement of the MMPs in development of the metastatic phenotype in solid tumors and the ability of the TIMPs to inhibit tumor cell invasion and metastasis both *in vitro* and *in vivo* is now well established. Previous studies indicate these gene products may also be involved in the progression of hematopoietic neoplasms (82, 136, 137, 206). They have also been implicated in leukocyte-endothelial cell adhesion, and migration during inflammation(185). As previous studies were performed in tissues or mixed cell populations, our aim in this study was to analyze the expression of these proteins in suspension cell cultures.

Using the sensitive zymogram technique, 92-kDa gelatinase (MMP-9) activity was only detected in the two Burkitt cell lines, Jijoye, and PA 682, peripheral blood T cells and in the tonsillar cell suspension. MMP-2 or interstitial collagenase activity was not detected by this method. Elevated MMP-9 expression, as assayed by *in situ* hybridization, has previously been observed in high-grade, large cell immunoblastic lymphomas and was associated with decreased survival (82). Zymogram detection of MMP-9 expression by the high-grade B-cell Burkitt lymphoma cell lines is consistent with these findings and indicates that this enzyme may play an important role in high-grade non-Hodgkin's lymphomas.

The actual tonsillar cell population producing MMP-9 may be B or T cells, although granulocytes and monocytes cannot be excluded. Although granulocytes and monocytes are known to express this enzyme (188), the 135- and 220-kDa forms of

gelatinase, which are typically expressed by these cells were not present, indicating they are unlikely sources for MMP-9. The ability of conditioned media from the tumor specific T cells to degrade basement membrane correlated with MMP-9 levels (268). Our studies support the concept that MMP-9 may play an important role in lymphoid degradation of basement membrane components. These findings support the role of this protease in normal lymphocyte migration from the vascular compartment to sites of inflammation, as well as invasion.

The current study shows that MMP-2 is not produced in isolated T-cell cultures, although the observed lack of expression may be due to other undefined differences in culture conditions. Expression of MMP-2 by stimulated T cells has been previously reported (194). In this study of normal donor T cells however, the B cells had not been completely removed from the lymphocyte culture preparation. The B cells could stimulate T cells to produce MMP-2 or they could be the actual source of this matrix metalloproteinase. In another study where T cells were isolated by fluorescence activated sorting and the purity of the T-cell population determined at 98%, the results were identical to ours in that MMP-2 was not detected, although MMP-9 was present basally and increased with stimulation (268). Other enzymes not detected by zymography under conditions used in these studies, such as plasminogen activators, may play an important role in basement membrane degradation during lymphoid cell invasion. Alternatively, lymphoid cells may produce MMPs upon interaction with basement membrane components, such as laminin, type IV collagen, or heparan sulfate proteoglycan, or in response to other tissue-specific interactions (eg, interaction with

endothelial cells) that are not present under *in vitro* tissue culture conditions. In fact, specific induction of MMP-2 has been observed in murine T cells that was mediated by vascular cell adhesion molecule (VCAM-1)-dependent adhesion to endothelial cells (192). Human T-cell synthesis and secretion of MMP-2 and MMP-9 are influenced by integrin-mediated adherence to basement membrane matrix (286). These findings suggest that these proteases contribute significantly to events associated with T cell transmigration across the subendothelial basement membrane. Thus, although MMP-2 and interstitial collagenase are not observed in the peripheral blood T cells and all of the cell lines studied under the culture conditions used, they may be produced by lymphomas and normal lymphocytes *in vivo*. Indeed, Shi et al., (287) reported that MMP-2 gelatinase was absent from peripheral leukocyte supernatants, whereas it was associated with all leukocytes cultured from endometrial tissue. A similar phenomenon has been observed for malignant breast epithelial cells cocultured with stromal fibroblasts. In this context, epithelial cells bind and activate MMP-2 that is synthesized and secreted by fibroblasts. This activity required the expression of a membrane type MMP (MT-MMP). Of note, the current study shows that MT1-MMP RNA is not expressed in any of the neoplastic B and T lymphoblastoid cell lines, or in peripheral blood T cells. A single report has demonstrated absence of MT1-, MT2-, MT3-MMP protein in an inflammatory lymph node by immunohistochemical analysis (288).

4.1.2 Expression of TIMPs

In the current study, TIMP-1 RNA transcripts and protein were expressed by the multipotential K562 cell line, the high-grade Burkitt B lymphoma cell lines, tonsillar cells, and peripheral blood T cells. TIMP-1 was not expressed in any of the neoplastic T cell lines. Expression of TIMP-1 by K562 has been previously reported and has been shown to possibly promote growth in these cells (127, 178). Although TIMP-1 expression by Burkitt cell lines has not been previously studied, it was observed in high-grade, large cell immunoblastic lymphomas and was associated with advanced stage disease (136). By *in situ* hybridization studies, the expression of TIMP-1 was localized to the stromal and endothelial cells although the method was not sufficiently sensitive to exclude lower levels of expression in the tumor cell. We have demonstrated that TIMP-1 is expressed not only by stromal cells, but also by high-grade neoplastic lymphoid cells of B lineage. This suggests that TIMP-1 may be of importance in the biology of high-grade B cell lymphomas. Indeed, recent studies have demonstrated that TIMP-1 has an anti-apoptotic effect in Burkitt lymphoma cells as well as in reactive B lymphocytes (138). Expression of TIMP-1 by tonsillar cells and peripheral blood T cells may indicate an association with the "activated" lymphoid phenotype or another role in normal lymphoid function.

High levels of TIMP-2 RNA transcript and protein expression were observed in the neoplastic T cell lines with lower levels of expression in normal peripheral blood T cells and hyperplastic tonsils. TIMP-2 expression occurred in response to activation with IL-2 and PHA in the peripheral blood T cells. TIMP-3 expression was not

detected in any of the lymphoid cells examined. TIMP-4 has recently been characterized (33) and its expression is not observed in the thymus or spleen and therefore is not likely to be expressed in lymphoid cells.

In summary, we have studied the expression of TIMP-1, TIMP-2, TIMP-3, MMP-2, MMP-9 and MT1-MMP in a variety of low-grade and high-grade hematopoietic cell lines, as well as normal T and B cells. MMP-9 production was demonstrated in the two Burkitt cell lines, tonsillar cell suspension, and normal peripheral blood T cells and may play an important role in lymphoid cell migration. MMP-9 production was not detected in the other lymphoid cell lines. This may be due to a requirement by lymphoid cells for interaction with components of the extracellular matrix or endothelial cells to induce or initiate gelatinase transcriptional activity. The results do demonstrate differential expression of the tissue inhibitors of matrix metalloproteinases, TIMP-1, and TIMP-2. TIMP-1 is expressed in the high-grade B cell Burkitt lymphoma lines, the multipotential K562 cells, tonsillar tissue, and activated T cells, while TIMP-2 expression is apparently restricted to T lymphoma cell lines and reactive T cells. The expression of these proteins in activated T cells will be discussed in the next section.

4.2 Effect of T Cell Activation on Expression of MMPs and TIMPs in Activated Peripheral Blood T Cells

Upon activation of peripheral blood T cells with IL-2 and PHA, we observed an increase in MMP-9 expression as well as the presence of activated forms of the

gelatinase (Figure 11A), a finding consistent with observations made by others (189, 268). MMP-9 is reported to be expressed at a basal level in cultures containing normal peripheral blood B and T cells (monocyte-depleted) and increases upon activation with PHA or IL-2 (195). Tumor specific T cells have been shown *in vitro* to secrete MMP-9 at basal levels with increased expression upon stimulation with IL-2 or tumor cells (194).

Activation of peripheral blood T cells by PHA and IL-2 induced the expression of TIMP-2 protein. In contrast, peripheral blood T cells secreted active TIMP-1 at a basal rate that did not increase significantly with activation. The inducible expression of TIMP-2 in activated T cells is unusual as most studies have shown that TIMP-2 expression is constitutive. Only a few studies have reported inducible expression of TIMP-2 in response to lipopolysaccharides and prostaglandin E2 in rat liver cells (200) and injured arteries (71). Although TIMPs are essentially interchangeable in their capabilities as inhibitors of MMPs, they are distinguished by the formation of specific complexes with different pro-MMPs. Conventional models derived from analysis of adherent cell lines involve MMP-2/TIMP-2 and MMP-9/TIMP-1 complexes as playing a critical role in activation of specific latent MMPs. The discordant expression of TIMPs and MMPs in lymphoid cells is puzzling and suggests that 1) MMPs and TIMPs may have cell-specific physiologic roles, and that 2) TIMPs may be functioning in a capacity aside from its metalloproteinase inhibitory function. Furthermore, the restricted expression of TIMP-1 in B cells and TIMP-2 in T cells indicates that they may have lineage-specific functions.

The growth-modulatory activities of TIMP-1 and TIMP-2 have not distinguished between different lymphoid cell lineages or state of differentiation. The role of TIMP-2 in growth modulation of T lymphocytes was the focus of the following studies.

4.3 The Role of TIMP-2 in T Cell Growth and Apoptosis

The presence of TIMP-1 and TIMP-2 in the absence of metalloproteinase expression in neoplastic lymphoma cell lines is an interesting observation. Although the MMPs studied may be expressed under different culture conditions, one would expect the regulation of expression of TIMP-1 and TIMP-2 might mirror that of the MMPs if the action of the TIMPs is merely inhibition of gelatinase activity. Therefore, the constitutive expression of the TIMPs without gelatinase suggests that they may have a growth factor activity, similar to that observed for TIMP-1 and TIMP-2 in the erythroid burst formation assay (69) and for TIMP-1 in K562 cells (127). This is further supported by the work of Hayakawa et al, (128, 134, 271) who observed that both TIMP-1 and TIMP-2 promoted growth of a number of human cell lines, including the following: the Burkitt cell lines Raji, Daudi, and Ramos; the human myelogenous leukemia line HL60; human lymphoblast cell line WIL2-NS; human breast adenocarcinoma line MCF7; human gingival fibroblasts; human skin epithelial cells; and human aortic smooth muscle cells.

The increased levels of TIMP-2 expression in neoplastic T cell lines compared with normal T cells is also consistent with an autocrine growth factor activity, such as

is observed in K562 cells. Alternatively, TIMP-1 and TIMP-2 may be coexpressed with and modulate the activity of other metalloproteinases that were not studied or may be coupled with MMP expression *in vivo*. Given this rationale, we first determined whether TIMP-2 affected the growth of T lymphocytes. Our initial experiment did not demonstrate an effect on growth promotion, but showed that T cells exposed to rTIMP-2 displayed increased numbers of apoptotic cells.

Programmed cell death, or apoptosis, is now recognized as playing a central role in development and tissue-specific functions in such diverse processes as regulation of immune responses and organogenesis (289, 290). In these processes, components of the extracellular matrix (ECM) (291) and in particular basement membrane act as survival factors and suppress apoptosis. Proteolytic modification of matrix organization or disruption of cell-matrix contacts can result in initiation of apoptosis or anoikis in epithelial cells, with induction of specific molecular effectors of apoptosis such as caspases (107, 108, 291). Together, these findings suggest that extracellular matrix proteases or their inhibitors (TIMPs) may directly influence growth and apoptosis of normal and neoplastic cells. **The present study was therefore undertaken to determine whether TIMP-2 regulates apoptosis in T cells.** The results of our study provide evidence for the contention that TIMP-2 is involved in regulating apoptosis through a novel, MMP inhibitory pathway and suggest that this protein plays a pivotal role in maintenance of T cell homeostasis. Apoptosis was measured by four independent methods including cell surface Annexin V staining, morphologic assessment, DNA fragmentation by agarose gel electrophoresis and cell viability

assays. All methods demonstrated that apoptosis was enhanced in cells exposed to rTIMP-2. Recombinant TIMP-2 exposure at 20 nM resulted in 35.7± 1.5 % T cell apoptosis compared to 15.5 ± 1.0% in control untreated cells. Unstimulated T cells demonstrated basal level of apoptosis (6.0 ± 0.4%) even with increasing concentrations of rTIMP-2; 6.2 ± 0.4 % at 5 nM, 6.7 ±1.1% at 20 nM and 7.7 ± 1.0% at 40 nM. Only activated T cells were susceptible to apoptosis when exposed to either TIMP-2 or BB-94. Thus, the pro-apoptotic effect of TIMP-2 and BB-94 is dependent upon the activation states of the T cells. Using a fragment of human FasL promoter capable of driving the expression of GFP to allow identification and analysis of FasL activity in Jurkat cells, Su et al., (237) have suggested that distinct populations of T cells with differential susceptibility to apoptosis exist and that this occurs according to different FasL production.

The pro-apoptotic effect of TIMP-2 was dose-dependent. Dose-response analysis demonstrated that initial effect on induction of apoptosis was seen at 5 nM rTIMP-2. A linear dose-response was seen up to 40 nM after which the effect appeared to plateau. Unstimulated T cells exhibited similar low levels of apoptosis (5.0 ± 5%; Figure 16) for the TIMP-2 treated and untreated cells. These data suggest that the TIMP-2 protein may be saturating a protein or molecule involved in inducing apoptosis. Receptors for TIMP-2 have not been identified to date although cell surface binding of TIMP-2 and pro-MMP-2/TIMP-2 complexes have been demonstrated in certain cell lines (177). Binding sites for rTIMP-1 have been identified in human keratinocytes, human erythroid precursors and K562 cells (127, 132). Secreted rTIMP-1-enhanced

green fluorescent protein (EGFP) has been shown to bind to the cell surface of MCF-7 breast carcinoma cells after which it translocated to the nucleus (181). It would be important to determine cell surface binding of rTIMP-2 on T lymphocytes in order to elucidate the mechanism involved in its pro-apoptotic function.

The effect of exogenous TIMP-2 on T cell apoptosis was not an immediate event. A time course analysis showed that induction of apoptosis by rTIMP-2 was observed initially at 24 hours and peaked at 36-48 hours and this effect was seen only with activated T cells. This coincides with the time that TIMP-2 protein is induced after T cell activation with PHA and IL-2 highlighting the physiologic relevance of the findings. This is similar to observations with TIMP-1 in B cells where its anti-apoptotic activity is specific for a state of differentiation and activation. After 52 hours all cells including cells under control conditions exhibited equal amounts of apoptosis (>45%). This suggests that the effect of rTIMP-2 on T cell apoptosis is transient and indicates the presence of a subset of T cells that are susceptible to its proapoptotic effect. Specific differentiation or activation-associated antigens or intercellular signaling molecules that are expressed during this phase of T cell activation may be involved. All four members of the TIMP family share similar structure-function properties and are approximately 40% identical at the amino acid level. The expression patterns and inhibitory specificities of the TIMPs differ, however suggesting that although all proteins inhibit the MMPs, they have some functional differences that may be useful in characterizing the cell type specificity of their physiologic role. Peripheral blood T cells were exposed to rTIMP-1 to determine if the effect on apoptosis was

mediated by the metalloproteinase inhibitory activity of TIMPs. The data presented show that TIMP-2, but not TIMP-1 induces apoptosis of activated T cells. As shown in Figure 18, TIMP-1 at concentrations that demonstrated maximum effect with TIMP-2 (40 nM) had no effect on T cell apoptosis. The lack of effect of TIMP-1 in inducing T cell apoptosis may be due to poorer inhibition of the enzyme involved; alternatively TIMP-1 may be functionally less specific than TIMP-2 in this setting.

Preliminary data suggested moreover that TIMP-1 treated T cells showed increased cell viability when compared to control cells. Although this was not further pursued it is an enticing observation as TIMP-1 has been shown to have a suppressive effect on Burkitt lymphoma cell apoptosis (138) and may be involved in the stimulation of proliferation of normal and malignant B cells.

Neutralization of secreted TIMP-2 with polyclonal rabbit anti-TIMP-2 antibody during activation of peripheral blood T cells decreased the numbers of apoptotic cells during the assay period. There was a 35% reduction of apoptotic cells in T cells exposed to neutralizing anti-TIMP-2 antibody compared to cells incubated with isotype control or anti-TIMP-1 antibody. Cells treated with antibody displayed a general increase in apoptosis compared to untreated T cells. The significance of this observation is uncertain. However, it suggests that the immunoglobulin component of the antibody may be changing the kinetics of T cell activation. Transmission microscopic analysis of cells demonstrated that the decrease of apoptotic cells was associated with increased numbers of cells showing blast morphology. Many of these cells exhibited activation associated markers CD71, CD69 and CD25 and HLA-DR by

flow cytometric immunotyping suggesting that anti-TIMP-2 may be slowing down the rate of apoptosis and allowing more cells to remain in the blast phase of activation before undergoing apoptosis.

Cells incubated with anti-TIMP-1 antibody showed a higher number of apoptotic cells at 24 hours compared to anti-TIMP-2 treated cells suggesting that TIMP-1 may have a different function in T cell homeostasis. This is consistent with previous observations that rTIMP-1 was not able to induce T cell apoptosis (Section 3.6 Figure 18). It has been demonstrated that, TIMP-1 can suppress apoptosis induced by a variety of stimuli including cold shock and Fas activation in B cells (138) but not serum starvation. These observations highlight the cell type specificity of TIMP function in lymphocyte homeostasis. These experiments do not allow us to conclude whether the apoptosis-inducing action of TIMP-2 depends upon its MP inhibitory function or on some other activity. The role of cell receptors for TIMP-1 and TIMP-2 may help elucidate the components of this complex process.

To determine whether the metalloproteinase-inhibitory activity is involved in the pro-apoptotic effect of rTIMP-2, we tested the effect of reduced and alkylated TIMP-2 in T cell viability and apoptosis. Chemical reduction and alkylation completely abrogates TIMP's ability to inhibit MMP activity (134). Compared to control peripheral blood Tcells treated with functional rTIMP-2 (37.3 ± 2.5%), those treated with reduced and alkylated rTIMP-2 (40nM) demonstrated reduced ability to induce T cell apoptosis (11.7 ± 1.5%) (Figure 20). These results support the hypothesis that the metalloproteinase inhibitory activity of TIMP-2 is required for induction of T

cell apoptosis. Together with data from Section 3.6 this experiment suggests that metalloproteinase inhibitory (MPI) function is required only in the setting of TIMP-2 in inducing T cell apoptosis. The non-MPI domain of TIMP-1 may define the specificity of this process, alternatively, the putative receptor for TIMP-1 in B cells may not be present or functional in this setting.

To determine whether the effect of rTIMP-2 was specific for reactive T cells, a panel of T cell lymphoma cell lines were exposed to varying concentrations of exogenous TIMP-2 for varying time periods. Two different high-grade lymphoblastic lymphoma cell lines Jurkat and Tsup were susceptible to rTIMP-2 mediated apoptosis. Concentrations required to induce apoptosis were similar to those for reactive T cells. Similar to what was observed with reactive peripheral blood T cells, the effect was demonstrated with preactivated cells and was not observed with unstimulated cells. Activation of Jurkat T cells with polyclonal activators such as PHA and Il-2 result in coexpression of Fas and Fas ligand on the cell surface (237).

Whether TIMP-2 has a pro-apoptotic effect on nonlymphoid cancer cells is debatable at this time. *In vitro* data showed that TIMP-2 inhibited growth of basic-FGF stimulated endothelial cells (142), however whether TIMP-2 directly induced apoptosis has not been demonstrated. The cell growth modulatory function of TIMP-2 may be cell-type specific as overexpression of the protein has been shown to protectapoptosis in melanoma cells (139).

To further determine whether the MPI activity of TIMP-2 is important for induction of T cell apoptosis, a panel of synthetic MPIs were used. BB-94, GM-

6001and KB-8301are low molecular weight broad-spectrum synthetic MPIs shown to inhibit not only tumor spread and metastasis (96, 97) but also tumor growth of human ovarian cancer xenografts (169). Furthermore, BB-94 can perturb the cell cycle in ovarian cancer cells with a block in G0/G1 and inhibition of clonogenicity (292). Their direct effects on cell apoptosis has not been addressed, although the biologic effect of BB-94 on lymphoid growth and function have been implicated in studies where BB-94 was shown to have an immunosuppressive effect in animal models of arthritis (176).

Both BB-94 and GM-6001 resulted in induction of apoptosis of preactivated peripheral blood T cells as well as T lymphoblastic lymphoma cell lines, Jurkat and Tsup. BB-94 caused a concentration-dependent induction of T cell apoptosis. Under these conditions, induction of apoptosis was seen at 5 uM with maximal effect achieved with 10 uM BB-94. Further increases in the concentration of BB-94 up to a maximum of 100 uM had no additional effect. GM-6001 demonstrated less potent effect on apoptosis at similar concentrations.

There are conflicting data in the literature regarding the effect of TIMP-2 in growth modulation. Hayakawa's studies showed that TIMP-2 enhanced the ^{3}H-thymidine uptake in a number of different cell types (128, 134). In these studies, however the concentration of TIMP-2 which stimulated growth was in the picomolar range. Furthermore, the growth of HL60 leukemia cells was inhibited by rTIMP-2 at 60 pM whereas Ramos, a Burkitt lymphoma cell line demonstrated growth stimulation at 30 pM but growth inhibition was noted at higher but unreported concentrations. The

effect of TIMP-2 at higher micromolar concentrations was not studied. It is thus likely that the effect of TIMP-2 is highly cell-type specific and concentration dependent.

Recent reports have shown that overexpression of TIMP-3 in some cell types results in increased apoptosis in non-hematopoietic cells (165, 172) including rat smooth muscle cells, colon cancer cells and melanoma cells. According to one report, TIMP-3 at an unspecified concentration did not have an apoptotic effect on Jurkat T cells and peripheral blood monocytes (157). It is important to point out that the time points used and the effective concentrations of recombinant proteins and transfected proteins were either not clearly delineated and were variable to allow definitive comparisons. These studies support the idea that TIMPs can exert different activities in addition to inhibition of MMP, including modulation of apoptosis. The mechanism for the effect of TIMP-2 and TIMP-3 on apoptosis was not addressed in these studies.

4.4 Potential Mechanism of TIMP-2 and Synthetic MPI Induction of T Cell Apoptosis

The results obtained thus far suggest that rTIMP-2 and synthetic MPIs induce apoptosis in human T lymphocytes. These results, however, do not provide any evidence with respect to the mechanism through which they induce apoptosis. Data using neutralizing TIMP-2 antibody, reduced and alkylated TIMP-2 and N-terminal domain mutant protein suggest that the metalloproteinase inhibitory activity of TIMP-2 is important in the process. Metalloproteinases have been implicated in the processing and cleavage of several cell surface receptors and molecules. Metalloproteinase inhibitor KB-8301 is a hydroxamic acid inhibitor of the MMPs that has been shown to

process Fas ligand (154). Fas ligand is initially expressed as a 40 kDa membrane bound protein (mFasL) on activated T cells and proteolytically processed to a 26 kDa soluble form (sFasL). TNF-α, TNF-receptor, IL-1β, L-selectin have also been shown to be targets of metalloproteinases (153-155, 157, 233, 238, 253, 254).

TNF-α and Fas ligand are important mediators of T cell apoptosis (215, 219, 222, 269, 277). TNF-α and Fas ligand impart differential susceptibility to activation-induced apoptosis. TNF-α is more important for T cells in lymph nodes and spleen whereas Fas ligand is important in peripheral blood T cells (278). We therefore hypothesized that TIMP-2 and synthetic MPIs induced activated T cell apoptosis by inhibiting the cleavage of Fas ligand and thereby enhancing the rate of apoptosis.

Treatment of peripheral blood T cells with 10 uM BB-94 and 20 uM KB-8301 resulted in a significant increase in the percentage of activated T cells with cell surface expression of Fas ligand (44.3 ± 4.7 %, 32.7 ± 3.7%, respectively) compared to PHA and IL-2 activated cells (7.8 ± 0.8%). No significant increase in cell surface Fas ligand expression was seen with 40 nM rTIMP-2 (14.2 ± 2.3%) (Figure 23). Importantly, the concentrations of BB-94 (10 uM) and KB-8301 (20 uM) required to demonstrate increased cell surface Fas ligand expression was significantly higher than that used for rTIMP-2. Notably, these are the concentrations required to demonstrate increased cell surface Fas ligand expression in previous reports suggesting metalloproteinase-mediated Fas ligand cleavage (155). As the data from dose response analysis demonstrated, TIMP-2 at these concentrations had saturated the effect on T cell apoptosis. Thus it can be interpreted that BB-94 acts to inhibit a broad spectrum of

metalloproteinases other than that which is inhibitable by TIMP-2. As BB-94 and KB-8301 are MPIs with broad substrate specificities it is possible that they inhibit a number of different metalloproteinases which may be responsible for the cleavage of Fas ligand. Additionally, as the concentrations of TIMP-2 used in this preliminary experiment were significantly lower than those used for synthetic MPIs and those used in the literature (1.5 uM for TIMP-3 inhibition of sIL-6R release from myeloma cells, (279), optimum concentrations may not have been used. Another explanation for these observations is that the enzyme responsible for Fas ligand cleavage may be a non-matrix-type metalloproteinase. Also, TIMP-2 may be inhibiting the shedding of other molecules such as TNF-R expressed on a subset of T cells.

By ELISA technique we were able to demonstrate decreased amounts of sFas ligand in conditioned media of peripheral blood T cells treated with rTIMP-2 and BB-94. BB-94 treatment resulted in a 68% reduction of sFas ligand in the conditioned media compared to 18% for rTIMP-2 treatment (Figure 24B). Western blot analysis of sFas ligand levels in conditioned media of cells treated with TIMP-2 or BB-94 was not informative and may reflect differences in antibody specificities (Figure 24A).

To further determine whether the Fas/FasL mediated apoptosis pathway is involved in the MPI mediated T cell apoptosis, T cells were treated with rTIMP-2 and BB-94 in the presence of the neutralizing anti-Fas antibody ZB4. This antibody binds to cell surface Fas receptor and inhibits apoptosis. Its binding would also prevent sFas ligand from binding to the Fas receptor on the cell surface and allow its accumulation in the conditioned media. Activated T cells incubated with anti-Fas ZB4 antibody

abrogated apoptosis induced by rTIMP-2 (Figure 25). These observations demonstrate that Fas/FasL pathway is involved in the T cell apoptosis induced by rTIMP-2. Interestingly, in contrast to its effect on TIMP-2 mediated apoptosis, ZB4 did not abrogate BB-94-mediated apoptosis although the percentage of apoptotic cells was decreased (Figure 26). These observations suggest that the enzyme responsible for the cleavage of Fas ligand is only partly inhibited by BB-94. Several enzymes may be responsible for the cleavage as BB-94 can inhibit a host of metalloproteinases. In addition, BB-94 may be inhibiting the cleavage of molecules involved in T cell apoptosis other than Fas ligand.

The abrogation of TIMP-2 mediated T cell apoptosis by ZB4 occurred concomitantly with increased levels of sFas ligand in the conditioned media as measured by ELISA. Conditioned media of T cells incubated with rTIMP-2 without ZB4 contained 0.8 ± 0.1 ng/mL of sFas ligand, whereas that incubated with rTIMP-2 and ZB4 had 4.7 fold higher levels of sFas ligand (3.8 ± 0.2 ng/mL). Similarly, cells incubated with BB-94 in the presence of ZB4 contained a higher amount of sFas ligand in the conditioned media (1.3 ± 0.1 ng/mL) than those incubated with BB-94 alone (0.4 ± 0.1 ng/mL) (Figure 27). Several explanations exist for the above observation. First, ZB4 may bind and saturate the cell surface Fas receptor and prevent the binding of soluble Fas ligand to its receptor. This would result in the accumulation of soluble Fas ligand in the conditioned media. Secondly, ZB4 may be stabilizing the soluble Fas ligand molecule preventing its degradation in the conditioned media.

The cleavage of Fas ligand in Ewing's sarcoma cells was found to be blocked by synthetic MPI only, with TIMP-1 or TIMP-2 having no effect (293). The conclusions from these studies are difficult to analyze as the concentrations of endogenous inhibitors were not always reported. The concentrations of synthetic MPI required for the effects were generally much higher (10-fold) than those used for TIMP proteins.

Although several studies have demonstrated the modulation of Fas ligand shedding and TNF-α-receptor shedding by natural and synthetic metalloproteinase inhibitors, this is the first report demonstrating a role in T cell homeostasis (See Figure 28). Although the data supporting a role for TIMP-2 in inhibiting the cleavage of Fas ligand are not conclusive, there is a suggestion that this may be partly responsible for the observation. Direct evidence for the pro-apoptotic effect of BB-94 and other synthetic MPIs have not been previously reported.

The results from the above studies indicate that the proapoptotic effect of TIMP-2 is secondary to MMP inhibition. The only exception to this is the lack of effect of TIMP-1. How can this discrepancy be reconciled? Because each TIMP has a unique pattern of gene expression and target enzyme preference (294), it is not unexpected that TIMP-1 and TIMP-2 would have differing profiles of biological activity as demonstrated in the above experiments. Other studies have reported this observation. TIMP-2 and synthetic MPI inhibited the shedding of membrane-bound TNFα receptors from two human cancer cell lines (Colo 20 and SW626) which was not seen with TIMP-1 (285). In this study, TIMP-2 was 10-100 times more potent for

inhibition of TNFα receptor shedding than the low molecular weight synthetic MPIs. Forced overexpression of TIMP-3 has been shown to inhibit the shedding of TNFR in DLD colon carcinoma cells (165). TIMP-3 is unique in that it is able to bind to matrix components via binding to heparan sulfate proteoglycans which are expressed at the cell surface. Binding to these glycosaminoglycans may localize TIMP-3 and enable interaction with cell surface metalloproteinases. There may also be additional motifs within TIMPs which contribute specificity to inhibit the shedding of certain molecules.

The mechanism involved in TIMP-1 promoting cell survival for cells does not seem to be uniform in that the work of Hayakawa (134) suggested the importance of the MMP inhibitory effect and preservation of cell-matrix contacts. The mechanism by which TIMP-1 inhibits apoptosis in B cells appears to be quite different as it was not secondary to MMP inhibition. (138). In addition, TIMP-2 or the synthetic inhibitor BB-94 failed to protect from apoptosis, even though the levels of BB-94 used should completely inhibit a wide spectrum of metalloproteinases, including MMPs. The ability of TIMP-1 to exert anti-apoptotic effects independently of its MMP inhibitory effect is consistent with other studies that demonstrated TIMP-1 cell growth modulatory effects unrelated to its ability to act as MMP inhibitor (135). We cannot exclude from these studies that TIMP-1 may be selectively blocking a metalloproteinase that blocks apoptosis. TIMP-1's inability to block the Fas ligand "sheddase" combined with its ability to block an antiapoptotic metalloproteinase might be sufficient to give it a very specific anti-apoptotic effect. This would still depend on the metalloproteinase

inhibition function however. Notably, TIMP-1 but not TIMP-2 can inhibit aggrecanase (ADAMTS-4)(295).

TIMP-2's failure to inhibit apoptosis in Burkitt lymphoma cell lines is in contrast to its observed anti-apoptotic activity in melanoma cell lines (139) but supportive of the pro-apoptotic effect on T cells presented in this study. These studies suggest that the effects of various TIMPs on apoptosis and cell growth may be tissue specific as well as being dependent upon stage of differentation and the cellular millieu. This is further supported by observation that TIMP-1 inhibited apoptosis in normal tonsillar B cells but not T cells (138, 296).

The results presented here show differences in the susceptiblity of Fas ligand cleavage to inhibition by TIMP-1 and TIMP-2 and synthetic metalloproteinases, BB-94 and GM-6001. This raises the possibility that cleavage of Fas ligand may be mediated by a number of different enzymes, although other explanations exist. This effect of metalloproteinases in Fas ligand regulation has important clinical implications for hematological disorders characterized by high serum soluble Fas ligand such as leukemias of natural killer (NK) or T cell-type, large granular lymphocytes (LGL) or NK-lymphomas (232, 256).

The close relationship between the shedding mechanisms involved in proteolytic cleavage of many different cell surface proteins is revealed through the use of a mutant chinese hamster ovary cell line, defective in the shedding of transfected L-selectin. TGF-α, IL-6 receptor, β-amyloid precursor protein and a number of unidentified endogenous proteins are present in the supernatants of parental, but not

mutant cells (252). Additionally, TACE-deficient cells are also defective in the shedding of a number of cell surface molecules other than TNF-α (297). Further studies identifying the enzyme for other metalloproteinase-cleaved cell surface molecules should help clarify the functional redundancy and overlap of the "sheddases".

Is there physiologically relevant data to support the findings of our studies? Therapeutic interventions acting via MMP inhibition have shown promise in a number of *in vitro* and *in vivo* tumor models (96, 97, 298, 299) including human clinical studies (300-302). Blood concentrations of synthetic MPI in human reach concentrations high enough to inhibit both TACE and TNF-α receptor sheddase (303, 304). Diminished receptor shedding could increase populations of TNF-α receptors at the cell surface and augment the apoptosis of tumor cells mediated through the "death domain" of TNFα-RI (305). Direct support for this concept can be found in the work of Williams et al., (254) which demonstrated that the cytotoxicity of TNF-α toward a human rhabdomyosarcoma cell line was enhanced 15-fold in the presence of a synthetic MPI that concomitantly reduced receptor shedding. An analogous process may be occuring in the current study where increased numbers of transmembrane Fas ligand augment the apoptosis of activated Fas positive cells. In support of this, increases in the cell concentration used in the assay which would effectively enhance cell-cell contact, enhanced T cell apoptosis. It has been demonstrated that membrane-associated TNF-α precursor is biologically active and can be up to 10 times more efficacious than solubie TNF-α in model cell systems (249, 306). Although it has been reported that soluble Fas

ligand is functional in inducing death signal, the differential efficacy of soluble vs membrane-bound Fas ligand has not been reported. As concentrations of soluble TNF-α receptors and Fas ligand are increased in cancer patients, it would be of interest to monitor these molecules in patients treated with MMPIs and to relate changes in receptor concentrations to anticancer efficacy and/or side effects.

The data from this study suggest that MMP-9 is not involved in the processing of Fas ligand or another protein involved in TIMP-2 mediated T cell apoptosis. The data presented herein raise several questions. What is the enzyme responsible for cleavage of Fas ligand? Is it TACE or a related metalloproteinase that belongs to the superfamily of metzincins? It is reasonable to propose that the enzyme involved in cleavage of Fas ligand could be a member of the adamalysin subfamily of metzincins.

Whether endogenous TIMPs can inhibit members of the ADAM family including TACE has been recently studied and remain controversial. TIMP-1 or TIMP-2 did not inhibit the processing of proTNF-α (240) whereas TIMP-2 had partial effect on shedding of L-selectin (307). TACE was not inhibited by TIMP-2 (285) suggesting that TNFα is probably not a major factor involved in TIMP-2 mediated T cell apoptosis. The shedding of IL-6 receptor in human myeloma cells was inhibited by TIMP-3 and hydroxamate-based synthetic MPI. TIMP-1 and TIMP-2 were shown to have no effect (279). TIMP-3 appears to be different from the function of TIMP-1 and TIMP-2 in that IL-6 receptor and L-selectin shedding is inhibited by the protein (307). In addition, TNF-α converting enzyme is inhibited by TIMP-3 (284).

The absence of MT1-MMP in T cells (Section 3.1.1) would exclude the involvement of this enzyme. However MT4-MMP and MT5-MMP are potential candidates. Future experiments to analyze the expression of MT4-, 5- and –6 MMPs would be informative as they share the characteristic of inhibition by TIMP-2 but not TIMP-1.

4.5 Clinical Implications of Observations

The data presented in this study show that endogenous TIMP-2 and synthetic MPIs have a role in normal immune regulation as well as cancer immunotherapy. The pharmacological action of synthetic and endogenous MPIs may involve manipulation of TNF-α activity and Fas ligand activity. Indeed, elevated sTNF-αR levels are found in cancer patients (308). Soluble forms of Fas ligand are elevated in certain types of leukemias which contribute to systemic tissue damage, including hepatic failure and neutropenia (11, 256) and melanomas (309). TNF-α and Fas ligand have been implicated in the pathogenesis of graft-versus-host disease, (GVHD) which is a major complication of allogeneic bone marrow transplantation. As suggested in this study and others, if MPIs block the shedding of biologically active molecules such as TNF-α and Fas ligand, it may have important physiological effects in immune mediated diseases such as GVHD, multiple sclerosis and rheumatoid arthritis. Recently, it has been demonstrated that a synthetic MPI (KBR-7785) that inhibits TNF-α and Fas ligand release can ameliorate the lethal acute GVHD model in mice (175) while

preserving the graft-versus-leukemia effect (310). Of interest, both CD4+ and CD8+ T cells of the host were increased in number in KB-R7785-treated mice supporting the notion that stabilizing of TNF-α and Fas ligand by synthetic MPI can manifest in modulating T cell homeostasis *in vivo*.

Matrix metalloproteinase inhibitors are also being tested for treatment of multiple sclerosis and rheumatoid arthritis (Clinical Trial at Sunnybrook and Women's Health Science Center). Potent MMPI was clinically effective in an animal model of multiple sclerosis, experimental autoimmune encephalophalomyelitis (EAE) in the SJL/J mouse (311). In addition, MMPIs inhibited TNF-α shedding and cytotoxicity in a human *in vitro* system with myelin basic protein peptide-specific CD4+ T cell clones. These studies highlight the potential for MMPIs as therapeutic agents in immune-mediated disorders. At a cellular level, these observations demonstrate the emerging concept of the diverse functions of metalloproteinases and TIMPs in the hematologic system.

4.5 Future Directions

Development of *in vivo* models to develop clinical applications of TIMP-2 and/or synthetic MPI as immunosuppressive agents are potential future studies to pursue. Analysis of inflammatory responses to bacterial or viral pathogens in TIMP-2 knockout mice can be achieved.

To address the mechanism of TIMP-2 induction of apoptosis, cell transfection studies or retroviral mediated gene transfer of TIMP-2 may be used. One drawback of the current study was the use of peripheral blood T cells which are a heterogenous

population of cells that have varying cell surface antigens and heterogenous activation states. Use of a homogenous cell population with higher expression of endogenous TIMP-2 may have yielded more conclusive data.

Another area of potential study is the determination of cell type specificity of TIMP function. Most recent data suggests that TIMP-3 may be quite different from TIMP-1 and TIMP-2. The structural determinants for these functional differences are unknown and are important to clarify. Most importantly, the multifunctional role of endogenous TIMPs and synthetic MPI must be considered when designing therapeutic agents for immune modulation and chemotherapy.

Conclusions

We have examined the expression of matrix metalloproteinases (MMPs) and their endogenous inhibitors, the tissue inhibitors of metalloproteinases (TIMPs), in human normal and neoplastic lymphoid cell lines. There is lineage specific expression of TIMPs in lymphoid cells. Although there is uniform expression of MMP-9 in cells of the lymphoid system, the expression of MMP-2 appears to be modulated by cell adhesion factors and other extracellular factors. The expression of MMP-9 and TIMP-2 is induced during T cell activation. Endogenous TIMP-2 and synthetic MPIs may have a role in normal immune regulation as well as cancer therapy. Evidence supporting this hypothesis includes the observations that (a) TIMP-2 is induced during T cell polyclonal activation; (b) neoplastic T cells express TIMP-2 discordantly with matrix metalloproteinase -9; (c) exposure to TIMP-2 and synthetic MPIs induces activation

induced apoptosis of T cells (d) expression of cell surface and soluble Fas ligand is modulated by SMPI and TIMP-2 (e) and blocking of Fas receptor by anti-Fas ZB4 antibody differentially abrogates the induction of apoptosis by these molecules. A schematic model of the potential role of TIMP-2 and synthetic metalloproteinases in modulating T cell apoptosis is presented in Figure 29. Further studies are required to confirm these findings in order to use these agents in the clinical setting.

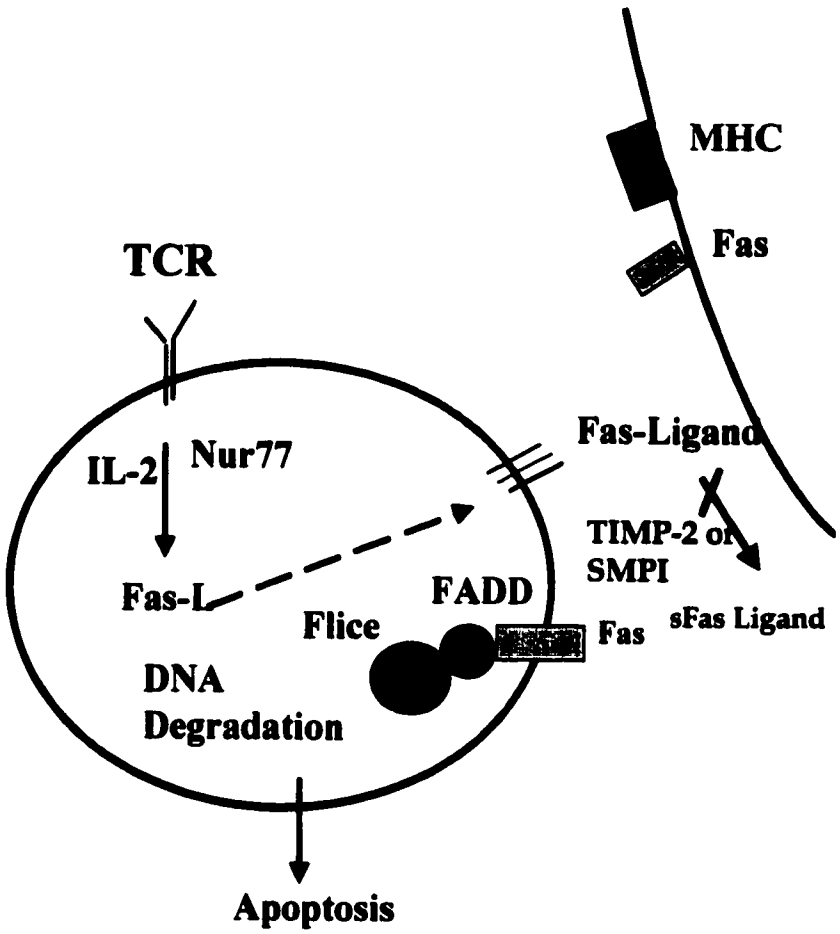


Figure 28. Schematic model of role of TIMP-2 and synthetic metalloproteinase inhibitors in modulating T cell apoptosis.

Postscript

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