EXPRESSION OF MATRIX METALLOPROTEINASES AND TISSUE INHIBITORS OF METALLOPROTEINASES IN THE HEMATOPOIETIC MICROENVIRONMENT

by

Mary Anne Viani

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Graduate Department of Laboratory Medicine and Pathobiology

University of Toronto

Abstract

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Mary Anne Viani
Department of Laboratory Medicine and Pathobiology
University of Toronto

Stem cells and progenitor cells within the hematopoietic microenvironment interact specifically with cell and ECM ligands present in the bone marrow. Matrix metalloproteinases (MMPs) and their inhibitors (TIMPs) are key regulators of ECM turnover. We hypothesize that MMPs and TIMPs play critical roles in regulating hematopoiesis. We analyzed the expression of functional gelatinases (MMP-2, MMP-9) and TIMPs (TIMP-1, TIMP-2) in long-term bone marrow cultures (LTBMC) by zymography, reverse zymography and Western blotting. Bone marrow progenitor cells constitutively express MMP-9 and TIMP-2 whereas expression of MMP-2 and TIMP-1 is correlated with the growth of the stroma. Stromal cell cultures were established to study cytokine regulation of MMPs and TIMPs. MMP-9, MMP-2 and TIMP-1 expression was induced by PMA, TNF-α and IL-1β yet no TIMP-2 was expressed. Combinations of PMA/TNF-α and GM-CSF/ IL-1β had additive effects on induction of MMP-9 expression. Tyrosine kinase inhibitors specifically inhibited cytokine-induced MMP-9, MMP-2 and TIMP-1 expression.

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Finally, I would like to thank my parents, Tony and Donna Viani, Rocco Miccoli, Sandra Viani and all my friends for their support and encouragement throughout my life and educational endeavours.

This thesis is dedicated to my parents, Tony and Donna Viani, and to Rocco Miccoli and Sandra Viani.

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List of Symbols, Abbreviations, Nomenclature

General:

GAPDH	glyceraldehyde 3-phosphate dehydrogenase
MMP	matrix metalloproteinase
TIMP	tissue inhibitor of metalloproteinase
ECM	extracellular matrix
MT-MMP	membrane-type matrix metalloproteinase
TACE	tumor necrosis factor-alpha converting enzyme
ADAM	a disintegrin and metalloprotease
SCF	stem cell factor
GMCSF	granulocyte/macrophage colony-stimulating factor
GCSF	granulocyte stimulating factor
TNF-α	tumor necrosis factor alpha
IL	interleukin
TGF-β	transforming growth factor-beta
EGF	epidermal growth factor
TPA	tetradecanoyl phorbol acetate
TRE	TPA responsive element
TIE	TGF-β inhibitory element
EPA	erythroid potentiating activity
PCR	polymerase chain reaction
ACTH	adrenocorticotropin
HGF	hepatocyte growth factor
PMA	phorbol myristate acetate
HPC	hematopoietic progenitor cells
BHK	baby hamster kidney
LTBMC	long-term bone marrow culture
BFU	blast forming unit
CFU	colony forming unit
uPa	urokinase plasminogen activator
DNA	deoxyribonucleic acid
RNA	ribonucleic acid
mRNA	messenger RNA
cDNA	complementary DNA

Unit of Measurement:

kDa	kiloDalton
kb	kilobase
°C	degrees Celsius
M	molar
mM	millimolar

μM	micromolar
mg	milligram
μg	microgram
ng	nanogram
L	liter
ml	milliliter
μL	microliter

Chemicals and Solutions:

BCA	bibinchoninic acid
BSA	bovine serum albumin
PBS	phosphate buffered saline
FCS	fetal calf serum
SDS	sodium dodecyl sulfate
PAGE	polyacrylamide gel
DMSO	dimethyl sulfoxide
EDTA	ethylenediaminetetraacetic acid
TRIS	tris-(hydroxymethyl)aminomethane

Chapter 1: Introduction

1.1 Overview of Hematopoiesis

Hematopoiesis is the formation and development of mature blood cells from stem cells. During embryonic development, hematopoiesis takes place in the yolk sac and progresses to the liver, spleen and bone marrow (Morrison SJ et al., 1995). In adult life, hematopoiesis is maintained primarily in the bone marrow. A hematopoietic stem cell is pluripotent and can differentiate into a number of cell types through different pathways generating cells such as erythrocytes, granulocytes, monocytes, mast cells, lymphocytes and megakaryocytes (Klein G, 1995). The ability for stem cells to differentiate and proliferate relies on their interaction with the surrounding microenvironment (Klein G, 1995; Ogawa M, 1993).

The hematopoietic microenvironment is composed of stem cells, progenitor cells, cytokines, stromal cells (fibroblasts, macrophages, endothelial cells, adipocytes) and a complex extracellular matrix (Mayani H et al., 1992). In adult bone marrow, hematopoiesis is regulated through cell-cell contact, cell-matrix contact or through secreted regulatory molecules, such as cytokines.

The stromal cells of the hematopoietic microenvironment have been shown to produce granulocyte/macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), IL-4, Il-6, Il-7, and erythropoietin (Kuby J, 1993). G-CSF, IL-3, IL-6 and stem cell factor (SCF) produced from the stromal cells stimulate proliferation and differentiation of hematopoietic progenitor/stem cells (Fukushima N and Ohkawa H, 1994; Ogawa M, 1993).

In addition to being stimulated by cytokines, stem cells and progenitor cells interact directly with the extracellular matrix and other adhesion molecules to aid in

their localization and differentiation in the microenvironment. Adhesion of stem cells and progenitor cells to extracellular components or stromal cells of the bone marrow microenvironment results in the co-localization of these cells and factors at a particular point in development (Verfaillie CM, 1998). This allows for the progenitor cells to be sequestered into proper niches for their development and proliferation. This further emphasizes that contact between progenitors and marrow stromal cell ligands is important in regulating hematopoiesis (Eaves CJ et al., 1991; Verfaillie CM and Catanzaro P, 1996). It has been hypothesized that cytokines act with the extracellular matrix molecules to help anchor stem cells in this hematopoietic microenvironment and thus regulate their function (Long MW et al., 1992). Long *et al.*, demonstrated that the extracellular matrix (ECM) protein thrombospondin and c-kit ligand induce signals that modulate hematopoietic stem cell function. Studies conducted by Simmons *et al* (1992) demonstrated that adhesion of CD34+ cells to marrow stromal cells is inhibited by monoclonal antibodies to integrin molecules. This indicates the potential importance of localizing early progenitor cells in the hematopoietic microenvironment (Simmons PJ et al., 1992). Non-cellular components of the ECM such as fibronectin, laminin, collagen and glycosaminoglycans are also important in localization of hematopoietic progenitor cells in the bone marrow (Coulombel L et al., 1988). It has been shown that type IV collagen and laminin are present in the stroma of long-term hematopoietic cell cultures (Zuckerman KS and Wicha MS, 1983). In addition, erythropoietic and granulopoietic progenitor cells interact differently with the ECM which has an effect on their differentiation (Coulombel L et al., 1988). It has also been shown that

progenitor cells growing in a stroma non-contact transwell are maintained and can differentiate (Verfaillie CM, 1992). Interestingly, more colony-forming cells were recovered from 5-week old cultures than from cultures in contact with the stroma. This suggests that the contact between the hematopoietic progenitor cells and the stroma may inhibit proliferation, as well as induce proliferation as seen in previous studies. It was demonstrated that fibronectin present in stroma can serve as an inhibitor to progenitor proliferation (Hurley RW et al., 1995).

The expression and function of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs), which play key roles in ECM turnover, cell growth and cell differentiation, have not been extensively studied in the hematopoietic microenvironment. As the hematopoietic microenvironment is a complex conglomerate of cytokines, progenitor cells, stromal cells, growth factors and extracellular matrix components, there is rationale to hypothesize that MMPs and TIMPs play important roles in hematopoiesis.

1.2 Overview of the Extracellular Matrix

The ECM serves to provide structural support and to be a source of growth factors important for cell proliferation. The ECM also provides support for the attachment of cells while they proliferate and differentiate. Thus the ECM can influence the formation and function of cells.

There are three groups of macromolecules that constitute the ECM: 1) the fibrous structural proteins such as collagens and elastins; 2) adhesive glycoproteins such a fibronectin and laminin and, 3) proteoglycans and hyaluronan. There is

substantial evidence that ECM turnover is regulated by matrix metalloproteinases (MMPs) and their endogenous inhibitors, tissue inhibitors of metalloproteinases (TIMPs) (Birkedal-Hansen H, 1995).

The extracellular matrix is an integral part of the hematopoietic microenvironment and in light of the regulatory control that MMPs and TIMPs exert over the ECM, it hypothesized that MMPs and TIMPs are integral in the function of the hematopoietic environment.

1.3 Matrix Metalloproteinases

1.3.1 Proteases

The degradation of the ECM may occur through one of four pathways: the matrix metalloproteinase (MMP) pathway; the plasmin-dependent pathway; the polymorphonuclear leukocyte serine proteinase pathway and a phagocytic pathway wherein material is digested by lysosomal cathepsins (Birkedal-Hansen H et al., 1993). The migration of cells across barriers requires degradation of the ECM. This involves serine proteases, specifically the plasminogen/plasmin system and the MMPs (Vassalli J-D and Pepper MS, 1994). MMPs are a family of zinc-dependent endopeptidases, which degrade components of the extracellular matrix such as collagen, laminin and proteoglycans. All MMPs share the following characteristics; 1) they have a catalytic mechanism that is dependent on a zinc ion at the active center for their ability to cleave one or more extracellular matrix components; 2) they are secreted as zymogens which are then activated by the removal of a 10kDa segment from the amino-terminus; 3) tissue inhibitors of metalloproteinases inhibit the activity

of MMPs; 4) MMPs require Ca^{2+} for full activity; and 5) they are inhibited by chelating agents such as EDTA (Mauviel A, 1993).

1.3.2 Classification

MMPs have been subgrouped into five classes based on their substrate specificity and protein structure: collagenases, stromelysins, gelatinases, membrane bound MMPs (MT-MMPs) and others. There are approximately 20 characterized MMPs (Table 1). The MMPs that are of interest to the current study are the gelatinases (discussed in section 1.4), MT-MMPs and TACE (ADAM-17) a member of the disintegrin and metalloprotease (ADAM) family.

The first MT-MMP was cloned in 1994 (Sato H et al., 1994). To date, six MT-MMP molecules have been cloned. These molecules are closely related and share 30-50% homology. Sato *et al.* also showed that MT-MMP expression at the cell surface induces specific activation of progelatinase A.

The ADAM family, as their name suggests, are comprised of both a potential adhesion disintegrin domain and a metalloprotease domain. The metalloprotease domain contains the conserved sequence which binds a zinc ion in a configuration required for catalytic activity (Millichip MI et al., 1998). The disintegrin-like domain of the ADAM family is likely to be a ligand for integrins or other receptors (Wolfsberg TG et al., 1995). ADAMs have common domain organization that allows these proteins to function in proteolysis, cell adhesion, cell signaling and cell fusion (Werb Z and Yan Y, 1999). Due to the potential signaling domain within the

Group	Members	MMP designation	Main substrate
Collagenase	Interstitial collagenase	MMP-1	Fibrillar collagens
	Neutrophil collagenase	MMP-8	Fibrillar collagens
	Collagenase-3	MMP-13	Fibrillar collagens
	Collagenase-4	MMP-18	Xenopus protein
Gelatinases	72-kDa Gelatinase A	MMP-2	Gelatin, types IV, V collagens, fibronectin
	92 kDa Gelatinase B	MMP-9	Gelatin, types IV, V collagens, fibronectin
Stromelysins	Stromelysin-1	MMP-3	Aggrecan, Collagens III,IV,V,IX,XI; gelatins; fibronectin; laminin; elastin
	Stromelysin-2	MMP-10	Aggrecan, Collagens III,IV,V,IX,XI; gelatins; fibronectin; laminin; elastin
	Matrilysin	MMP-7	Laminin, non-fibrillar collagens, fibronectin
	Stromelysin-3	MMP-11	α proteinase inhibitor (serpin)
MT-MMPs	MT1-MMP	MMP-14	Pro-MMP-2, collagens, gelatin
	MT2-MMP	MMP-15	Pro-MMP-2, collagens, gelatin
	MT3-MMP	MMP-16	Pro-MMP-2, collagens, gelatin
	MT4-MMP	MMP-17	Pro-MMP-2, collagens, gelatin
	MT5-MMP	MMP-24	Not known
	MT6-MMP		Not known
Others	Metalloelastase	MMP-12	Elastin
	Enamelysin	MMP-20	Not known
	Xenopus MMP	MMP-?	Not known
	Not known	MMP-19	Aggrecan

Table1: Members of the matrix metalloproteinase (MMP) family (Yong VW et al., 1998)
MMP-? Refers to MMPs where the numerical designation has not yet been assigned.

cytoplasmic component of MT-MMPs and ADAMs, these molecules are likely to play important roles in hematopoiesis.

1.3.3 Structural Features

All MMPs are secreted as proenzymes, which require cleavage for their activation. In addition, all MMPs have sequence similarities and share common domains. The structure of the MMP proteins consist of several domains (Figure1). Primarily there is a signal peptide domain which directs the translation product to the endoplasmic reticulum (Hewitt RE et al., 1996) followed by an amino-terminal propeptide domain. This propeptide domain is approximately 80 amino acids and has a conserved PRCG(V/N)PD sequence. The removal of this propeptide region results in an active MMP enzyme. The cysteine within the conserved sequence, also known as the "cysteine switch", is bound to the catalytic zinc ion to maintain the proenzyme structure (Van Wart HE and H., 1990). Alternatively, MT1-MMP, MMP-11 and MMP-14 are cleaved by intracellular furin through a RRKR sequence (reviewed in Toi M et al., 1998).

The catalytic domain is approximately 170 amino acids and contains the zinc-binding site, HEXXHXXGXXH. There is also a conserved methionine that forms a "Met-turn" structure. This domain contains a five stranded β-sheet, three α-helices and some bridging loops (Nagase H and Woessner JF, 1999). There is a second zinc ion associated with the catalytic domain but little is known about its function. The catalytic zinc, at the active site, is coordinately bound by three conserved

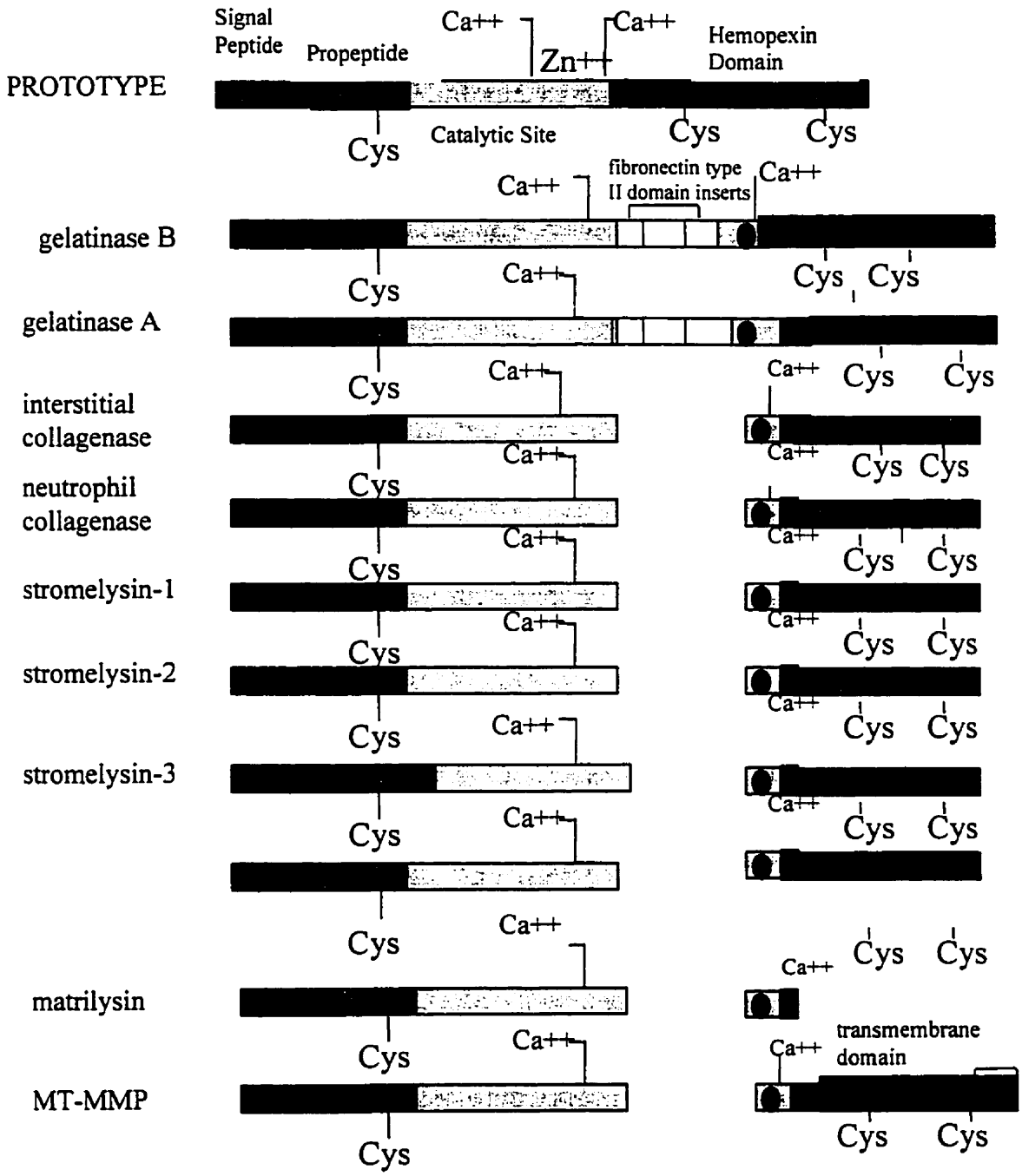


Figure 1: Domain Structure of the Matrix Metalloproteinases (MMPs). The MMP family has a five-domain structure. Three tandem fibronectin types II repeats are characteristic of the gelatinases. Matrilysin lacks an hemopexin-like domain. MT-MMPs possess a transmembrane domain.

histidine residues and two or three calcium ions to maintain stability (Massova I et al., 1998).

The third domain conserved between most MMPs is the hemopexin-like domain at the carboxy-terminal end. This domain is approximately 210 amino acids and has a disk shape with a four bladed propeller structure (Nagase H and Woessner JF, 1999). This domain is also a requirement for the cleavage of triple helical interstitial collagens and for the cell surface activation of pro-MMP-2 by MT-MMPs (Bode W et al., 1999). MMP-7, which is the smallest of the MMP molecules, lacks the hemopexin domain. There is a hinge region linking the catalytic domain and the hemopexin domain. This region is proline rich and is suggested to play a role in substrate specificity (Birkedal-Hansen H et al., 1993). In addition to the domains outlined previously, MT-MMPs also possess a transmembrane domain which anchors these molecules to the cell surface.

MMPs genes are highly conserved and each contains nine exons and ten introns. The genes range in size from 8-12 kbp. MMP-7 lacks exons 7-10 and half of exon 6. The 7-10 region encodes for the hemopexin domain and the partial exon 6 region encodes for the hinge region (Birkedal-Hansen H et al., 1993).

1.3.4 Regulation

Together, the MMPs are capable of digesting all protein components of the ECM. Due to their highly destructive potential, MMPs must be tightly regulated. The regulation of MMPs is a complex process that occurs at many different levels: 1) MMPs are regulated at the bioactivation level wherein they are secreted as inactive

proenzymes which require cleavage of the propeptide domain for activation, 2) MMPs are regulated at the transcriptional and translational level, 3) MMPs undergo post-translational modifications and 4) MMPs are regulated by endogenous inhibitors, tissue inhibitors of metalloproteinase (TIMPs).

As mentioned earlier, MMPs are secreted as zymogens and must be cleaved in order to become active (Figure 2). Interstitial collagenase, stromelysin and type IV collagenases (gelatinases) can be activated *in vitro* by organomercurial compounds (Yong VW et al., 1998). There are four mechanism by which MMPs can be activated *in vivo*: 1) the extracellular activation by non-MMPs, 2) the extracellular activation by other MMPs, 3) the intracellular activation of MT-MMP and stromelysin-3 by furin, and 4) the MT-MMP activation of MMP-2 (reviewed in Corcoran ML et al., 1996). Endogenous activation of MMPs is carried out by plasmin (Mauviel A, 1993) through a urokinase plasminogen activator-dependent (uPA) pathway (Yong VW et al., 1998) (Figure 3). In this system, uPA converts plasminogen to plasmin that is capable of activating collagenase enzymes. Other MMPs may activate each other as seen in the case of matrilysin being able to activate stromelysin-1 and proMMP-9 (Corcoran ML et al., 1996). The activation of MMPs by furin occurs before the enzyme is secreted. Furin is a serine protease which recognizes the RXKR sequence that is found in stromlysin-1, -2 and -3 and MT-MMPs (Corcoran ML et al., 1996).

The MT-MMP family has been identified in many cell extracts from various organs and tissues by Northern Blot analysis. It has been demonstrated that MT1-MMP is regulated by cytokines such as TNFα, IL-1β, epidermal growth factor (EGF)

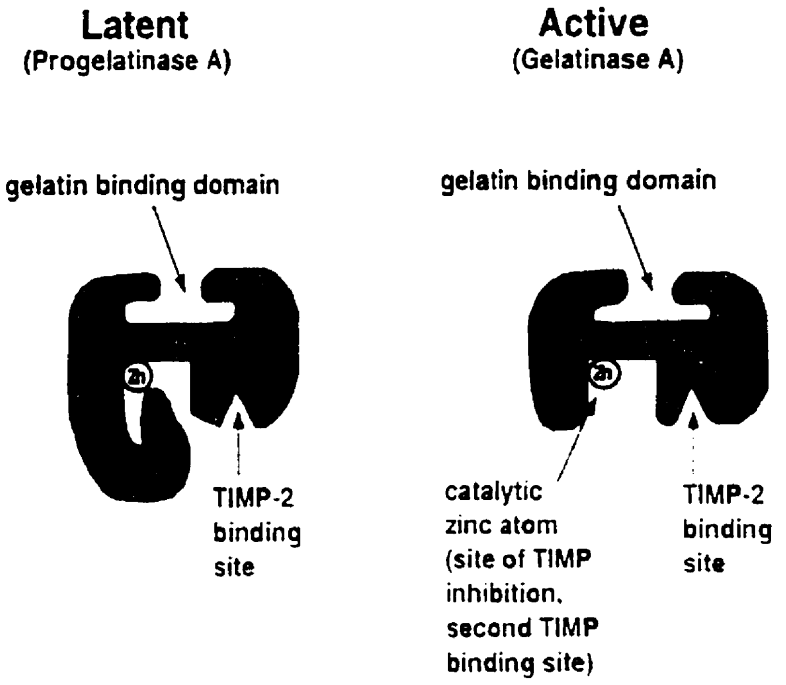


Figure 2: Activation of MMP-2.
Progelatinase has three essential domains: the pro-domain with a conserved cys residue which interacts with the Zn atom to maintain inactivity; the gelatin binding domain; and the TIMP-2 binding site at the carboxy-terminus. This enzyme can be activated through removal of the pro-domain. Upon cleavage of the pro-domain, another TIMP binding site becomes available.

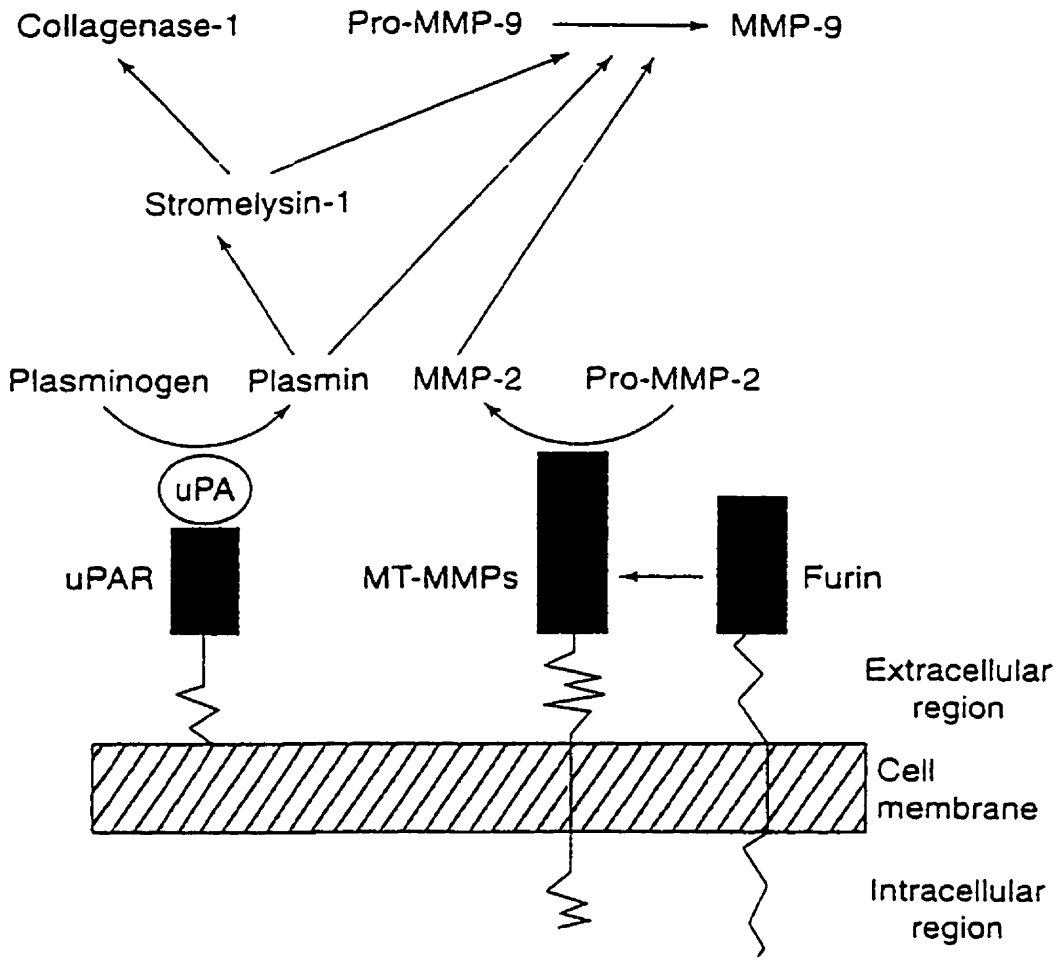


Figure 3: Activation Mechanism of Matrix Metalloproteinases.
The activation of many MMPs is initiated by the cleavage of plasminogen into plasmin. The cleavage of plasminogen to plasmin is catalyzed by urokinase plasminogen activator (uPA) which is anchored by its receptor, uPAR. Plasmin activates several MMPs such as stromelysin-1, collagenase-1 and pro-MMP-9. MMP-2 is activated by MT-MMPs which are in turn activated by furin proteinases.

in synovial cells and basic fibroblast growth factor (bFGF) in human embryonic lung fibroblasts (reviewed in Knauper V and Murphy G, 1998).

It was also found that MT-MMPs are expressed in stromal components of human carcinomas, suggesting a role in tumor progression (Okada A et al., 1995; Yoshizaki T et al., 1997). It has been suggested that there is cooperation between stromal cell and tumor cells for the production of MT-MMP and that this increased expression correlates with invasiveness in cervical cancer cells (Gilles C et al., 1996).

One of the first characterized ADAMs was ADAM17 or tumor necrosis factor-α converting enzyme (TACE)(Black RA et al., 1997). TACE is a 'sheddase' that cleaves the membrane bound proTNF-α into its soluble form. TACE has been shown to be up-regulated in arthritis-affected cartilage as compared to normal cartilage. TNF-α is a pivotal cytokine in the arthritic disease process (Patel IR et al., 1998). TACE knockout mice surprisingly showed no phenotype of lacking TNF-α but they showed the phenotype of a mouse lacking epidermal growth factor (EGF) receptor (Werb Z and Yan Y, 1999). This was attributed to the participation of TACE in making ligands available for EGF receptors. Of note, TACE is inhibited by TIMP-3 but not by other TIMPs (Amour A et al., 1998).

1.4 Gelatinases

1.4.1 Structure

Gelatinases are a subgroup of the matrix metalloproteinases that include 72-kDa gelatinase/Gelatinase A/MMP-2 and 92-kDa gelatinase/gelatinase B/MMP-9. These two enzymes can degrade gelatin, fibronectin, types IV and V collagen and

elastin (Allan JA et al., 1995). Within the catalytic domain of the gelatinases there is the addition of three repeats of a fibronectin-type II domain insert (reviewed in Nagase H and Woessner JF, 1999 and Birkedal-Hansen H et al., 1993). This fibronectin-like gelatin-binding domain is thought to contribute to the substrate specificity of these enzymes (Strongin AY et al., 1993).

The two gelatinases are similar in many ways including their high affinity for latent and activated gelatin. The main difference is that MMP-9 has a 54 amino acid hinge region that is homologous to the α2 chain of type V collagen. (reviewed in Birkedal-Hansen H et al., 1993).

1.4.2 Regulation

As with the other MMPs, gelatinases are regulated transcriptionally, post-transcriptionally, through activity of TIMPs and by cleavage into their active form. MMP-2 and MMP-9 are differentially regulated and this is apparent throughout the literature. MMP-2 is constitutively expressed in many cell types and is not inducible by agents such as tetradecanoyl phorbol acetate (TPA) or IL-1 (Hewitt RE et al., 1996). MMP-9, on the other hand, is very responsive to growth factors and cytokines. The promoter region of MMP-2 is different from that of other MMPs. It lacks a TATA box (Yu AE et al., 1998) and an AP-1 site or TPA responsive element (TRE) (Overall CM et al., 1991) whereas MMP-9 has two such elements in its promoter region (Birkedal-Hansen H et al., 1993). The promoter region of MMP-2 also lacks an upstream TGF-β inhibitory element (TIE) that other MMPs possess.

This supports the findings that TGF-β does not inhibit MMP-2 production yet it has the opposite effect and stimulates its expression (Overall CM et al., 1991).

Gelatinases are also regulated by the presence of TIMPs. Like other MMPs, MMP-2 is secreted as a zymogen and requires cleavage for activity. MMP-2 is found to preferentially complex with TIMP-2 and not TIMP-1. It is the interaction between the carboxy-terminal domains of MMP-2 and TIMP-2 that act in the binding of these two molecules (reviewed in Yu AE et al., 1998).

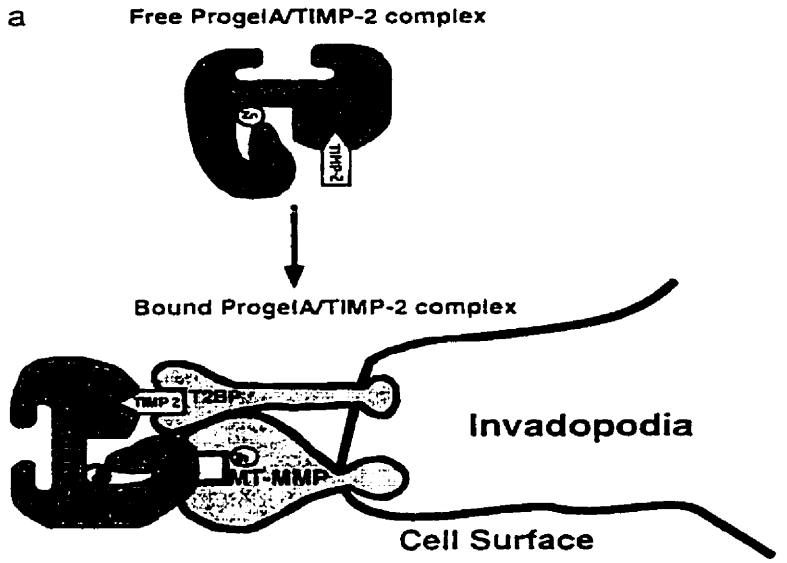
The proenzyme activation of gelatinases is another area where regulation of activity can occur. It has been shown that MT-MMPs are responsible for the cleavage of MMP-2 into its active form at the cell surface (Kinoshita T et al., 1996). Further investigation revealed that MT-MMP complexes with TIMP-2 which then sequesters and binds MMP-2 (Imai K et al., 1996). This trimeric complex then results in the activation of MMP-2 (Figure 4). It has also been suggested that MMP-2 is activated by another membrane-associated protein. Binding of pro-MMP-2 to $\alpha_v\beta_3$ integrin results in its autoproteolytic activation (reviewed in Yu AE et al., 1998 and Hewitt RE et al., 1996).

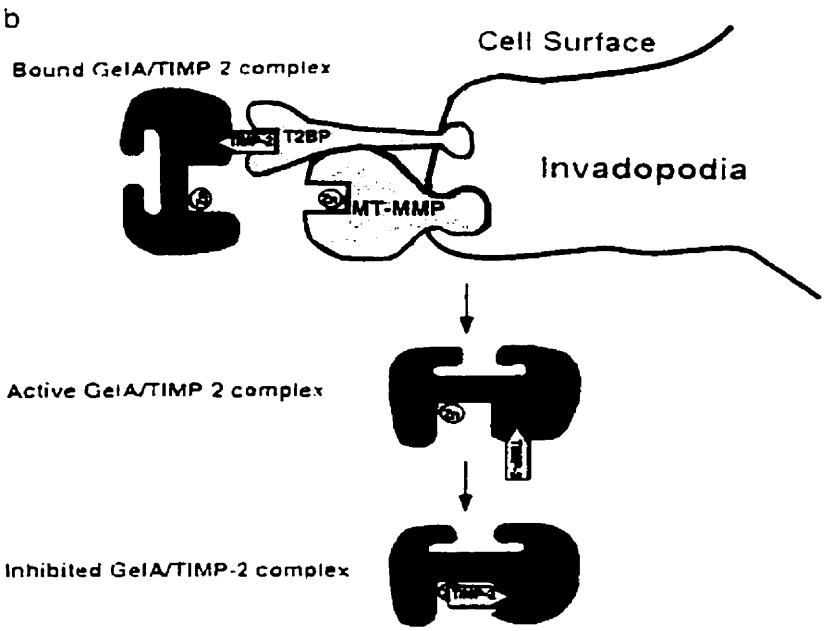
Unlike MMP-2 which is constitutively expressed in many cell types, MMP-9 is normally expressed in trophoblasts, osteoclasts, neutrophils, and macrophages (Vu TH and Werb Z, 1998) although its expression can be induced in a variety of cells by growth factors, cytokines, ECM molecules and other stimuli.

Figure 4: Activation of MMP-2 through Interactions with MT1-MMP and TIMP-2.

(a) Attachment of progelatinase A-TIMP-2 complex to a cell surface receptor. Activation of progelatinase A (MMP-2) occurs at the cell surface at the tip of projections on invading cells called invadopodia. For activation to occur, the complex must bind to MT-MMP. The attachment is mediated by TIMP-2, which binds either another MT-MMP molecule or TIMP-2-binding protein (T2BP) that is closely associated with MT-MMP. TIMP-2 binding is thought to help orient the complex for activation by MT-MMP.

(b) Once activated the gelatinase A-TIMP-2 complex detaches from the cell surface and carry out biological functions. This active state may be short lived as the complex can rearrange wherein TIMP-2 can inhibit the active site of gelatinase A.


a
Free ProgelA/TIMP-2 complex
TIMP-2
Bound ProgelA/TIMP-2 complex
TIMP 2
T2BP
MT-MMP
Invadopodia
Cell Surface


b
Cell Surface
Bound GelA/TIMP 2 complex
T2BP
MT-MMP
Invadopodia
Active GelA/TIMP 2 complex
Inhibited GelA/TIMP-2 complex

1.5 The Tissue Inhibitors of Metalloproteinases

TIMPs are a family of secreted proteins whose major function is to inhibit and regulate the activity of secreted MMPs. Their control of MMP activity is essential for normal physiologic processes such as wound healing, nerve growth, ovulation, bone remodeling, and other. The imbalance of MMP and TIMP expression contributes to pathologic conditions such as tumor invasion and metastasis, arthritis, and periodontal disease, as well as others (Parks WC and Mecham RP, 1998). There are at least four known TIMPs to date: TIMP-1, TIMP-2, TIMP-3 and TIMP-4 (Table 2). TIMP proteins differ in their structure, expression and biochemical properties (Edwards DR et al., 1996). TIMPs are different in their level of glycosylation in that TIMP-1 and -3 are glycosylated whereas TIMP-2 is not (Guedez L et al., 1996). Although TIMPs are different, they are similar in their ability to inhibit MMPs, in their genomic organization and their conserved NH_2-terminal domain (Apte SS et al., 1995). TIMPs bind to MMPs in a 1:1 ratio resulting in loss of MMP activity (Stetler-Stevenson WG, 1997).

TIMP-1 and TIMP-2 preferentially form non-covalent complexes with proMMP-2 and proMMP-9 (Howard EW et al., 1991) respectively, to block possible cleavage into their active enzymatic forms. Both TIMP-1 and TIMP-2 bind with active MMP-2 to inhibit its activity. TIMP-2 can also interact with MT1-MMP forming a complex that inhibits the activation of proMMP-2 (Kinoshita T et al., 1996).

TIMP-3 is another member of the TIMP family yet unlike TIMP-1 and TIMP-2, TIMP-3 is secreted and bound to the extracellular matrix (Leco KJ et al., 1994).

Property	TIMP-1	TIMP-2	TIMP-3	TIMP-4
MMP inhibition	Yes, all	Yes, all	Yes	Yes
Protein size	28.5 kDa	21 kDa	24 kDa	22 kDa
Localization	Diffusable	Diffusable	ECM bound	Diffusable
Expression	Inducible	Constitutive?	Inducible	Constitutive?
EPA	Yes	Yes	?	?
Pro-MMP complex	MMP-9	MMP-2	MMP2/9	MMP-2
Major tissue sites	Bone, ovary	Placenta	Kidney, Brain	Heart

Table 2: Properties of Tissue Inhibitors of Metalloproteinases.

TIMP-3 has been shown to promote detachment of mature cells from the ECM and to accelerate morphological changes in transformed cells. In addition, up-regulation of TIMP-3 has been associated with the blockage of cell cycle at G_0 phase during differentiation of HL-60 leukemia cells (Gomez DE et al., 1997).

TIMP-4 possesses all the homologous characteristics of the other TIMPs. TIMP-4 structure shares 37% sequence identity with TIMP-1 and 51% with TIMP-2 and -3. TIMP-4 is tissue specific showing abundant transcript expression in the human heart and very low levels in kidney, placenta, colon, and testes whereas no transcripts were found in liver, brain, lung, thymus or spleen (Greene J et al., 1996). TIMP-4 has shown inhibition of tumor growth and metastasis of human breast cancer cells (Wang M et al., 1997).

1.5.1 Structure/Function

TIMPs range in size from 21-28.5 kDa. Characteristic of all TIMPs is a conserved 12 cysteine residue that folds into the protein forming 6 disulfide bonded loops in a two domain structure (Figure 5)(Edwards DR, in press; Edwards DR et al., 1996; Gomez DE et al., 1997). The NH_2-terminal domain comprises three of the loops. This domain folds independently of the carboxy-terminus and it is said to be sufficient for MMP inhibition (Edwards DR et al., 1996; Murphy G et al., 1991). The NH_2-terminal domain in all TIMPs contains a consensus sequence, VIRAK, which is essential for MMP inhibitory activity. In addition, it is believed that a 29 amino acid

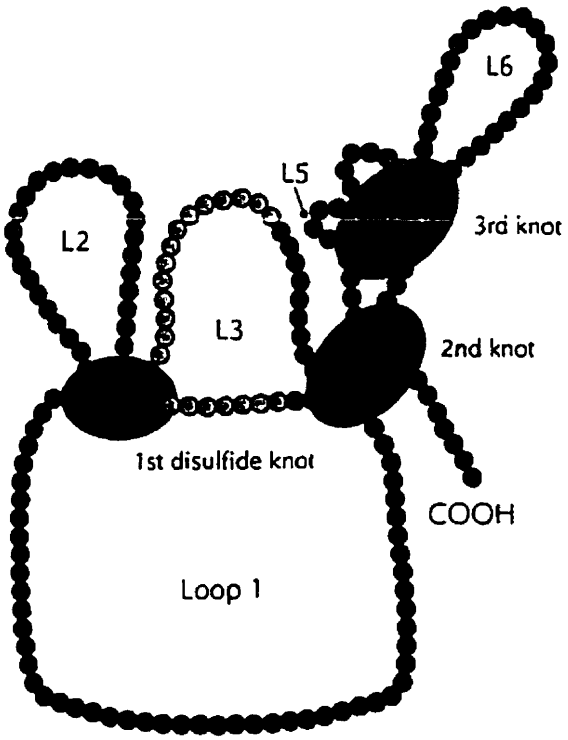


Figure 5: Diagram of the structure of TIMP-1.
The positions and structure of the six loops (L) and the three disulfide knots are shown. The NH_2-terminal domain is comprised of loops 1, 2 and 3. This domain is sufficient for MMP inhibition.
(adapted from Birkedal-Hansen, 1995)

sequence is cleaved to produce the mature protein (Gomez DE et al., 1997). Structural studies have shown that the NH_2-terminal domain is a 5-stranded β-sheet that folds over into a barrel form. This barrel formation is similar to the oligonucleotide/oligosaccharide-binding (OB) fold exhibited by bacterial enterotoxins and nucleases and yeast apartyl-tRNA synthesase (Williamson RA et al., 1994). X-ray crystallographic studies of the MMP-3/TIMP-1 complex indicate that TIMP-1 is elongated and fills the entire active site cleft of the MMP molecule (Gomis-Ruth FX et al., 1997) (reviewed in Edwards DR, in press). In addition, it has been demonstrated that the sequence between cysteine 3 and 13 is important in MMP inhibition (O'Shea M et al., 1992).

Although studies involving the digestion of the carboxy-terminus of TIMP-1 protein indicated that this section was not necessary for inhibition of MMPs (Williamson RA et al., 1993), it is important in the formation of complexes with gelatinases. The carboxy-terminal domain of TIMPs interacts with the hemopexin-like domain in the carboxy-terminal end of MMPs (Murphy G and Knauper V, 1997; Overall CM et al., 1999). It has been shown that TIMP-1 and TIMP-3 bind to the hemopexin-like carboxy-terminal domain of MMP-9 and that TIMP-2, TIMP-3 and TIMP-4 bind with MMP-2 (Bigg HF et al., 1997; Butler G S et al., 1999; Butler GS et al., 1998; O'Connell JP et al., 1994). The interactions of the carboxy-terminal domains serve to dock the TIMP protein with an active MMP and accelerate the interaction of the amino-terminal domain of the TIMP molecule and the catalytic site of MMP, thus blocking activity. Therefore, it can be conferred that the NH_2-terminal domain of TIMP has the inhibitory function, while the carboxy-terminal domain

maintains binding specificity (Yu AE et al., 1998). In addition, the association of the two carboxy-termini results in blocking the cleavage of the pro-gelatinases into their active form (reviewed in Edwards DR, in press). In fact, pro-MMP-9 is less likely to be activated by other MMPs when it complexes with TIMP-1 (Itoh Y and H., 1995). In a very interesting turn of events, the pro-MMP-2/TIMP-2 complex interacts with the membrane type MMPs in order to become activated. The amino-terminal domain of TIMP-2 inhibits MT1-MMP, on the cell surface. This association creates a receptor-like structure which can bind pro-MMP-2 (Yu AE et al., 1998). The specificity of TIMP-2 binding with pro-MMP-2 may be due to a highly acidic carboxy-terminus of TIMP-2 which is absent in TIMP-3 and TIMP-4, therefore rendering them incapable of forming the receptor-like complex to activate pro-MMP-2 (Edwards DR, in press). It has been demonstrated that MT1-MMP is tightly regulated by TIMP-2 and TIMP-3, whereas TIMP-1 is not an effective inhibitor (Will H et al., 1996).

1.5.2 TIMPs as Growth Modulating Factors

The main function of TIMPs known to date is to inhibit MMPs. In modifying the function of MMPs, TIMPs in turn play vital regulatory roles in ECM degradation, wound healing, and other physiologic activities. It wasn't until the discovery of the erythroid-potentiating activity of TIMP-1 and TIMP-2 that it became apparent that TIMPs had an even more diverse role (Stetler-Stevenson WG et al., 1992). There have been many reports of TIMP growth promoting activity in a variety of cell types

(reviewed in Corcoran ML et al., 1996; Denhardt DT et al., 1993; Edwards DR, in press; Edwards DR et al., 1996; Gomez DE et al., 1997).

Investigations revealed that TIMP-1 had proliferative effects on the human keratinocytes (Bertaux B et al., 1991), Burkitt's lymphoma cell line Raji and gingival fibroblasts (Hayakawa T et al., 1992). Hayakawa *et al.* cultured gingival fibroblasts and Raji cells in synthetic media lacking serum and observed that neither cell line was able to grow. Upon the addition of rTIMP-1, both cell lines grew almost to the level of being supplemented with 10% FCS, thus supporting their hypothesis that TIMP-1 had growth promoting properties. In addition, Raji cells do not secrete MMPs, therefore suggesting that the TIMP-1 proliferative activity on these cells is independent of MMP inhibition (Hayakawa T et al., 1992). It was soon realized that TIMP-2 also possessed the EPA sequence and in addition to TIMP-1, had the ability to support the growth of erythroid precursors (CFU-E and BFU-E) as well as the K562 human erythroleukemia cell line (Avalos BR et al., 1988). Antibodies developed to block the EPA region of TIMP-1 and TIMP-2 were capable of preventing proliferative activity of the TIMPs (Stetler-Stevenson WG et al., 1992). This suggests that TIMP-1 and TIMP-2 both possess a similar epitope for mediating the growth of hematopoietic stem cells (Corcoran ML et al., 1996; Stetler-Stevenson WG et al., 1992). Following the discovery of the TIMP-2 EPA sequence, it was reported that the SV40-transformed human fibroblast cell line, HSF4-T12, secreted TIMP-2 into conditioned media which stimulated growth of normal human foreskin fibroblasts (Nemeth JA and Goolsby CL, 1993). TIMP-2 was subsequently shown to promote growth of Raji cells (Hayakawa T et al., 1994) and Hs68 fibroblasts and

HT1080 fibrosarcoma cells (Corcoran ML and Stetler-Stevenson, 1995). Corcoran *et al.* demonstrated that, in the absence of serum or exogenous growth factors, rTIMP-2 stimulated growth of normal dermal fibroblasts and fibrosarcoma cells by promoting adenylate cyclase to produce cAMP which, in turn, activates cAMP-dependent protein kinase (PKA). The pituitary hormone adrenocorticotropin (ACTH) induces the expression of TIMP-2 through the cAMP pathway as well (Quirin N et al., 1999). It has been suggested that another mechanism by which TIMP-2 acts to stimulate growth is through a co-factor effect with insulin (Nemeth JA et al., 1996). As mentioned earlier, the actions of TIMPs on MMPs is independent of its growth promoting activity since inactivation of TIMP-2 through the addition of alanine to the amino-terminal domain still promotes fibroblast growth (Wingfield PT et al., 1999) and akylation and reduction of TIMP-2 does not effect its growth promoting abilities (Hayakawa T et al., 1994).

In addition to the mitogenic effects of TIMPs within normal physiologic conditions, they may also promote growth of tumor cells. Elevated expression of TIMP-1 has been associated with increased malignancy in human lymphomas (Kossakowska AE et al., 1991; Stetler-Stevenson WG, 1997) and with more advanced disease in non-small cell lung cancer (Thomas P et al., 2000). As will be discussed in the next section, increase in TIMP expression also leads to suppression of tumor growth. This discrepancy indicates that in some tumors TIMPs promote growth whereas in other tumors it suppresses growth (Edwards DR, in press).

TIMPs have also been linked to other cellular functions such as cell motility/migration. TIMP-1 and TIMP-2 both inhibit endothelial cell migration

(Johnson MD et al., 1994; Murphy AN et al., 1993). Murphy *et al.* demonstrated that TIMP-2 could inhibit the *in vitro* proliferation of bFGF-induced human microvascular endothelial (HME) cells. Interestingly, cell adhesion to the tissue culture flask was stimulated by TIMP-2 and cell migration was inhibited. Using A2058 human melanoma cells transfected with retroviral TIMP-2, it was seen that there was increased cell adhesion with increased TIMP-2 secretion and decreased cell adhesion with decreased TIMP-2 secretion (Ray JM and Stetler-Stevenson WG, 1995). Similarly, TIMP-1 overexpression in human hepatoma cells suppressed cell migration, enhanced cell-cell contact and, interestingly, increased MMP-2 and MMP-9 expression (Roeb E et al., 1999). TIMP-1 complexes with procathepsin-L and stimulates steroidgenesis of Leydig cells and ovarian granulosa cells (Boujrad N et al., 1995; Corcoran ML et al., 1996). Finally, another activity of TIMP-1 is its accumulation in the nuclei of human gingival fibroblasts (Gin-1 cells) in a cell cycle-dependent manner (Zhao W-Q et al., 1998).

1.6 MMPs and TIMPs in Cancer and Metastasis

Metastasis is the spread of cancer to other sites originating from the primary site. The steps of metastasis are: escape of cells from the primary tumor, intravasation/entry of cells into the lymphatic or blood circulation, survival and transport in circulation, arrest in distant organs, extravasation/cells escaping the circulation, and cell growth to form a secondary tumor (Chambers AF and Matrisian LM, 1997). As ECM modulators, it is not surprising that MMPs and TIMPs play a role in metastasis and tumor growth. Elevated levels of various MMPs have been

seen in different malignancies and have been related to tumor behaviour (reviewed in Jones JL and Walker RA, 1997). MMPs are predominately expressed by surrounding stromal cells in response to various cytokines and growth factors that tumor cells secrete (Shapiro SD, 1998). MMP-2 knockout mice have demonstrated low primary tumor growth and decreased metastasis of Lewis Lung carcinoma cells (Itoh T et al., 1998).

MMPs have been implicated in tumor invasion and metastasis, therefore the regulatory role of TIMPs was analyzed in these settings as well. In some tumors, TIMP expression was found to inhibit tumor progression and invasion. Inoculation of LMC19 cells transfected with TIMP-1 or TIMP-2 into nude mice resulted in suppression of tumor growth, invasion and metastasis (Kawamata H et al., 1995). Over-expression of TIMP-2 in tumor cells *in vivo* by retroviral-mediated gene transfer in nude mice demonstrated a significant inhibition of tumor growth and local invasion (Imren S et al., 1996). In addition, a study of various tumor cell lines indicated that increased TIMP-3 expression correlates with decreased metastatic potential. Hepatocyte growth factor (HGF)-induced TIMP-3 expression mediates phenotypic changes and loss of TIMP-3 may enhance invasion of certain tumors (Castagnino P et al., 1998). In many cases, TIMP overexpression has been demonstrated to reduce invasion and metastasis yet, as discussed earlier, this is not always the case. For example, increased TIMP-1 plasma concentration has been indicated as a potential marker of malignant progression of prostate cancer (Jung K et al., 1997).

The ability of MMPs to degrade Il-1β and to cleave TNF-α into a soluble form, in addition to TIMP-3 inhibiting TNF-α activation, can lead one to believe that

MMPs and TIMPs may also regulate the availability of cytokines in tumors thus presenting another level of control (Westermarck J and Kahari V-M, 1999).

1.6.1 Angiogenesis

Angiogenesis is the formation of new vessels from existing endothelial-lined vessels. This differs from vasculogenesis in that the vessels are formed from pre-existing vessels rather than from the differentiation of stem cells (Stetler-Stevenson WG, 1999). For the formation of these new vessels, degradation of the existing ECM is necessary, as is the proliferation and migration of endothelial cells and synthesis of a new matrix. Although this process is required for various physiological activities, it is also necessary for the development and metastasis of tumors. As MMPs and TIMPs are key players in ECM turnover, they also are players in angiogenesis. Schnaper *et al.* have demonstrated, using an *in vitro* angiogenesis model, that the balance between MMPs and TIMPs controls endothelial cell morphology. They found that TIMPs inhibited endothelial cells elongated morphology and tube formation. The use of MMP-2 antibodies also produced this same effect. *In vivo* studies support the role of MMP-9 in angiogenesis. MMP-9 null mice exhibit 10% shorter bones than those of wild type mice. In addition, these mice have a delay in angiogenesis that is recovered when MMP-9 is supplemented (Vu TH et al., 1998).

1.7 Cytokine Regulation of MMPs and TIMPs

The bone marrow microenvironment is believed to sustain hematopoiesis by the action of cytokines secreted by bone marrow cells (Robledo MM et al., 1998). It

is through the interaction of these components that hematopoietic cells differentiate and proliferate. Two sets of molecules help maintain the steady state of hematopoiesis. These two molecular classes are cytokines and cell adhesion molecules (Levesque J-P et al., 1996).

Direct contact between hematopoietic cells and stroma has been shown not to be a necessity for proliferation and differentiation *in vitro* (Verfaillie CM, 1992) while secreted factors from the bone marrow are essential (Jazwiec B et al., 1998). Jazwiec *et al.* demonstrated that the addition of exogenous cytokines to endothelial/hematopoietic progenitor co-cultures significantly increased cell proliferation. Cytokines play different roles in the regulation of hematopoiesis. Cytokines such as the colony stimulating factors and erythropoietin act as stimulators while others such as tumor necrosis factor-α (TNF-α) and transforming growth factor-β (TGF-β) act as inhibitors (Mayani H et al., 1992). Studies of normal bone marrow specimens demonstrating steady-state hematopoiesis have shown the presence of IL-6, IL-1β, stem cell factor (SCF), and others (Gupta P et al., 1998). GM-CSF, IL-3, IL-6 and SCF are produced from hematopoietic stromal cells and have been demonstrated to stimulate proliferation and differentiation of hematopoietic stem cell and progenitor cells (Ogawa M, 1993).

MMPs are regulated at many levels including the transcriptional level. MMP expression is controlled at this level by various cytokines and growth factors through positive or negative regulatory elements of its genes (Ries C and Petrides, 1995). The expression and secretion of MMPs and TIMPs are influenced by cytokines.

The study of cytokine regulation on MMPs has been reported for many cell types. In a brief summary, phorbol myristate acetate (PMA) increases MMP-9 activity in many cells including smooth muscle cells yet has no effect on MMP-2 (Fabunmi RP et al., 1996). PMA, as well as TNF-α, have also been shown to increase MMP-9 secretion in CD4+ T cells (Johnatty RN et al., 1997). Human fibroblast cells and rat bone cells have been shown to increase the level of MMP-2 expression by stimulation with TGF-β (Overall CM et al., 1991). A study of the head and neck squamous cell carcinoma cell lines indicated that TNF-α had a large effect on MMP-9 secretion whereas IL-1β, IL-6 and interferon-γ had slight to no effect (Mann E et al., 1995). In monocytes, MMP-9 expression is upregulated by IL-1β, TNF-α and GM-CSF (Saren P et al., 1996; Zhang Y et al., 1998). Most recently, it has been reported that IL-6, IL-1β, TGF-β, or TNF-α do not regulate MMP secretion by bone marrow stromal cells (Barille S et al., 1997). TIMP-1 expression is stimulated by growth factors IL-1β, TNF-α and TGF-β, as well as PMA (Birkedal-Hansen H et al., 1993).

Growth factors that stimulate mobilization of progenitor cells into the circulation are GM-CSF and IL-6. Recently, it has been reported that peripheral blood CD34+ cells strongly expressed MMP-2 and MMP-9 whereas bone marrow CD34+ cells did not. In addition, CD34+ cells of the bone marrow were induced to secrete MMP-2 and MMP-9 by G-CSF, SCF, GM-CSF, M-CSF, IL-8, and TNF-α (Janowska-Wieczorek A et al., 1999). Within this study, it was also noted that there was a positive correlation between MMP-2 and MMP-9 secretion and CD34+ cell migration using an *in vitro* matrigel invasion assay.

TIMP-1 and TIMP-3 genes are inducible at the transcriptional level in response to stimuli such as growth factors, cytokines, tumor promoters and inflammatory mediators, whereas TIMP-2 and TIMP-4 genes are constitutively expressed (reviewed in Edwards DR, in press; Edwards DR et al., 1996). Phorbol esters and IL-1β stimulate both MMP and TIMP-1 expression. Other stimulators of TIMP-1 are TGF-β1, EGF, IL-1, and oncostatin. TNF has a stimulatory effect at low concentrations and suppression at high concentrations on TIMP-1 (reviewed in Gomez DE et al., 1997).

1.8 Signaling Mechanisms in Cytokine Stimulation of MMPs and TIMPs

MMP and TIMP gene expression is regulated by cytokines. It has been demonstrated that IL-1, TNF-α, and phorbol esters induce the expression of MMPs and of TIMPs. On the other hand, TGF-β has been shown to reduce the expression of MMP-1 and MMP-3 while it induces the expression of MMP-2 and MMP-9 in cultured fibroblasts (Mauviel A, 1993). Cytokines act to induce proliferation and differentiation through their interaction with cell surface receptors. Once the receptors are activated they induce a signaling cascade which in turn causes the expression of certain targeted genes. One major signaling cascade is the mitogen activated protein kinases. These kinases are activated by a variety of stimuli such as cytokines, growth factors and environmental stresses (reviewed in Zafarullah W, 1998).

Recently, it has been shown that MMP-9 expression is dependent on a tyrosine kinase pathway in mammary tumor cells and on p38 MAPK in PMA-induced

secretion of MMP-9 in a human squamous cell carcinoma cell line (Aguirre-Ghiso J et al., 1998; Simon C et al., 1998). Another study demonstrated that the MAPK pathway regulates the expression of MMP-9 in breast epithelial cells (Reddy KB et al., 1999). Collagen-dependent induction of MMP-13 in dermal fibroblasts required p38 activity (Ravanti L et al., 1999). In addition, tyrosine kinase and mitogen-activated protein kinase inhibitors suppressed oncostatin M -induced TIMP-3 expression suggesting that these cascades are involved in the signaling of oncostatin M leading to TIMP-3 expression (Zafarullah W, 1998). Protein kinase inhibitors were also found to reduce the production of MT1-MMP in a fibroblast monolayer culture (Li L et al., 1998).

1.9 Rationale

The bone marrow microenvironment consists of a heterogeneous population of stromal cells, progenitor cells, cytokines and ECM molecules. Cellular interactions between immature hematopoietic progenitor cells and stroma are critical in the regulation of hematopoiesis. These interactions most likely are involved in many processes of hematopoiesis such as localization of primitive hematopoietic stem cells within the bone marrow and regulation of the release of mature hematopoietic cells into the circulation (Kieran K et al., 1999).

Although cell adhesion molecules have been studied during hematopoiesis, studies of MMPs and TIMPs are scarce. As multifunctional proteins that are critical for regulation of cell motility, adhesion, proliferation and differentiation, they are potentially key players in the hematopoietic microenvironment. In particular, they

may be important in regulating proliferation and differentiation of hematopoietic progenitor cells (HPC). Due to the degradative ability of MMPs towards components of the ECM, it is easy to speculate that MMPs and TIMPs may aid in the release of the HPC into the peripheral blood after maturation.

We hypothesized that MMPs and TIMPs are expressed in the bone marrow microenvironment, as will be shown through the use of an *in vitro* model of the long-term bone marrow culture. In addition, it is hypothesized that cytokines play an integral role in the control of MMP and TIMP expression in bone marrow.

Recent studies have focused on the expression of MMPs and TIMPs in leukemic cells. It was found that gelatinases, TIMP-1 and TIMP-2 were up regulated by leukemic cells suggesting their role in acute myeloid leukemia (AML)(Janowska-Wieczorek A et al., 1999). Another study investigating bone marrow mononuclear cells demonstrated continuous production of MMP-9 and TIMP-1 and up-regulation of MMP-2 in leukemic cells (Birkedal-Hansen H et al., 1993). These studies report leukemic cell MMP and TIMP expression but to date no studies have investigated expression in normal human LTBMC or stromal cells.

Prior to formulating specific hypothesises regarding function of MMPs/TIMPs in growth modulation or matrix physiology, our goal was to obtain a comprehensive expression profile of MMP-2, MMP-9 and TIMPs during the LTBMC. We extended this study to determine the regulation of expression of these proteins by key cytokines implicated in hematopoiesis.

Furthermore, potential signal transduction mechanisms involved in this process are identified.

Hypothesis

1. MMPs and TIMPs (MMP-2, MMP-9, MT-1-MMP, TACE, TIMP-1, TIMP-2, TIMP-3 and TIMP-4) are expressed in cells of the bone marrow microenvironment.
2. Cytokines regulate the expression of MMPs and TIMPs in bone marrow stromal cells.

1.10 Objectives

There are four aims of this research project:

1. To analyze the expression of MMP-2, MMP-9, MT-1-MMP, TIMP-1, TIMP-2, TIMP-3, and TIMP-4 in long term bone marrow cultures.
2. Establishment of bone marrow stromal cells for analysis of MMP and TIMP expression.
3. To determine the regulation of MMPs and TIMPs by cytokines which are important for the hematopoietic microenvironment (GM-CSF, TNF-α, IL-1β, IL-6, TGF-β, EGF and PMA) in the bone marrow stromal cell populations.
4. To determine the mechanism of cytokine-induced MMP and TIMP expression of bone marrow stromal cells.

Chapter 2: Materials and Methods

2.1 Long-Term Bone Marrow Cultures

LTBMCs were achieved as previously demonstrated by Dexter *et al.* (1977). Briefly, normal bone marrow samples were kindly provided from Dr. Messner's laboratory (Princess Margaret Hospital, Toronto). The mononuclear cells were separated by density gradient centrifugation using 60% Percol or Ficoll-Paque (Amersham Pharmacia Biotechnology Inc., New Jersey). Cell viability of the bone marrow sample was calculated using trypan blue dye exclusion and cell counts were obtained. LTBMCs were established by seeding 1-5 x 10^6 cells per T75 flasks in myelocult myeloid long-term culture medium for primitive human hematopoietic cells (StemCell Technologies Inc., Vancouver). Myelocult was supplemented with 1% hydrocortisone, 1% anti-bacteria/anti-micotic solution and fresh 10% L-glutamine. The cultures were maintained at 5% CO_2 and 37°C for three weeks. To maintain growth of the LTBMCs, half the media was removed and replaced with fresh media on days 7 and 14. Serum free conditions were established by removing the myelocult media, washing the cells twice with 1x Dulbecco's phosphate-buffered saline (Gibco BR Life Technologies, Maryland) and replacing the media with α-MEM. Cultures were incubated for 16 -18 hours in serum-free conditions before collecting the conditioned media. Conditioned media was collected on days 3, 6, 11, 16, and 21. The total amount of protein in the conditioned media samples was determined by a bicinchoninic acid (BCA) protein assay (see section 2.4).

2.2 Isolated Stromal Cell Cultures

Stromal cells were established in culture by seeding 1 x 10^8 bone marrow cells in T150 flask in Myelocult media supplemented with 10% l-glutamine, 1% hydrocortisone, and 10% antibiotic/antimicrobial (Gibco BRL Life Technologies, Maryland). After two hours the non-adherent cells were removed and the adherent cells were washed twice with phosphate buffered saline (PBS). The cultures were then supplemented with fresh media and grown at 5% CO_2 and 37°C for 21 days. At days 7 and 14 half the media was replaced with fresh media to sustain growth. When the stromal layer reached 80% confluency, it was exposed to serum free media and the conditioned media was collected for analysis of MMP and TIMP protein expression.

2.3 Collection of Protein Samples from Cultures

Conditioned media was collected at specified time points by centrifugation (Beckman GS-6R centrifuge) at 500 g for 10 minutes and then stored at -20°C until use.

2.4 Protein Concentration Determination

Protein concentrations of conditioned media and cell lysates were determined by the BCA protein assay (Pierce Chemical Company, Illinois). One hundred microliters of each standard or sample was combined with 2.0 ml of BCA working reagent (50:1 BCA reagent A to BCA reagent B). Following mixing, the samples were incubated for 30 minutes at 37°C and subsequently cooled to room temperature.

The absorbance was measured at 562 nm versus a water reference. The protein concentrations were then determined from a BSA standard curve prepared at the same time as the samples.

2.5 Zymogram Analysis

Gelatinolytic activity was ascertained by electrophoresis in 12% polyacrylamide gels (30% acrylamide; 1.5M Tris pH8.8; 10% SDS; 10 % ammonium persulfate; TEMED) containing freshly solubilized pig gelatin (1mg/ml). Aliquots of conditioned media were combined in a 3:1 ratio with 4x gel loading buffer (200mM Tris pH6.8; 4% SDS; 0.1% bromophenol blue; 40% glycerol) and electrophoresed at 30mA per gel for 1 hour. Following electrophoresis, the SDS was extracted from the gel with wash buffer containing Triton X-100 (2.5% Triton X-100; 50mM Tris pH7.5; 5mM $CaCl_2$) three times for a duration of 15 minutes. The gels were placed in incubation buffer (50mM Tris pH7.5; 5mM $CaCl_2$) for 15-18 hours. Following incubation, the gels were stained in 30% methanol, 10% acetic acid, 0.5% Coomassie blue R-250 (Gibco BR Life Technologies, Maryland) for 3 hours and destained in 10% acetic acid 30% methanol mix until the desired intensity was obtained. Gels were dried using the Novex gel drying system following manufacturer's instructions (Novex, San Diego).

2.6 Reverse Zymogram Analysis

TIMP activity was assessed by reverse zymography, which involves electrophoresis of polyacrylamide gel with baby hamster kidney (BHK) culture-

conditioned media as a source of matrix metalloproteinase. Briefly, 12% Tris glycine SDS-PAGE gels were prepared with 1mg/ml gelatin and 1 ml BHK culture-conditioned media/10ml SDS-PAGE gel as gelatinase. BHK cells are genetically engineered to produce Gelatinase A and B and the conditioned media from BHK-105 cells is utilized for TIMP-3 detection and BHK-72 cells for TIMP-1 and TIMP-2 detection. The conditioned media from the BHK cell cultures was concentrated 10-fold using Centricon-10 concentrators (Amicon, Massachusetts). Aliquots of conditioned media were combined in a 3:1 ratio with non-denaturing 4x gel loading buffer (200mM Tris pH6.8; 4% SDS; 0.1% bromophenol blue; 40% glycerol) and electrophoresed at 30mA per gel for 1 hour. Following electrophoresis, the SDS was extracted from the gel with wash buffer containing Triton X-100 (2.5%Triton X-100, 50mM Tris pH7.5) twice for 1 hour. The gels were placed in incubation buffer (50mM Tris pH7.5; 5mM $CaCl_2$) for 24 hours. Following incubation, the gels were stained in 30% methanol, 10% acetic acid, 0.5% Coomassie blue R-250 (Gibco BR Life Technologies, Maryland) for 3 hours and destained in 10% acetic acid:30% methanol mix until the desired intensity was obtained. Gels were dried using the Novex gel drying system following manufacturer's instructions (Novex, San Diego).

2.7 Preparation of Cell Lysate for Western Blot Analysis

Non-adherent cells were obtained and pelleted at 10,000 g for 5 minutes. The media was removed from the adherent layer and the cells were washed twice with PBS. The adherent layer was lifted off by treatment with 0.6ml of ice cold RIPA buffer containing 1XPBS, 1% nonidet P-40 (BDH Chemicals), 0.5% sodium

deoxycholate, 0.1% SDS with freshly added protease inhibitor cocktail (Sigma, St. Louis). The culture dish was then washed with 0.3ml of the RIPA buffer described above. This lysate was then combined with the non-adherent cells collected earlier. The mix was then passed through a 22-gauge needle to homogenize the lysate and then incubated on ice for 60 minutes. The cell lysate was microcentrifuged at 10,000xg for 10 minutes at 4°C. The supernatant was collected and stored at -20°C for future use.

2.8 Western Blot Analysis

Western blots were performed by treating the condition media in a 1:1 ratio with 2X SDS lysis buffer (100nM Tris pH6.8; 4% SDS; 20 % glycerol; 0.2% bromophenol blue; 200mM β-mercaptoethanol) and boiling the samples for 2 minutes. After boiling, the samples were electrophoresed on a 12% SDS-PAGE for 1-1.5 hours at 20mA per gel and transferred to a nitrocellulose membrane (Gibco BRL Life Technologies, Maryland) for 1.5 hours at 15 volts using Trans-blot SD semi-dry transfer cell (Biorad, California). Once transferred, the membrane was blocked for 2 hours using 5% non-fat dry milk in PBS with 1% Tween-20 (PBS-T) for 1 hour. The membrane was incubated with the primary antibody (1:2000 dilution) in blocking buffer for 1 hour and washed 4 times in PBS-T. The membranes were incubated in 1:2500 dilution of goat-anti-mouse-horseradish peroxidase-conjugated secondary antibody for 30 minutes to one hour. The membranes were washed four times in PBS-T and developed using enhanced chemiluminescence detection system for HRP-labeled secondary (Amersham Pharmacia Biotechnology Inc., New Jersey)

as per manufacturer's instructions. The blots were exposed to Kodak film for one minute. The antibodies used in this study are outlined in Table 3.

MMP/TIMP	Antibody	Source
MMP-2	MMP-2 (Ab-1)	Calbiochem, San Diego, California
MMP-9	MMP-9 (Ab-1)	Calbiochem, San Diego, California
MT1-MMP	MT1-MMP (Ab-1)	Calbiochem, San Diego, California
TIMP-1	TIMP-1 (Ab-1)	Calbiochem, San Diego, California
TIMP-2	TIMP-2 (Ab-1)	Calbiochem, San Diego, California
TIMP-3	TIMP-3	Medicorp, Montreal, Quebec
TACE	TACE (K-20)	Santa Cruz Biotechnology, Santa Cruz, California

Table 3: List of Antibodies used for detection of MMPs and TIMPs by western blot analysis

2.9 Reverse Transcriptase (RT)-Polymerase Chain Reaction (PCR)

Total RNA was extracted using Trizol reagent (Gibco BRL Life Technologies, Maryland) from cells of days 3, 6, 11, 16, and 21 of the LTBMC and stromal cells from cytokine stimulation experiments. The RNA transcripts within the samples were then reverse transcribed using RT-PCR pre-amplification superscriptase system (Gibco BRL Life Technologies, Maryland). Briefly, 1-2μg of total RNA was combined with 100ng of Random Hexamer primers, 4μL 5X First Strand Buffer, 2μL of 0.1M DTT and 1μL of 10mM dNTP mix (10mM each of dATP, dGTP, dCTP, and dTTP). Following a 2-minute incubation at 42°C, 200 units of Superscript II was added and incubated for another 50 minutes. Heating at 70°C for 15 minutes inactivated the reaction. This RT reaction was then subsequently used for PCR analysis. Previously, some primer sets were acquired from another laboratory but were not optimizing very well, therefore new primer sets were designed using the VectorNTi software (Table 4) for TIMP-3, TIMP-4, TACE and MT1-MMP.

Gene	Sense Primer	Antisense Primer	Annealing Temperature	Product Length
MMP-2	GGC CCT GTC ACT CCT GAG AT	GGC ATC CAG GTT ATC GGG GA	ND	473 bp
MMP-9	CAA CAT CAC CTA TTG GAT CC	CGG GTG TAG AGT CTC TCG CT	ND	480 bp
MT1-MMP	ACA AGT TTG GGG CTG AGA TCA AGG	TGA TGT CGG CCT GCT TCT CAT G	59°C	228 bp
TACE	GGC AGT CTC TCC TAT TCC TGA CCA	TCC ACC ACC ACG ACC TTG AAA	57.5°C	295 bp
TIMP-1	GCG GAT CCA GCG CCC AGA GAG ACA CC	TTA AGC TTC CAC TCC GGG CAG GAT T	ND	650 bp
TIMP-2	GGC GTT TTG CAA TGC AGA TGT AG	CAC AGG AGC CGT CAC TTC TCT TG	ND	457 bp
TIMP-3	CTT CTG CAA CTC CGA CAT CGT	CAT CTT GGT GAA GCC TCG GTA	54°C	127 bp
TIMP-4	TGC CAC TCG GCA CTT GTG ATT C	GGC TGT TGG CTT CTA GTT TCA CAC C	59°C	171 bp
GAPDH	CGG AGT CAA CGG ATT TGG TCG	AGC CTT CTC CAT GGT GGT GAA GAC	59°C	306 bp

Table 4: List of Primer sequences used for analysis of MMP and TIMP expression in LTBMC. (ND= not determined).

PCR reactions were performed using one tenth of the cDNA, 10X PCR buffer, 0.25μL of 10mM dNTP mix (10mM each of dATP, dGTP, dCTP, and dTTP), 0.4μM of each primer, and 0.25 U of Taq polymerase (Gibco BRL Life Technologies, Maryland). The cDNA was denatured for 5 minutes at 94°C and then 25-35 cycles of: 50 seconds denaturation at 94°C, 1 minute of annealing at specific temperatures, and 50 seconds of extension at 72°C were performed. The final extension was for 5

minutes. The PCR products were then analyzed by electrophoresis on a 1% agarose gel with ethidium bromide incorporated.

2.10 Quantification of MMP and TIMP Activity

The protein bands of Western blot analysis, zymography, or reverse zymography, as well as the product from RT-PCR were quantified by scanning the image using an Epson ES-1000C scanner and Adobe Photoshop version 5.0. Images were converted to TIFF format and analyzed using Molecular Dynamics ImageQuant version 3.3.

2.11 Cytokine Stimulation of Bone Marrow Stromal Cells

The establishment of stromal cell population was achieved as outlined previously. Once the stromal layer was grown to confluency, the cells were removed from the flask by treatment with trypsin and plated in 48-well plate at a concentration of 2×10^5 cells/ml. The cells were left to grow to 80% confluency (~12hrs) and then exposed to serum-free media with 10ng/ml of either IL-1β, IL-6, GM-CSF, TGF-β, EGF, TNF-α (Medicorp Inc., Quebec) or 5ng/ml of PMA (Gibco BRL Life Technologies, Maryland) and incubated for 24 hrs. All cytokines were dissolved in dimethyl sulfoxide (DMSO). The conditioned media was then collected and stored at -20°C for use by zymography and reverse zymography analysis. In addition, the cells were harvested with Trizol reagent for RNA isolation and subsequent RT-PCR analysis.

Once it was noted which cytokines had the most effect on the stromal cell MMP and TIMP expression, these cytokines were combined in treatment of the stromal cells. Therefore, bone marrow stromal cells were grown as previously indicated and subsequently incubated with combinations of TNF-α+ PMA, TNF-α+ GM CSF, TNF-α+ IL-1β, IL-1β + GM CSF, and IL-1β+ EGF for 24 hrs. The conditioned media and cells were harvested and stored at -20°C for future use.

2.12 Inhibition of Cytokine-Stimulated MMP and TIMP Activity

The establishment of stromal cell population was achieved as outlined previously. Once the stromal layer was grown to confluency, the cells were removed from the flask by treatment with trypsin and plated in 48-well plate at a concentration of 2×10^5 cells/ml. The cells were left to grow to 80% confluency (~12hrs). The cells were washed twice with PBS and then exposed to serum-free media containing one of the following inhibitors PD98059 (100μM), SB203580 (50μM), Genistein (100μM) or Herbimycin A (15μM) for 2 hrs. Then PMA, TNF-α, IL-1β, TNF-α+ PMA, TNF-α+ GM CSF, TNF-α+ IL-1β, IL-1β + GM CSF, and IL-1β+ EGF were added to cells with and without inhibitor for a 24 hr incubation. The conditioned media was then collected for analysis by zymography and reverse zymography. In addition, the cells were harvested with Trizol reagent for RNA isolation and subsequent RT-PCR analysis.

2.13 Quantification of TIMP-1 by ELISA

To quantify the level of TIMP-1 in the conditioned media of cells treated with cytokines and inhibitors an ELISA method (BIOTRAK, Amersham Pharmacia, New Jersey) was used following the manufacturer's instructions.

2.14 Statistical Analysis

Results of the densitometric analyses and ELISA assay were represented as bar graphs containing error bars representing the standard deviation. Significant differences between samples or time points were determined using the Student's *t*-test (Microsoft Excel, Redmond, WA) and a P value of less than 0.05 was considered statistically significant.

Chapter 3: Results

3.1 Analysis of MMP and TIMP Expression in LTBMC and Isolated Stromal Cell Populations

In this report, we limited our studies to gelatinase A (MMP-2), gelatinase B (MMP-9), membrane-type matrix metalloproteinase 1 (MT1-MMP), and tumor necrosis factor-alpha convertase enzyme (TACE). MMP-2 and MMP-9 are of interest in this research since they are well characterized and have been studied in other systems such as the lymphatic system (Montgomery AM et al., 1993), thymus system (Aoudjit F et al., 1997) and smooth muscle cells (Fabunmi RP et al., 1996). **In addition, the combined activity of MMP-2 and MMP-9 is thought to be responsible for a significant amount of ECM degradation.** MT1-MMP is of interest because of its association with the membrane and its associated activation of MMP-2 (Tokuraku M et al., 1995). MT1-MMP, due to its membrane bound properties, is more likely to be directly involved in signal transduction pathways within the cell. TACE is a member of the ADAMs group of proteins and is also known as ADAM-17. TACE is of interest in these studies because it has been demonstrated to cleave the membrane-bound proTNF-α molecule into its soluble form (Amour A et al., 1998). In addition, TNF-α is involved in the regulation of hematopoietic differentiation and stimulation of MMP-9 in many cell lines (Ismair MG et al., 1998). More recently, it has been shown that TIMP-3 inhibits TACE activity (Amour A et al., 1998) suggesting a correlation between TACE and metalloproteinase inhibitors.

3.1.1 Morphology of the LTBMC

The LTBMC is a culture system used to simulate the hematopoietic microenvironment. This culture is a very dynamic and evolving process. Therefore, in order to study the expression profile of MMPs and TIMPs in the LTBMC many time points during the culture process were analyzed. Photomicrographs were obtained from each of the time points of the LTBMC (Figure 6). On day 1 of the LTBMC the cells appear round and suspended within the media. As the LTBMC progresses some cells begin to adhere to the bottom of the culture flask and elongate (as seen on day 3, 6 & 11). Progenitor cells that give rise to BFUs and CFUs form small to medium sized clusters on top of the adherent layer. The stromal/adherent layer of the LTBMC becomes increasingly dense as the culture progresses. As seen on day 16 & 21, there is the establishment of a confluent stromal layer.

3.1.2 Differential Gelatinase Expression in LTBMC

The LTBMCs were established and exposed to serum free conditions for 12-18 hours before collection of the conditioned media. The conditioned media was collected on days 3, 6, 11, 16, and 21. The total protein concentration in the samples was determined using the BCA protein assay system. Conditioned media containing 15 μg of total protein was subjected to zymogram and reverse zymogram analysis. Analysis was performed on bone marrow from 5 different individuals, which were collected and cultured independently.

Gelatin zymography of LTBMC supernatants (Figure 7) demonstrated two dominant bands of gelatinolytic activity with molecular weight of 72 kDa and 92

kDa. The activity at 72kDa corresponds to the molecular weight of MMP-2 zymogen and 92kDa corresponds to MMP-9 zymogen. Figure 8A depicts a graphical representation of MMP-2 expression in all five samples throughout the LTBMC (inset shows the average MMP-2 expression).

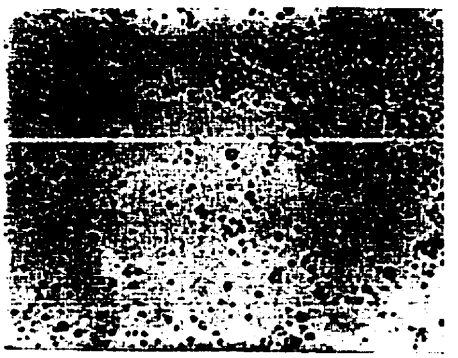
MMP-2 expression has a distinct pattern characteristically seen in all patients. The average MMP-2 expression is low (2600 IDU/μg protein) in the early stages of the LTBMC and then peaks around day 11 or 16 (10000 IDU/μg protein) followed by a plateau. There is a greater than 4-fold increase in MMP-2 expression between day 3 and day 11 of the LTBMC (p-value <0.005). In addition, MMP-2 expression was higher at the end of the LTBMC (day 21=9400 IDU/μg protein) than at the commencement (day 3=2600 IDU/μg protein)($p<0.05$) of the LTBMC.

In contrast, MMP-9 activity was seen throughout the LTBMC at a constant level between the range of 10000-12000 IDU/μg protein (Figure 8B). It can also be noted that the initial expression of MMP-9 on day 3 (10693 IDU/μg protein) is significantly higher than that of MMP-2 (2605 IDU/μg protein)($p<0.005$). Logarithmic transformations of the data were performed on the averages of MMP expression (Figure 9). The slope of the MMP-2 activity line between day 3 and 11 is 13.097 and the slope of MMP-9 is 0.00819. From this it can be concluded that there is differential expression of MMP-2 and MMP-9 within the LTBMC. This would indicate that throughout hematopoietic development, there are differences in MMP expression that may affect the development, proliferation and motility of mature blood cells.

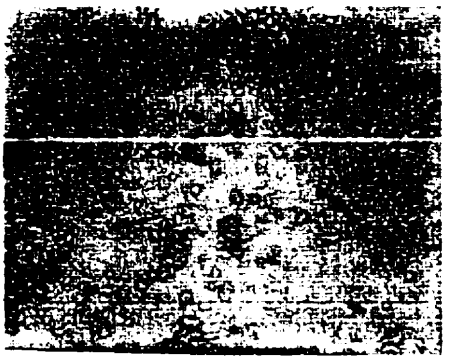
Some interesting correlations can be made between the growth of the LTBMC and the expression of MMP-2 and MMP-9. The increase in MMP-2 expression coincides with the increase in the stromal cell population. The increase in MMP-2 expression can be attributed to a growing stromal cell population suggesting that these cells are the predominant source of MMP-2. Another explanation is that the cell-cell and cell-solid support contact may induce the expression of MMP-2 by either the stromal cells or the progenitor cells.

Figure 6: Photomicrographs of the LTBMC.
Photomicrographs were taken at a magnification of 1.8x of the LTBMC at various time points. On day 1, most of the cells are suspended. As the LTBMC progresses from day 3-11 some cells began to adhere to the flask and elongate. On days 16 and 21 there is a confluent stromal/adherent cell layer and the appearance of progenitor cell colonies or hematopoietic islands.

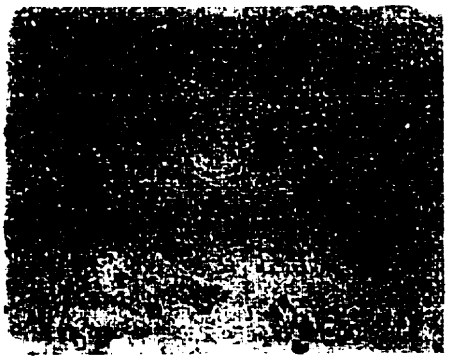
Day 1



Day 3

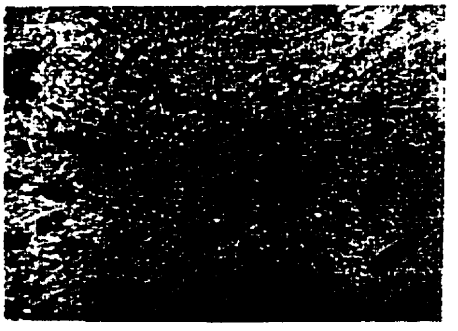


Day 6



Day 11

Day 16



Day 21

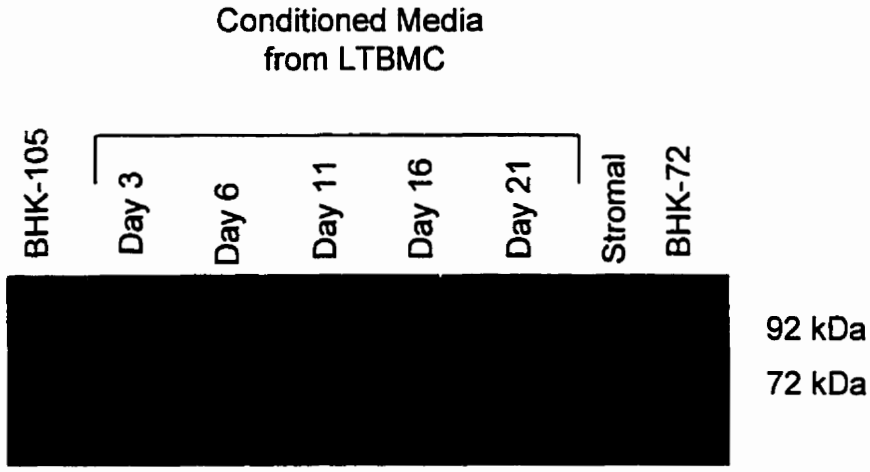
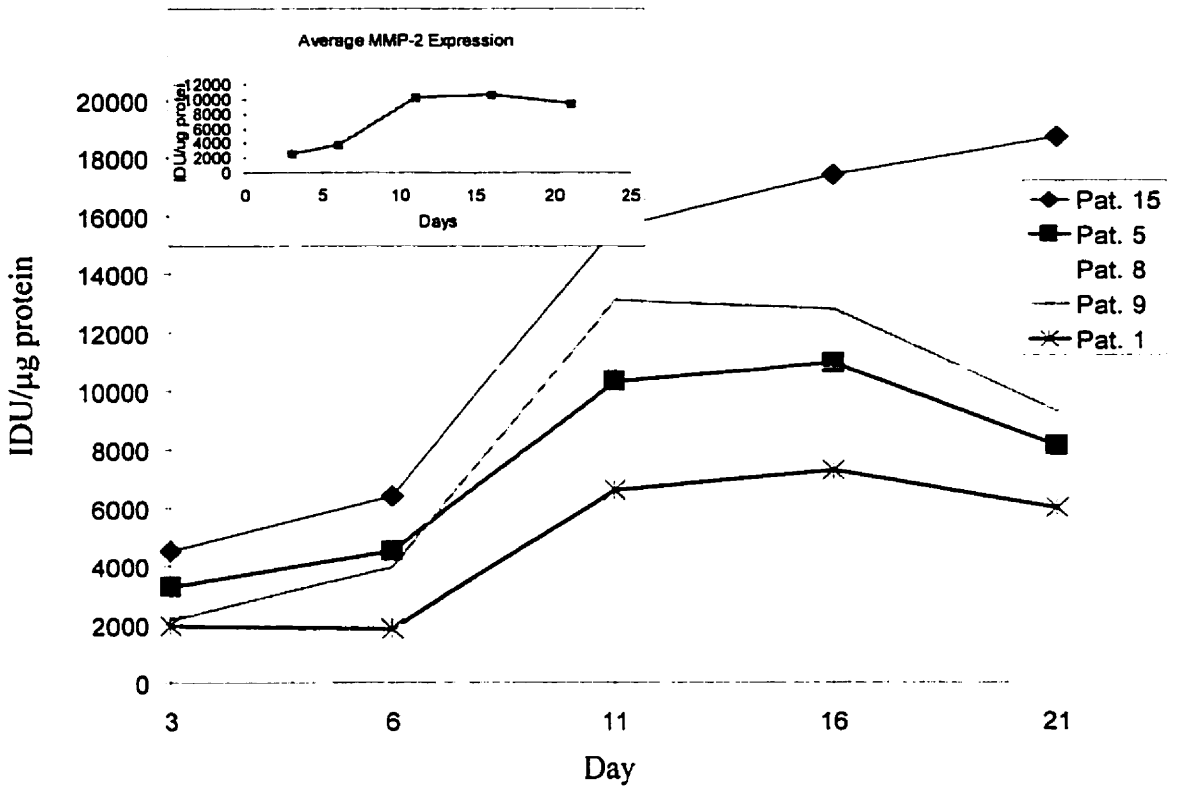


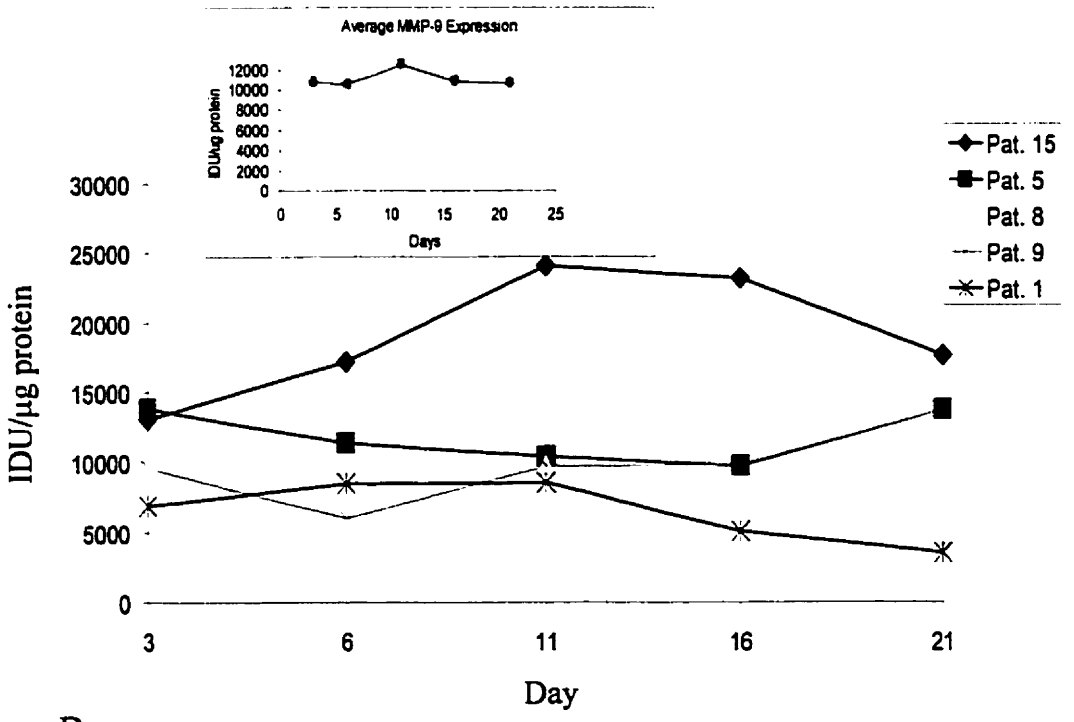
Figure 7: Zymogram analysis of conditioned media collected from the LTBMC of normal bone marrow samples on days 3, 6, 11, 16 and 21.

Mononuclear cells were separated from normal bone marrow by density gradient using Ficoll. These cells were cultured in Myelocult media supplemented with 1% hydrocortisone, 1% ntibacterial/antimicrobial solution and 10% fresh L-glutamine. The cultures were grown at 5% CO_2 and 37°C for 21 days. The conditioned media and cells were harvested on days 3, 6, 11, 16 and 21. Fifteen micrograms of total protein was subjected to gelatin substrate zymography. A representative experiment is shown here. The conditioned media obtained from BHK-105 cells was used as a positive control for MMP-9 expression and BHK-72 conditioned media as a positive control for MMP-2 expression. Conditioned media from isolated stromal cell cultures was collected and analyzed by zymography.

Figure 8: Densitometric analysis of conditioned media collected from the LTBMC of normal bone marrow samples on days 3, 6, 11, 16 and 21.
Mononuclear cells were separated from normal bone marrow by density gradient using Ficoll. These cells were cultured in Myelocult media supplemented with 1% hydrocortisone, 1% antibacterial/antimicrobial solution and 10% fresh L-glutamine. The cultures were grown at 5% CO_2 and 37°C for 21 days. The conditioned media and cells were harvested on days 3, 6, 11, 16 and 21. Fifteen micrograms of total protein was subjected to gelatin substrate zymography. MMP-2 increases in expression in the beginning of the LTBMC and subsequently plateaus (A). In contrast, there is constitutive MMP-9 expression throughout the LTBMC (B). The experiment was performed on 5 different bone marrow samples. The average of 5 samples are displayed in the inset of each graph.


Average MMP-2 Expression
IDU/ug protein
Days
IDU/µg protein
Day
Pat. 15
Pat. 5
Pat. 8
Pat. 9
Pat. 1

A.


Average MMP-9 Expression
IDU/ug protein
Days
IDU/µg protein
Day
Pat. 15
Pat. 5
Pat. 8
Pat. 9
Pat. 1

B.

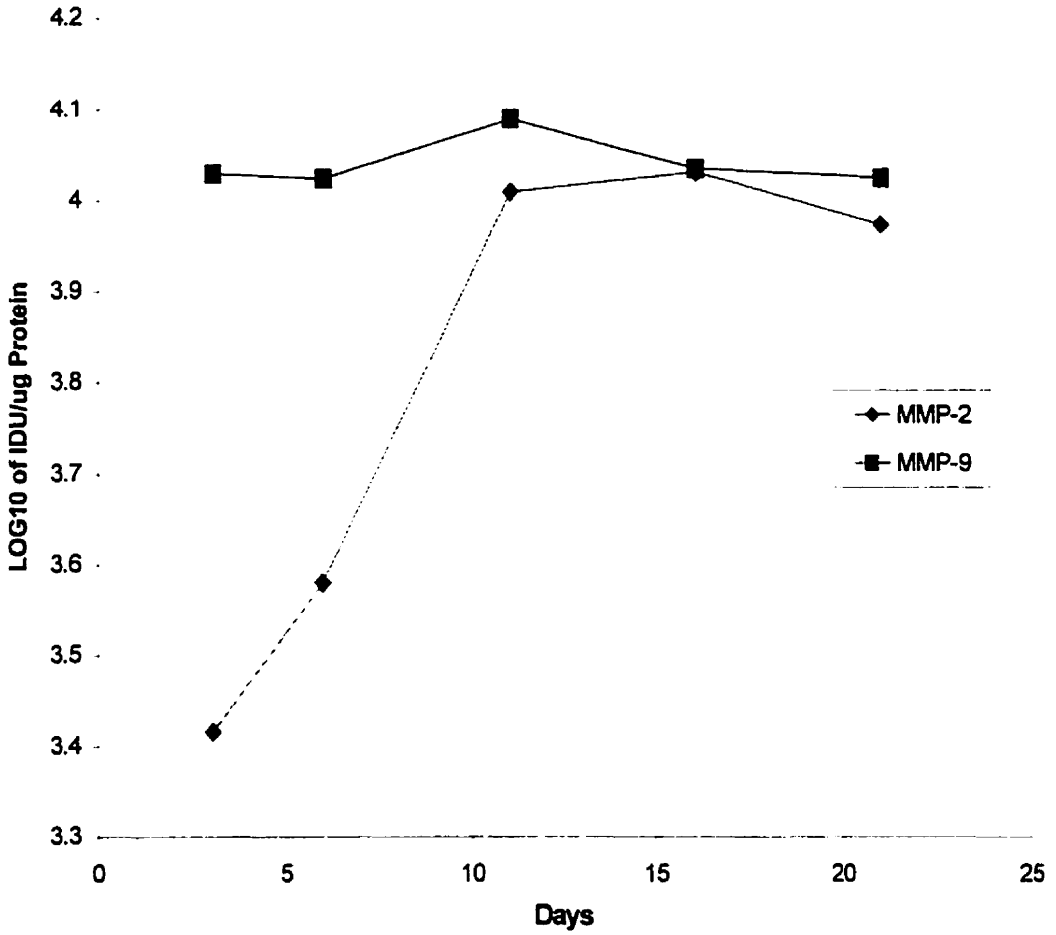


Figure 9: Logarithmic transformation of LTBMC data.
The LTBMC data was transformed logarithmically and plotted. The slope of the MMP-2 activity line is 13 whereas the slope for MMP-9 is 0.008 between days 3-11. The slope of MMP-9 is near zero and therefore reflects little change over the course of the LTBMC. MMP-2 slope indicates a significant change in expression between day 3 and 11. This further emphasizes the differential expression of MMP-2 and MMP-9.

3.1.3 Inhibition of Gelatinase Activity by Calcium Ion Chelator, EDTA

In order to confirm that the enzymatic activity seen by zymography was due to MMPs, two identical zymogram gels were run using conditioned media from the LTBMC. One gel was processed in incubation buffer and the other in incubation buffer containing 10mM EDTA. EDTA is an ion chelator used to determine the Ca2+ dependence of zinc dependent metalloproteinases. Figure 10 demonstrates that incubation with EDTA completely abrogates the enzymatic activity and further supports the identity of the gelatinases as MMPs.

3.1.4 Differential TIMP Expression in LTBMC

To analyze the activity of the endogenous inhibitor of MMPs, reverse zymograms were prepared utilizing conditioned media obtained from BHK cell lines that over-express the 72kDa gelatinase (BHK-72) and 92kDa gelatinase (BHK-105) incorporated within the gelatin-substrate gel.

Reverse zymography demonstrated that the level of TIMP-1 activity was basal (1100 IDU/μg of protein) until day 16 and 21 at which time the activity significantly increased to 2700-2800 IDU/μg of protein (Figure 11A&B). There is a greater than two fold increase in TIMP-1 expression from the beginning of the LTBMC until the end. In contrast, there was a basal level of TIMP-2 expression (1100-1400 IDU/μg of protein) throughout the LTBMC. Figure 11B depicts the increase in activity of TIMP-1 on day 16 and 21 of the LTBMC and the steady state level of TIMP-2 activity throughout the LTBMC as determined by densitometry. TIMP-1 expression is increased with the establishment of the stromal layer, an observation similar to that

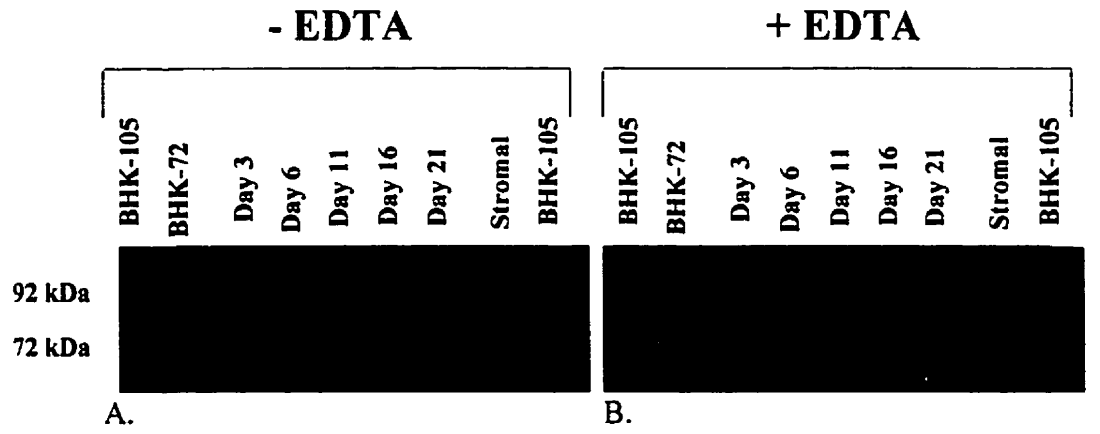
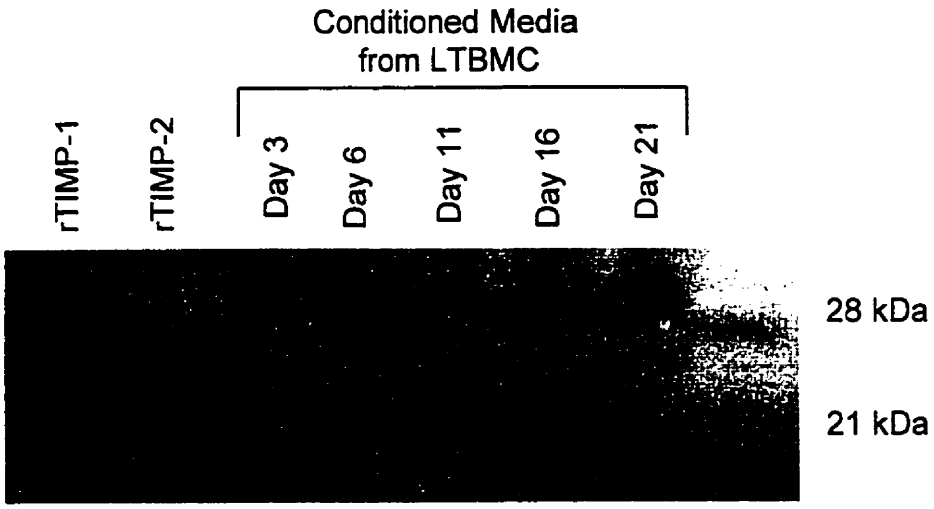


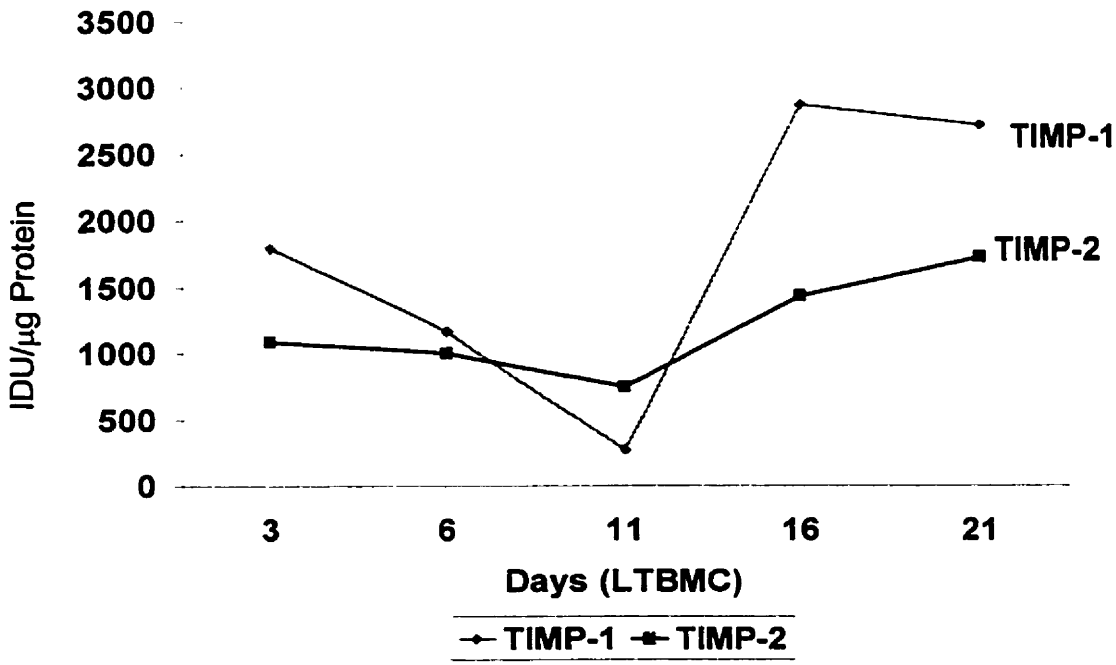
Figure 10: Analysis of EDTA treatment of zymogram gels containing conditioned media from days 3, 6, 11, 16 and 21 of the LTBMC. Mononuclear cells were separated from normal bone marrow by density gradient using Ficoll. These cells were cultured in Myelocult media supplemented with 1% hydrocortisone, 1% antibacterial/antimicrobial solution and 10% fresh L-glutamine. The cultures were grown at 5% CO_2 and 37°C for 21 days. The conditioned media and cells were harvested on days 3, 6, 11, 16 and 21. Fifteen micrograms of total protein was subjected to gelatin substrate zymography(A). Incubation of the gelatin zymogram with 10mM EDTA resulted in inhibition of the gelatinase activity (B). This provides support for the gelatinase being an MMP.

Figure 11: Reverse zymography of conditioned media collected from LTBMC at days 3, 6, 11, 16 and 21.

Mononuclear cells were separated from normal bone marrow by density gradient centrifugation using Ficoll. These cells were cultured in Myelocult media supplemented with 1% hydrocortisone, 1% antibacterial/antimicrobial solution and 10% fresh L-glutamine. The cultures were grown at 5% CO_2 and 37°C for 21 days. The conditioned media and cells were harvested on days 3, 6, 11, 16 and 21. Fifteen micrograms of total protein was subjected to reverse zymography (A). Densitometric analysis indicates that TIMP-2 is consistently expressed throughout the LTBMC whereas TIMP-1 varies in expression. TIMP-1 expression is very low in the beginning of the LTBMC and increases during the final days. Recombinant TIMP-1 and TIMP-2 were used as positive controls for the reverse zymogram analyses.


Conditioned Media
from LTBMC
rTIMP-1
rTIMP-2
Day 3
Day 6
Day 11
Day 16
Day 21
28 kDa
21 kDa

A.


3500
3000
2500
2000
1500
1000
500
0
IDU/µg Protein
3
6
11
16
21
Days (LTBMC)
TIMP-1
TIMP-2
TIMP-1
TIMP-2

B.

of MMP-2 expression.

BHK-105 conditioned media was used to detect the activity of TIMP-3 whereas BHK-72 was used to detect TIMP-1 and TIMP-2 activity. The reverse zymogram for TIMP-3 showed no expression of TIMP-3 throughout the LTBMC. This result was not unexpected since most secreted TIMP-3 is tightly bound to the ECM and usually not detectable in the conditioned media.

3.1.5 Confirmation of MMP and TIMP Expression by Western Blot Analysis

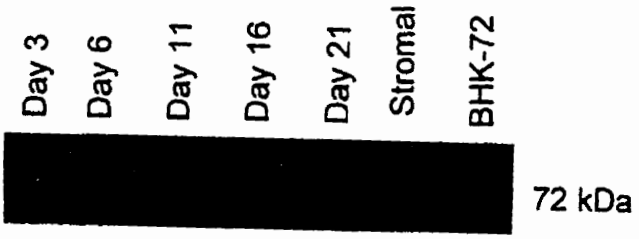
To confirm the gelatinolytic activity as being that of MMPs and TIMPs, Western blot analysis was performed. The conditioned media from the LTBMC for days 3, 6, 11, 16, and 21 and stromal cell population were used for Western blot analysis of MMP-2, MMP-9, TIMP-1 and TIMP-2. Figure 12 shows a western blot using the human anti-MMP-2 antibody. The Western blot analysis confirms MMP-2 expression in the LTBMC. In addition, the pattern of MMP-2 expression in the Western blot analysis also indicates an increase from the beginning to the end of the culture. The isolated stromal cell population showed solely MMP-2 protein expression. Western analysis for MMP-9 (Figure 13) shows a higher level of MMP-9 protein at day 11 and then subsequent decrease in protein presence for the remainder of the LTBMC. The western blot analysis of MMP-9 and MMP-2 further emphasizes that the enzymatic activity seen in the zymogram analysis is indeed MMP-2 and MMP-9 activity.

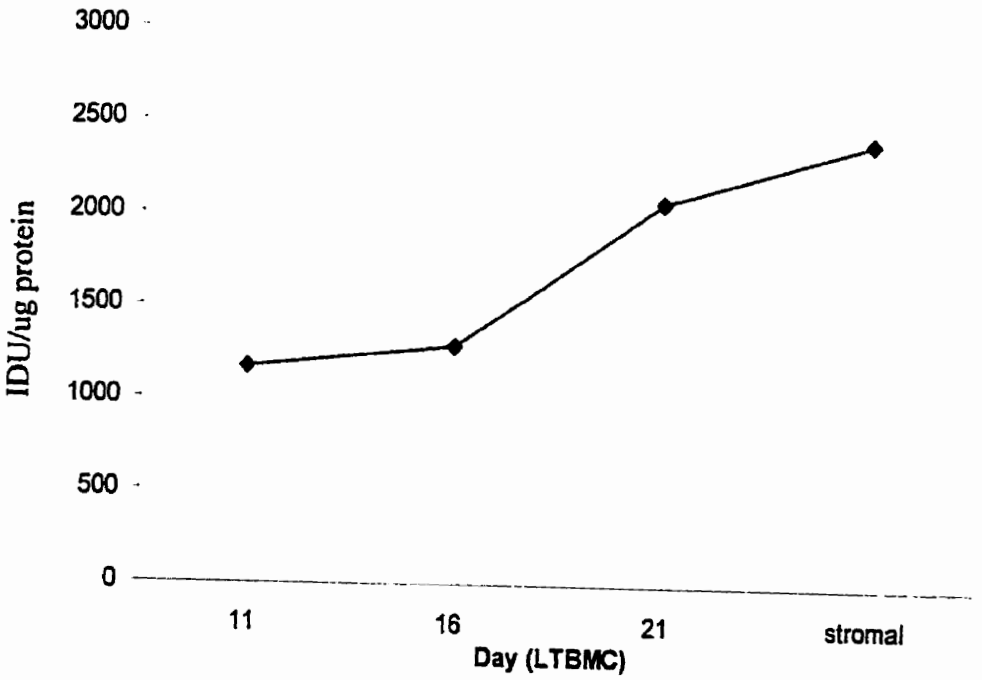
Western blot analysis of TIMP-1 and TIMP-2 reveals that the pattern of expression of TIMP-1 and TIMP-2 is similar to that of the reverse zymogram (Figure

Figure 12: Western Blot analysis of MMP-2 activity in the conditioned media harvested on days 3, 6, 11, 16, and 21 of the LTBMC.

Mononuclear cells were separated from normal bone marrow by density gradient using Ficoll. These cells were cultured in Myelocult media supplemented with 1% hydrocortisone, 1% antibacterial/antimicrobial solution and 10% fresh L-glutamine. The cultures were grown at 5% CO_2 and 37°C for 21 days. The conditioned media and cells were harvested on days 3, 6, 11, 16 and 21. Fifteen micrograms of total protein was subjected to Western Blot analysis (A). This analysis indicates the MMP-2 activity is very low at the beginning of the LTBMC and then increases during days 16 and 21. This is consistent with the findings of the zymographic analysis of the LTBMC. Densitometric analysis confirms the increase of MMP-2 activity throughout the LTBMC (B). Conditioned media from BHK-72 cells was used as a positive control for MMP-2 activity.

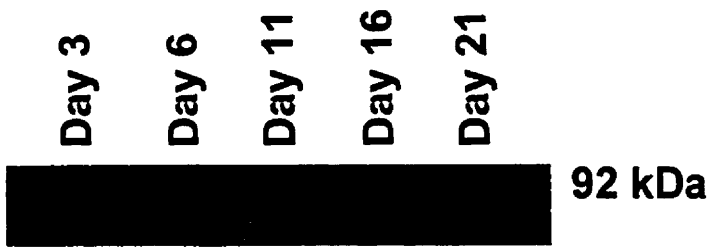
A.


Day 3
Day 6
Day 11
Day 16
Day 21
Stromal
BHK-72
72 kDa

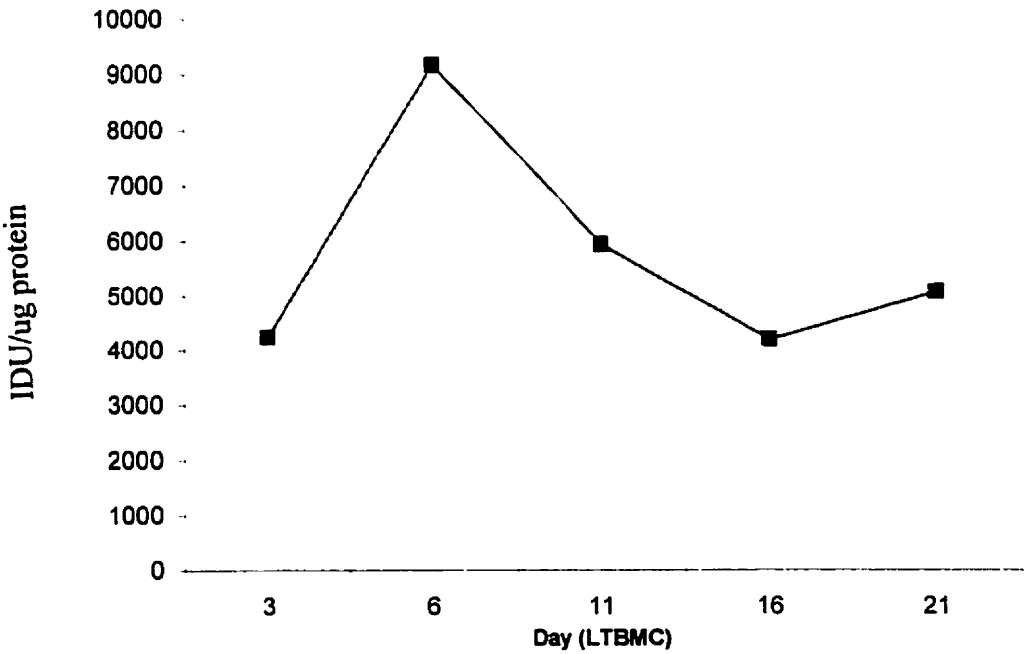

3000
2500
2000
1500
1000
500
0
IDU/ug protein
11
16
21
stromal
Day (LTBMC)

B.

Figure 13: Western Blot analysis of MMP-9 activity in the conditioned media harvested on days 3, 6, 11, 16, and 21 of the LTBMC.
Mononuclear cells were separated from normal bone marrow by density gradient using Ficoll. These cells were cultured in Myelocult media supplemented with 1% hydrocortisone, 1% antibacterial/antimicrobial solution and 10% fresh L-glutamine. The cultures were grown at 5% CO_2 and 37°C for 21 days. The conditioned media and cells were harvested on days 3, 6, 11, 16 and 21. Fifteen micrograms of total protein was subjected to Western Blot analysis (A). There is constitutive expression of MMP-9 during the LTBMC. This analysis confirms the presence of MMP-9 protein with the conditioned media of the LTBMC at the specified days.

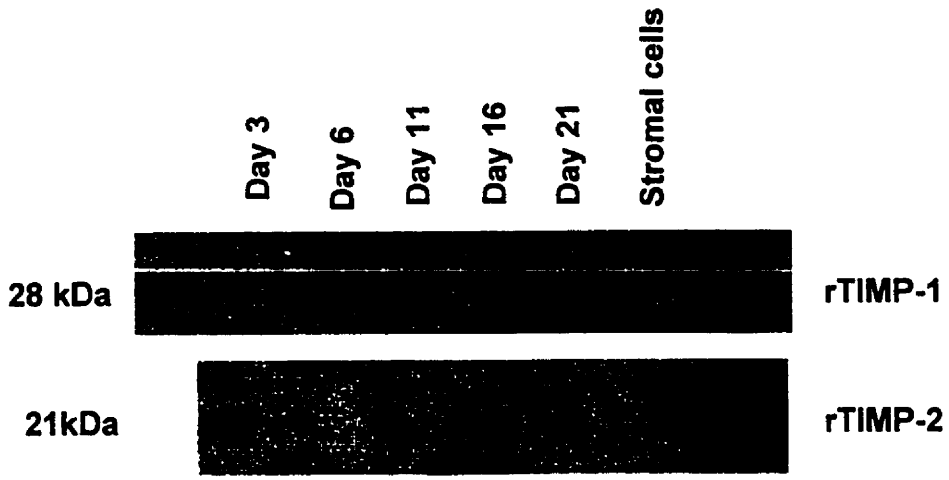

Day 3
Day 6
Day 11
Day 16
Day 21
92 kDa

A.

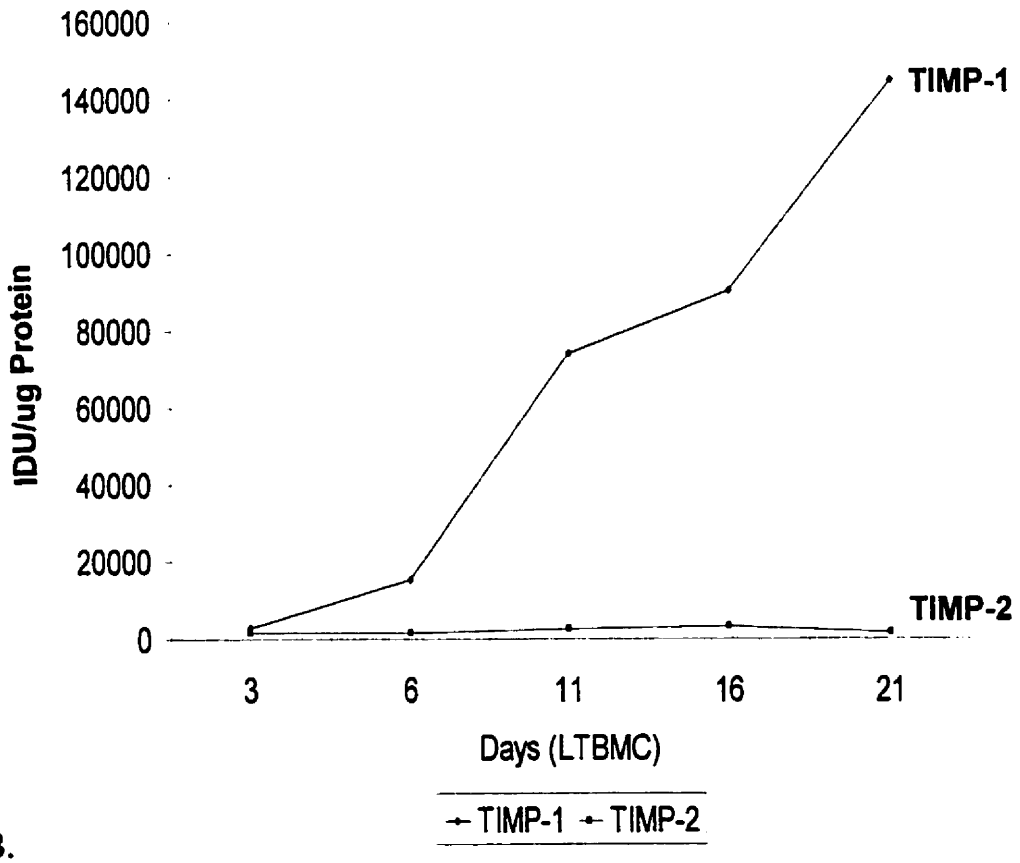

IDU/ug protein
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
3
6
11
16
21
Day (LTBMC)

B.

Figure 14: Western Blot analysis of TIMP-1 and TIMP-2 activity in the conditioned media harvested on days 3, 6, 11, 16, and 21 of the LTBMC.
Mononuclear cells were separated from normal bone marrow by density gradient using Ficoll. These cells were cultured in Myelocult media supplemented with 1% hydrocortisone, 1% antibacterial/antimicrobial solution and 10% fresh L-glutamine. The cultures were grown at 5% CO_2 and 37°C for 21 days. The conditioned media and cells were harvested on days 3, 6, 11, 16 and 21. Fifteen micrograms of total protein was subjected to Western Blot analysis (A). TIMP-1 activity was seen to increase in the later stages of the LTBMC. TIMP-2 activity was constant throughout the LTBMC. Both of these findings are consistent with the reverse zymogram analysis (B). Recombinant TIMP-1 and TIMP-2 were used as positive controls for the Western blot analysis.


Day 3
Day 6
Day 11
Day 16
Day 21
Stromal cells
28 kDa
rTIMP-1
21kDa
rTIMP-2

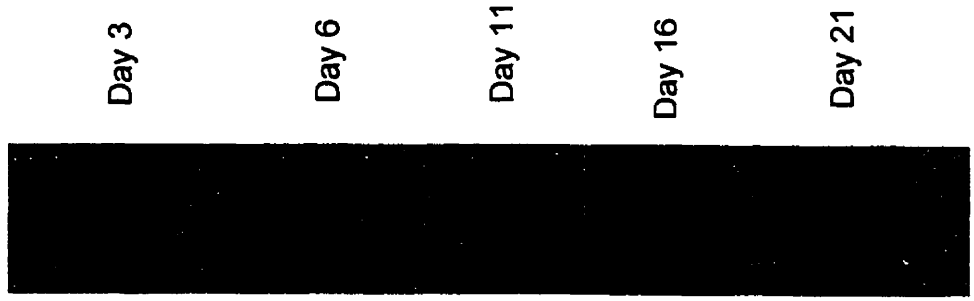
A.


160000
140000
120000
100000
80000
60000
40000
20000
0
IDU/ug Protein
TIMP-1
TIMP-2
3
6
11
16
21
Days (LTBMC)
TIMP-1
TIMP-2

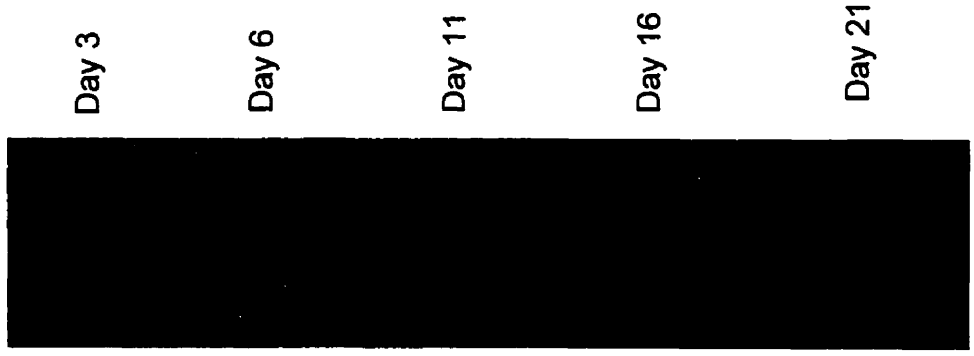
B.

14). TIMP-1 protein is highest during day 16 and 21 of the LTBMC whereas level of TIMP-2 expression remains constant throughout the culture. The isolated stromal cell population demonstrated TIMP-1 expression and no TIMP-2 expression. Western blot analysis was used to assess the expression of TIMP-1 in the LTBMC and found that, as reverse zymography indicated, TIMP-1 expression is highest on day 16 and 21. The p-value of the activity on day 3 versus day 21 was <0.05. Therefore there was a significant change in TIMP-1 expression during the LTBMC.

A LTBMC was established and the cell lysates at day 3, 6, 11, 16 and 21 were collected for use in a Western blot analysis using antibodies for MT1-MMP and TACE. Figure 15A illustrates the Western blot analysis for MT1-MMP. It can be seen that there is a relatively constant level of MT1-MMP expression throughout the LTBMC. In addition Figure 15B, illustrates TACE western blot analysis and also shows a constant level of TACE expression throughout the LTBMC. Western blot analysis of stromal MT1-MMP and TACE would be an important analysis to investigate.



A.



B.

Figure 15: Western blot of MT1-MMP and TACE. A. Western blot analysis of MT1-MMP shows a constant level of expression throughout the LTBMC. B. Western blot analysis of TACE indicates constitutive expression throughout the LTBMC.

3.1.6 Analysis of MMP and TIMP mRNA by RT-PCR

Total RNA from the LTBMC samples were reverse transcribed and PCR amplified with various MMP and TIMP primers. Expression of the housekeeping gene GAPDH was demonstrated in all LTBMC samples at a constant level (Figure 16).

Figure 17 depicts the expression of TIMP-3 throughout the LTBMC. There is a high level of expression throughout the LTBMC except on day 6 where there is a visible decrease in TIMP-3. There also appears to be a peak of expression of TIMP-3 on day 11. TACE is expressed within the LTBMC as well and again showed a decrease in expression on day 6 (Figure 18). TIMP-4 expression showed the most variability over the LTBMC than any other gene (Figure 19). TIMP-4 has a low level of expression early in the LTBMC but increases during day 16 and 21. This is a similar pattern to that of TIMP-1 and may also be correlated with stromal cell development. MT1-MMP showed little expression in the LTBMC yet some is seen on day 16 (Figure 20). The RT-PCR results obtained so far are preliminary.

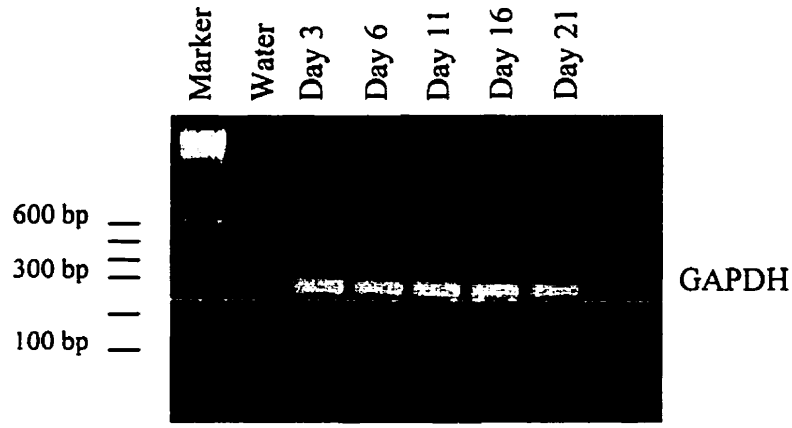


Figure 16: RT-PCR analysis of housekeeping gene GAPDH revealed a constant level of transcript expression over the LTBMC. The PCR reaction was run for 40 cycles at an annealing temperature of 59°C.

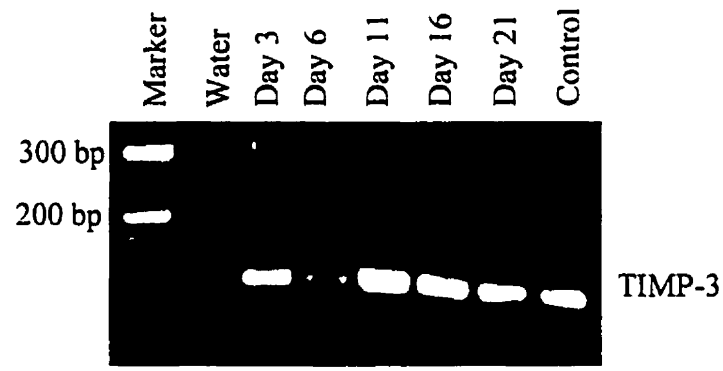


Figure 17: RT-PCR analysis of TIMP-3 transcript. The PCR reaction was run for 35 cycles at an annealing temperature of 54°C. There is a slight decline in TIMP-3 expression from Day 11 onward in the LTBMC. There is also a decline in expression on Day 6.

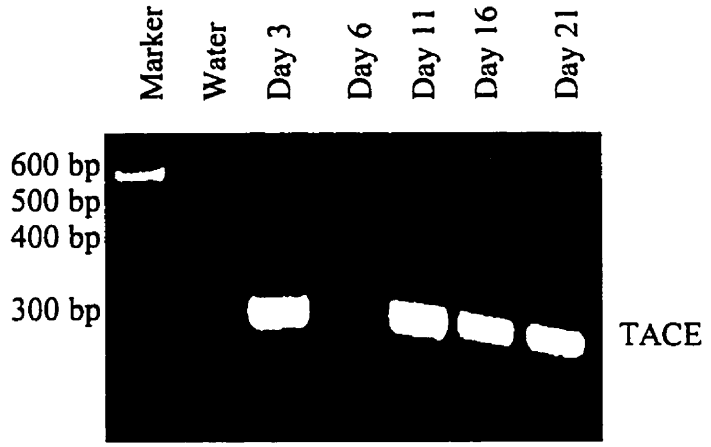


Figure 18: RT-PCR analysis of TACE transcript. The PCR reaction was run for 40 cycles at an annealing temperature of 57°C. There is a decrease in expression from Day 11 onward. In addition, there is a decrease in TACE transcript on Day 6.

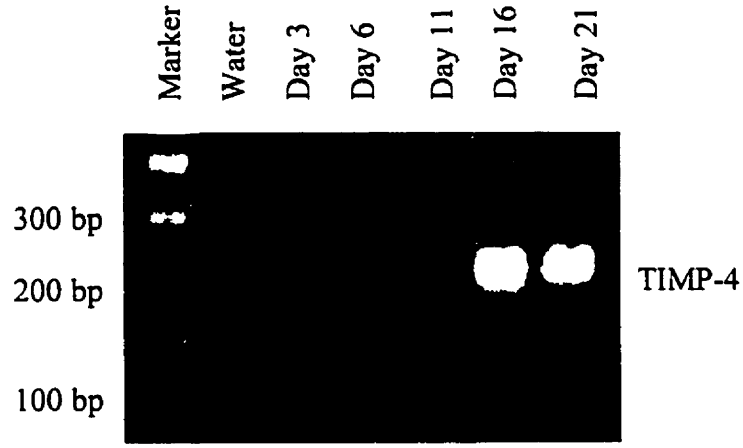


Figure 19: RT-PCR analysis of TIMP-4 expression. The PCR reaction was run for 40 cycles at an annealing temperature of 59 °C. There is a high lelel of expression of TIMP-4 during Day 16 & 21 of the LTBMC. The expression correlates with MMP-2 and TIMP- 1 expression patterns

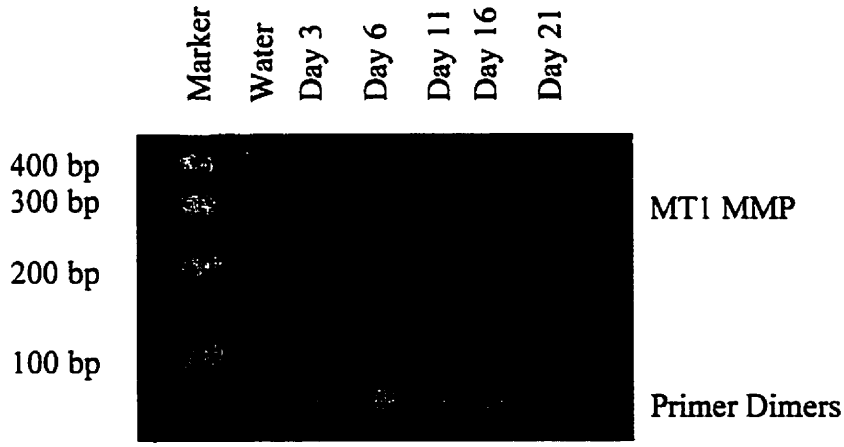


Figure 20: RT-PCR of MT1-MMP expression in LTBMC. The PCR reaction was run for 40 cycles at an annealing temperature of 59°C. There is minimal expression of Day 16 otherwise no detection of MT1-MMP transcript

3.1.7 Summary

Through zymography, reverse zymography and Western blot analysis we show evidence for the expression of functional gelatinase and TIMP activity by cells of the LTBMC. The patterns of expression of MMP-2 and MMP-9 are quite distinct during the course of the LTBMC. MMP-2 expression is increased through the culture whereas MMP-9 remains largely unchanged. In addition, there is also differential expression of TIMP-1 and TIMP-2. TIMP-1 expression is apparent in the later stages of the LTBMC while TIMP-2 expression is constant throughout. This addresses the primary objective of this proposal in that MMP and TIMP activity is present and they vary throughout the LTBMC. The data from five different bone marrow samples indicate that there is a consistent pattern of expression for MMP-2 and MMP-9 although significant individual variability in absolute values was evident. Logarithmic transformations were thus used to illustrate the differences in MMP-2 and MMP-9 expression. The slope of MMP-2 between days 3-11 was 13 while that of MMP-9 was 0.008. It is clear that there is differential expression of gelatinases and TIMPs during the period of the LTBMC.

3.2 Cytokines Regulate MMP and TIMP Expression in Bone Marrow Stromal Cells

In this investigation, the following growth factors and cytokines GM-CSF, IL-6 and IL-1β were selected for their well-characterized effects and their role in mobilization of progenitor cells into peripheral blood. EGF has been shown to up-regulate MMP secretion in fibroblasts as well as in breast and colon carcinoma cells (Parks WC and Mecham RP, 1998). Therefore, its role in bone marrow MMP and TIMP secretion is of interest. TNF-α, TGF-β and PMA also have been shown to regulate MMPs and TIMPs in other systems and thus were studied.

Cytokines play a pivotal role in the stimulation and regulation of MMPs and TIMPs as well as playing a key role in hematopoiesis. Therefore, the analysis of MMP and TIMP regulation by cytokines within the hematopoietic microenvironment will provide some insight into the mechanism by which progenitor cells are released into circulation. In this study, the bone marrow stromal cells were isolated and then subsequently stimulated with various cytokines to evaluate their effect on MMP and TIMP expression.

3.2.1 PMA, TNF-α and IL-1β Induce MMP-9 Expression in Bone Marrow Stromal Cells

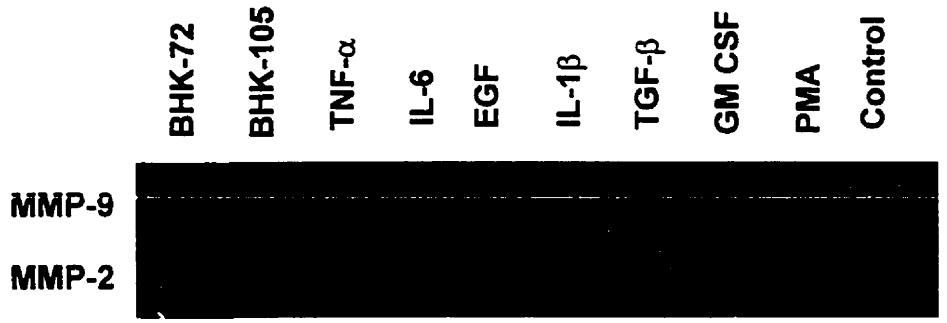
Conditioned media containing equal amounts of protein (15μg) from cytokine stimulated stromal cells (as outlined in section 2.1) of five different bone marrows samples were analyzed by zymography after 24 hr exposure (Figure 21). Conditioned media from the untreated stromal cells demonstrated MMP-2 and MMP-9 activity.

PMA, TNF-α and IL-1β induced the expression of MMP-9 as compared to the control unstimulated stromal cells. In addition, TNF-α- stimulation of bone marrow stromal cells demonstrated an increase MMP-2 expression of 1.6 fold (the p-value between the control and the stimulated was <0.05) and a 2-fold increase in activated MMP-2 (the p-value between the control and stimulated was <0.005) expression.

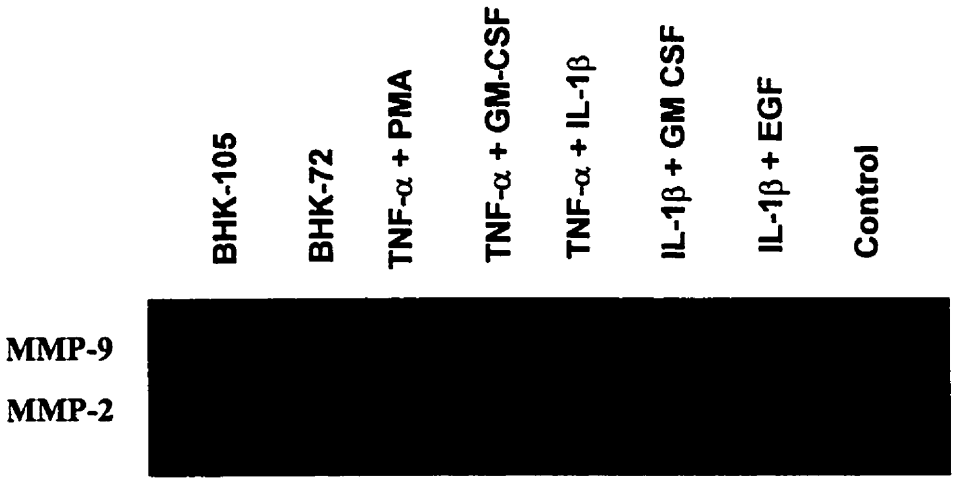
To determine potential additive effects on MMP and TIMP activity, combinations of cytokines were tested on the stromal cell cultures. Figure 21B depicts the gelatinolytic activity of stromal cell conditioned media after 24hr exposure to combinations of cytokines. Figure 22 depicts the average densitometric values of MMP-9, MMP-2 and activated MMP-2 expression for five different bone marrow samples. MMP-9 activity was induced 2-fold upon stimulation of the stromal cells with TNF-α. TNF-α also induced activated MMP-2 by nearly 2-fold as well. IL-1β induced both activated MMP-2 and MMP-9 expression by more than 1.5-fold. It can be seen that all cytokine combinations caused a dramatic increase in MMP-9 expression. The combination of TNF-α+PMA, TNF-α+IL-1β, and TNF-α+GMCSF all induced a 2.5-fold or greater expression of MMP-9 ($p<0.05$). The expression of MMP-2 was increased by all cytokine combinations except that of IL-1β+EGF.

Figure 21: Zymographic analysis of MMP-2 and MMP-9 activity after cytokine and growth factor stimulation.

Stromal cells were established in culture by seeding 1 x 10^8 bone marrow cells in T150 flask in Myelocult media supplemented with 10% L-glutamine, 1% hydrocortisone, and 10% antibiotic/antimicrobial. After two hours the non-adherent cells were removed and the adherent cells were washed twice with PBS. The cultures were then replaced with fresh media and grown at 5% CO_2 and 37°C for 21 days. At days 7 and 14 half the media was replaced with fresh media to sustain growth. . Once the stromal layer was grown to confluency, the cells were removed from the flask by treatment with trypsin and plated in 48-well plate at a concentration of 2 x 10^5 cells/ml. The cells were left to grow to 80% confluency (~12hrs) and then exposed to serum-free media with 10ng/ml of either IL-1β, IL-6, GM-CSF, TGF-β, EGF, TNF-α or 5ng/ml of PMA and incubated for 24 hrs (A). Another experiment exposed the cell to combinations of TNF-α (10ng/ml) + PMA (5ng/ml), TNF-α (10ng/ml) + GM CSF (10ng/ml), TNF-α (10ng/ml) + IL-1β (10ng/ml), IL-1β (10ng/ml) + GM CSF (10ng/ml), and IL-1β (10ng/ml) + EGF (10ng/ml) for 24 hrs (B). The conditioned media was then collected and used for analysis by zymography. Five different experiments were conducted in duplicate. Representative experiments are shown in (A) and (B).


BHK-72
BHK-105
TNF-α
IL-6
EGF
IL-1β
TGF-β
GM CSF
PMA
Control
MMP-9
MMP-2

A.


BHK-105
BHK-72
TNF-α + PMA
TNF-α + GM-CSF
TNF-α + IL-1β
IL-1β + GM CSF
IL-1β + EGF
Control
MMP-9
MMP-2

B.

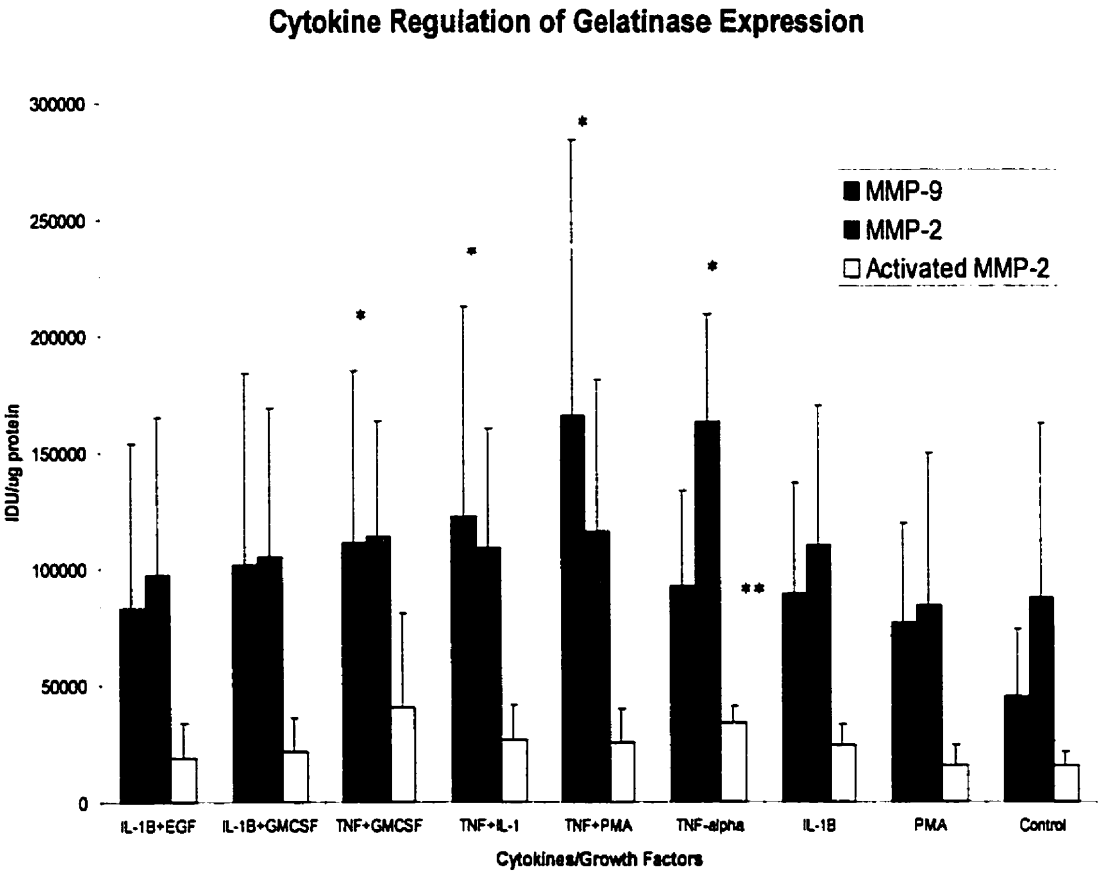


Figure 22: Quantitative analysis of MMP-2 and MMP-9 expression upon stimulation with cytokines and growth factors.

Stromal cells were cultured and stimulated by cytokines and growth factors as previously outlined. MMP-9 activity was induced upon stimulation with PMA, IL-1β, and TNF-α. MMP-2 expression was increased mostly by TNF-α. IL-1β and TNF-α induced activated MMP-2. All the cytokine combinations induced MMP-9. Specifically, the combination of TNF-α + IL-1β resulted in > 6-fold increase in MMP-9 activity. Activated MMP-2 was induced by the combinations of TNF-α + IL-1β and TNF-α + GM CSF. The experiment was performed on five samples in duplicate. The error bars represent the standard deviation between all five samples. (* represents $p<0.05$)(** represents $p<0.005$)

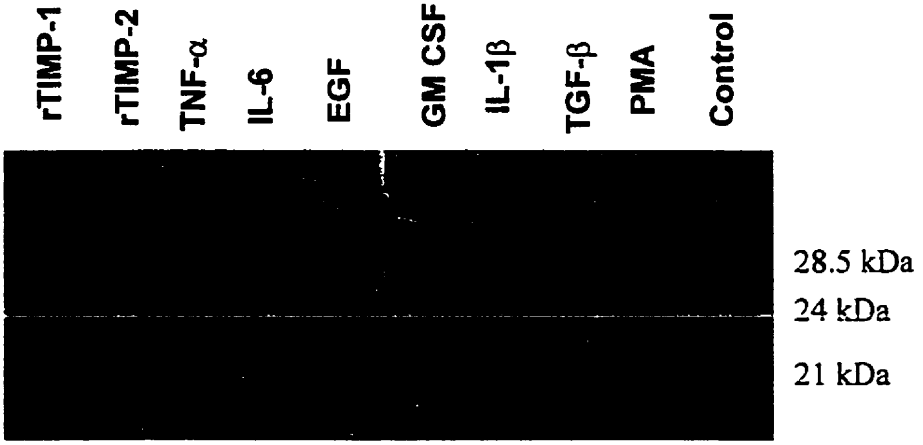
3.2.2 Cytokines Induce TIMP-1 Expression in Bone Marrow Stromal Cells

Reverse zymography was used to ascertain the increase in TIMP expression due to the stimulation of stromal cells by cytokines. The conditioned media from untreated stromal cells showed TIMP-1 expression only. Upon stimulation with cytokines alone or in combination, a significant increase in TIMP-1 expression was seen (Figure 23A&B). PMA up-regulated TIMP-1 2-fold over the control. The most significant increase was caused by the combination of TNF-α+PMA with over 2.5-fold increase in TIMP-1 expression (1400000 IDU/μg protein)(Figure 24 A&B). All of the cytokine combination regimes induced TIMP-1 at least a 2-fold and resulted in expression ranging from 1000000-1400000 IDU/μg protein.

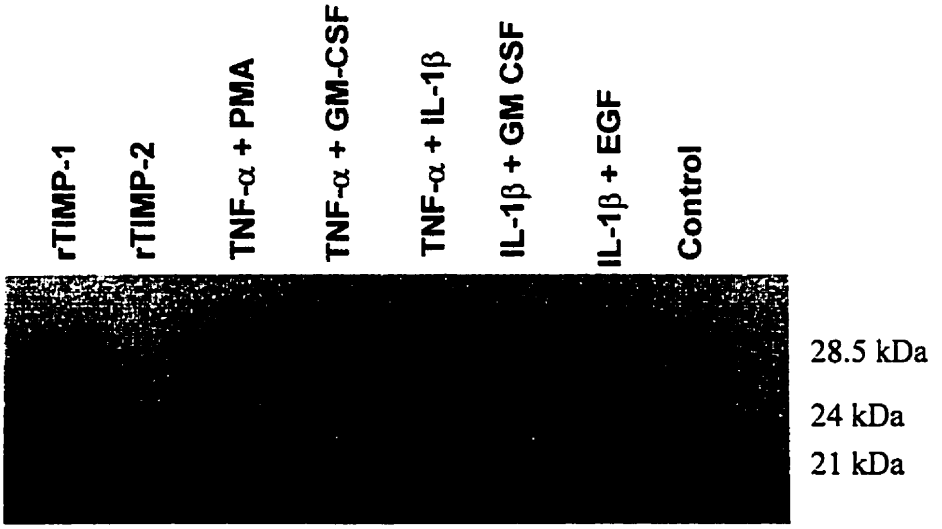
To aid in the quantification of TIMP-1 protein, a commercial ELISA was used for subsequent experiments. TIMP-1 ELISA analysis (Figure 25) demonstrated that PMA and PMA+TNF-α treatment induced 6000 ng/ml of TIMP-1 ($p<0.005$) versus the control of less than 1000 ng/ml. In addition, the other cytokines tested demonstrated a 2.5-fold induction of TIMP-1 ($p<0.05$). The findings by reverse zymography were in general similar to those obtained by TIMP-1 ELISA.

Figure 23: Reverse Zymographic analysis of TIMP-1 expression upon stimulation with cytokines and growth factors.

Stromal cells were established in culture by seeding 1 x 10^8 bone marrow cells in T150 flask in Myelocult media supplemented with 10% l-glutamine, 1% hydrocortisone, and 10% antibiotic/antimicrobial. After two hours the non-adherent cells were removed and the adherent cells washed twice with PBS. The cultures were replaced with fresh media and grown at 5% CO_2 and 37°C for 21 days. At days 7 and 14 half the media was replaced with fresh media to sustain growth. . Once the stromal layer was grown to confluency, the cells were removed from the flask by treatment with trypsin and plated in 48-well plate at a concentration of 2 x 10^5 cells/ml. At 80% confluency (~12hrs) and the cells were exposed to serum-free media with 10ng/ml of IL-1β, IL-6, GM-CSF, TGF-β, EGF, TNF-α or 5ng/ml of PMA and incubated for 24 hrs. In addition, stromal cells were treated with combination of cytokines such as TNF-α (10ng/ml) + PMA (5ng/ml), TNF-α (10ng/ml) + GM CSF (10ng/ml), TNF-α (10ng/ml) + IL-1β (10ng/ml), IL-1β (10ng/ml) + GM CSF (10ng/ml), and IL-1β (10ng/ml) + EGF (10ng/ml) for 24 hrs. The conditioned media was then collected and used for analysis by reverse zymography. Representative experiments are shown in (A) and (B). Recombinant TIMP-1 and TIMP-2 were used as positive controls for reverse zymography analysis.


rTIMP-1
rTIMP-2
TNF-α
IL-6
EGF
GM CSF
IL-1β
TGF-β
PMA
Control
28.5 kDa
24 kDa
21 kDa

A.


rTIMP-1
rTIMP-2
TNF-α + PMA
TNF-α + GM-CSF
TNF-α + IL-1β
IL-1β + GM CSF
IL-1β + EGF
Control
28.5 kDa
24 kDa
21 kDa

B.

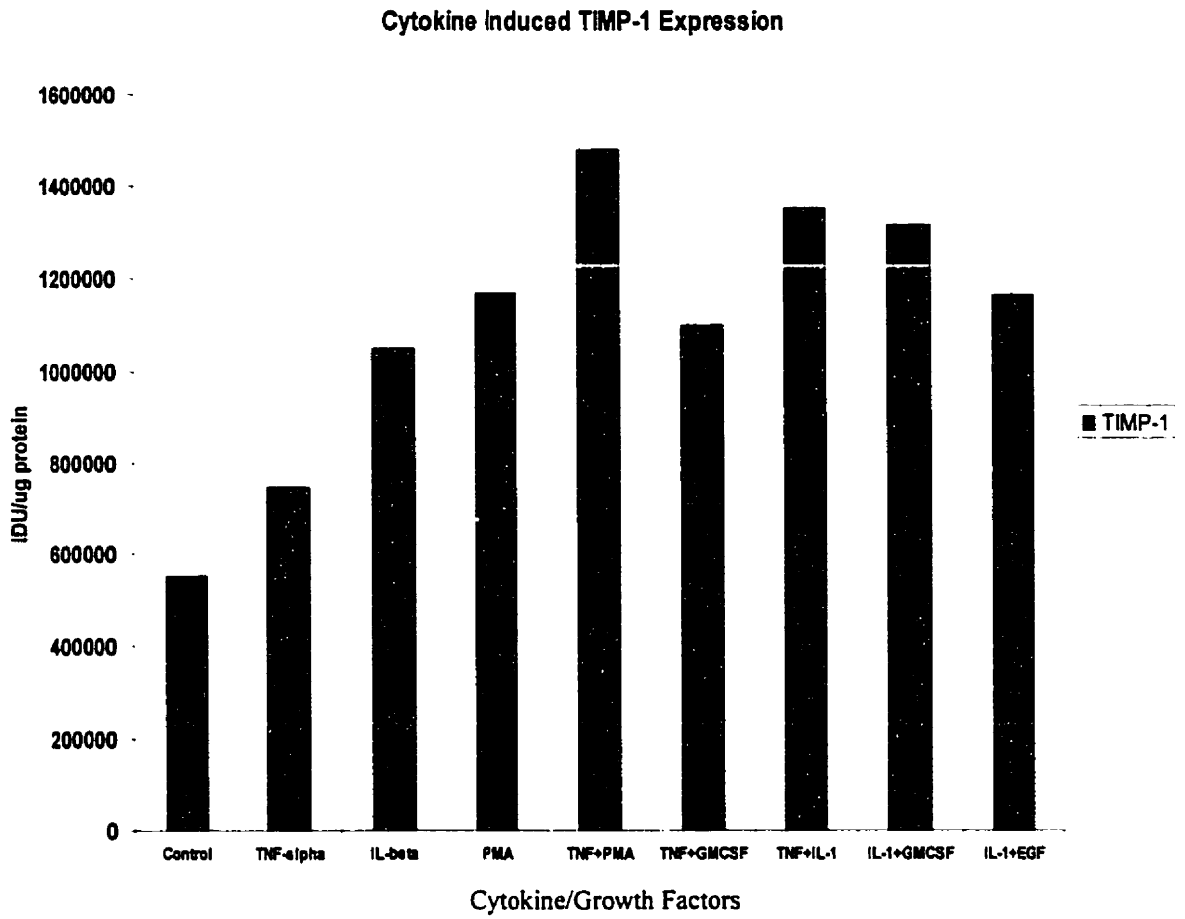


Figure 24: Quantitative analysis of reverse zymography for TIMP-1 expression upon stimulation with cytokines and growth factors. Stromal cells were established in culture as previously mentioned. The conditioned media was then collected and used for analysis by reverse zymography. The cytokines studied induced a higher level of TIMP-1 activity. PMA+TNF-α induced TIMP-1 the most with 1450000 IDU/μg protein yielding a 2-fold increase in expression over untreated TIMP-1 expression. All cytokine combinations had dramatic effects of the induction of TIMP-1. All combinations, except TNF-α + GM CSF, had >2-fold increases in TIMP-1 production.

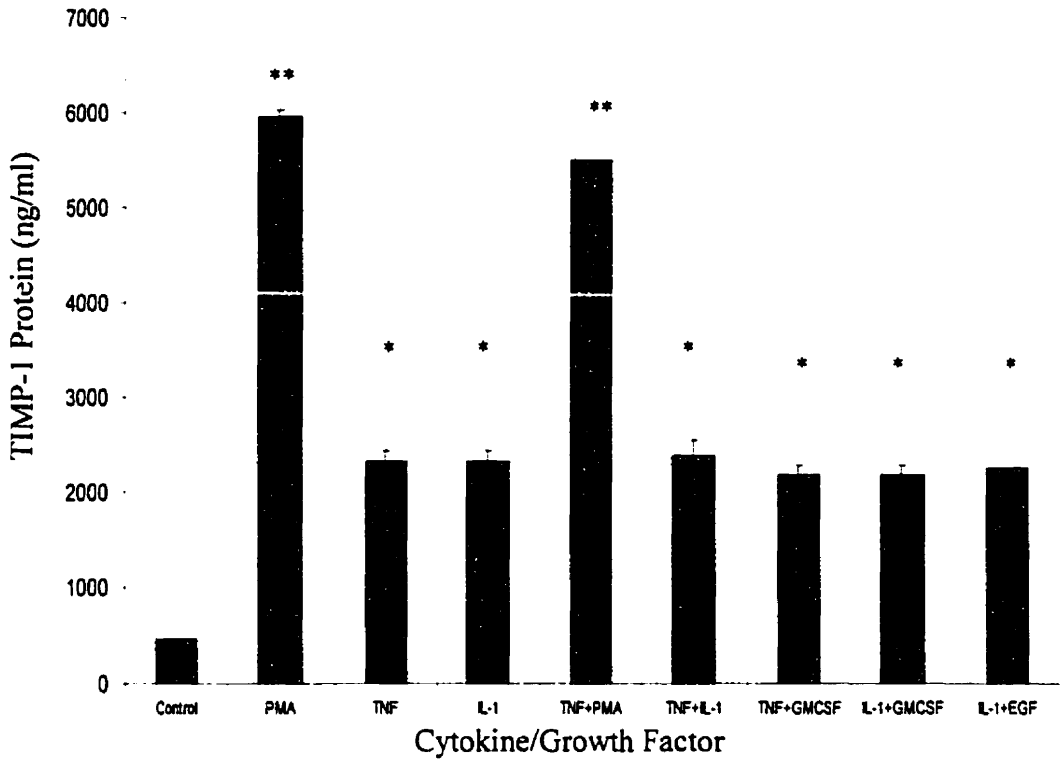


Figure 25: Quantitative analysis of TIMP-1 expression by ELISA.
Stromal cells were established in culture by seeding 1 x 10^8 bone marrow cells in T150 flask in Myelocult media supplemented with 10% l-glutamine, 1% hydrocortisone, and 10% antibiotic/antimicrobial. After two hours the non-adherent cells were removed and the adherent cells washed twice with PBS. The cultures were replaced with fresh media and grown at 5% CO_2 and 37°C for 21 days. At days 7 and 14 half the media was replaced with fresh media to sustain growth. . Once the stromal layer was grown to confluency, the cells were removed from the flask by treatment with trypsin and plated in 48-well plate at a concentration of 2 x 10^5 cells/ml. At 80% confluency (~12hrs) and the cells were exposed to serum-free media with 10ng/ml of IL-1β, IL-6, GM-CSF, TGF-β, EGF, TNF-α or 5ng/ml of PMA and incubated for 24 hrs. In addition, stromal cells were treated with combination of cytokines such as TNF-α (10ng/ml) + PMA (5ng/ml), TNF-α (10ng/ml) + GM CSF (10ng/ml), TNF-α (10ng/ml) + IL-1β (10ng/ml), IL-1β (10ng/ml) + GM CSF (10ng/ml), and IL-1β (10ng/ml) + EGF (10ng/ml) for 24 hrs. The conditioned media was then collected and used for analysis by ELISA. TIMP-1 ELISA analysis demonstrated that TIMP-1 expression was induced >6-fold upon stimulation with PMA or PMA+TNF-α. In addition, all other cytokines had at least a 2.5-fold induction of TIMP-1. TIMP-1 concentrations ranged from 500ng/ml for the control to 6000ng/ml for the greatest induction.
(* represents $p<0.05$)(** represents $p<0.005$)

Control cells did not express TIMP-2 and it was not induced by single cytokine stimulation. However, there appears to be an induction of TIMP-2 activity that is slightly visible in Figure 23B. This will need to be further investigated. There is the appearance of a band that is between the size of TIMP-1 and TIMP-2. It is speculated that this band may be TIMP-3 since its molecular weight (24kDa) falls within this range. In addition, TIMP-3 is regulated in a similar fashion as TIMP-1 and this seems to be the case demonstrated in the reverse zymogram gel. Western blot analysis was performed on the cell lysates of the LTBMC for TIMP-3 expression but due to a weak antibody no conclusive results were obtained. RT-PCR can be used to test the hypothesis that this band represents TIMP-3.

3.2.3 Summary

The expression of MMPs and TIMPs by bone marrow stromal cells is regulated by cytokines. MMP-9 and TIMP-1 expression is significantly affected by cytokine stimulation. MMP-2 and TIMP-2 are not as dramatically affected by cytokine stimulation. Furthermore an activated form of MMP-2 is seen upon stimulation with TNF-α or IL-1β, or in combination. This suggests that the cytokines could be stimulating the cleavage of MMP-2 after inducing the zymogen form.

3.3 Mechanism of Cytokine Stimulation of Bone Marrow Stromal Cells

Signal transduction is a process wherein extracellular stimuli bind to membrane associated receptors and induce an intracellular signaling cascade. These intracellular signals result in cellular responses such as proliferation and growth. One of the most important intracellular pathways invoked is the mitogen-activated protein kinase (MAPK) pathway. Other pathways involved in the regulation of cell growth and differentiation are PI-3 kinase, inositol-lipid (IP_3), cyclic adenosine monophosphate (cAMP), the JAK/STAT signaling system and the stress kinase system (Cross T et al., 2000).

From section 3.2.2 we provide evidence that cytokines induce the expression of MMP-2, MMP-9 and TIMP-1 in bone marrow stromal cells. Combinations of cytokines evoked an augmented response resulting in even more gelatinase and TIMP-1 expression. To determine the mechanism by which the cytokines stimulate MMP and TIMP expression, tyrosine kinase inhibitors Genistein and Herbimycin A, and MAPK kinase inhibitor PD98059 and p38-specific MAPK inhibitor SB203580 were used to analyze their effect on cytokine-mediated gelatinase and TIMP expression.

3.3.1 Selective Inhibition of MMP-9 by Tyrosine Kinase Inhibitors

Figure 26 illustrates the gelatinolytic activity of the conditioned media acquired from cytokine stimulation of bone marrow stromal cells in the presence or absence of genistein. A significant decrease in MMP-9 expression was observed when stromal cells were exposed to all cytokine regimes in the presence of genistein

(Figure 27A). This experiment was performed in duplicate in two independent experiments. For the combination of TNF-α+PMA there was a 4-fold decrease in MMP-9 activity upon incubation with genistein ($p<0.005$). Genistein also had an inhibitory effect on cytokines, Il-1β, TNF-α+ Il-1β, and TNF-α+ GMCSF by 1.8, 2.5 and 2.2-fold respectively ($p<0.05$). MMP-2 activity was also moderately reduced upon incubation with genistein (Figure 27B). Treatment of the bone marrow stromal cells with cytokines plus Herbimycin A also resulted in a decrease in MMP-9 activity in comparison to the control untreated cells (Figure 28&29A). Again, incubation with Herbimycin A had the most significant effect on TNF-α+PMA induced MMP-9 expression (a 3-fold decrease)($p<.05$). In most cytokine treatment regimes, there was no significant effect of Herbimycin A on MMP-2 activity (Figure 29B). Treatment of the bone marrow stromal cells with cytokines plus or minus PD98059 showed inhibitory effects on MMP-9 activity (Figure 32&33A). PMA ($p<0.05$) and TNF-α+ IL-1β stimulation of MMP-2 activity was inhibited by PD98059 yet little inhibition was seen for the other treatments (Figure 33B). When a specific p38 MAPK inhibitor, SB203580, was tested (Figure 30) there was no effect on the induction MMP-2 or MMP-9 activity with or without inhibitor (Figure 31 A&B).

3.3.2 Inhibition of Cytokine-Induced TIMP-1 Expression by Genistein

From our preliminary studies presented in Section 3.2.2, we demonstrated that cytokines induced the expression of TIMP-1 specifically with no effect on TIMP-2. The effect kinase inhibitors on cytokine mediated TIMP-1 expression was determined in the following experiments by using an ELISA system. Figure 34 shows the

expression of TIMP-1 upon stimulation in the presence or absence of various inhibitors. We have demonstrated in section 3.3.1 that genistein had the most dramatic effect on cytokine-induced MMP-9 expression and therefore analyzed its effect on TIMP-1. PMA-mediated expression of TIMP-1 was decreased nearly 6-fold (from 6000ng/ml to 1000 ng/ml) by the tyrosine kinase inhibitor, genistein ($p<0.0005$)(Figure 34A). The combination of TNF-α+PMA was equally affected, demonstrating a 6-fold decrease as well ($p<0.005$). TNF-α, IL-1β, TNF-α+ IL-1β, TNF-α+GMCSF and IL-1β+GMCSF demonstrated 2-fold decreases in TIMP-1 expression by Genistein (all p-values were<0.05).

The MAPK inhibitor inhibited PMA-mediated TIMP-1 expression by 50% ($p<0.005$) (Figure 34B). However, cytokine induction by TNF-α, IL-1β, TNF-α+ IL-1β, TNF-α+GMCSF and IL-1β+GMCSF was unaffected by PD98059.

3.3.3 Summary

Incubation of stromal cells with cytokines in the presence or absence of inhibitor helps delineate which intracellular mechanism may be involved. From the current study, both tyrosine kinase inhibitors, genistein and Herbimycin A, block MMP-9 and TIMP-1 induction. p38 MAPK inhibitor, SB203580 had relatively no effect on the induction of MMPs. PD98059, which is a MEK inhibitor, had little to no effect on MMP activity yet it did inhibit the PMA and TNF-α+PMA induction of TIMP-1. Our studies show that cytokine stimulation of MMP-2, MMP-9 and TIMP-1 are regulated through tyrosine kinase pathway and not through the MAPK pathway, although some TIMP-1 activation may occur through MEK indicating some overlap in signal mediators. These findings are preliminary and further confirmation is needed.

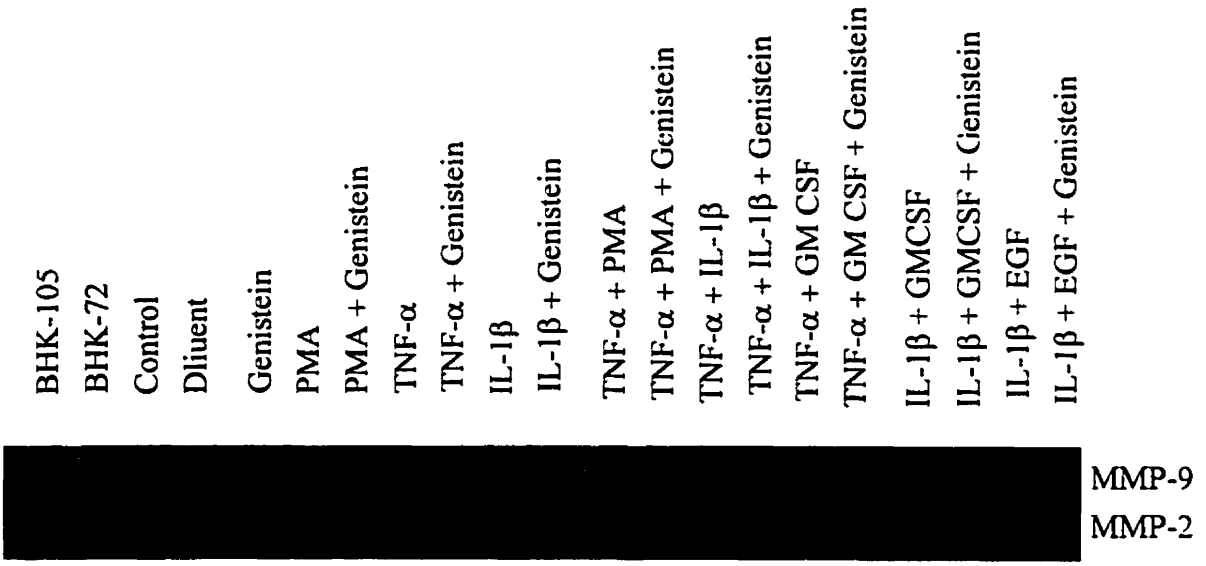
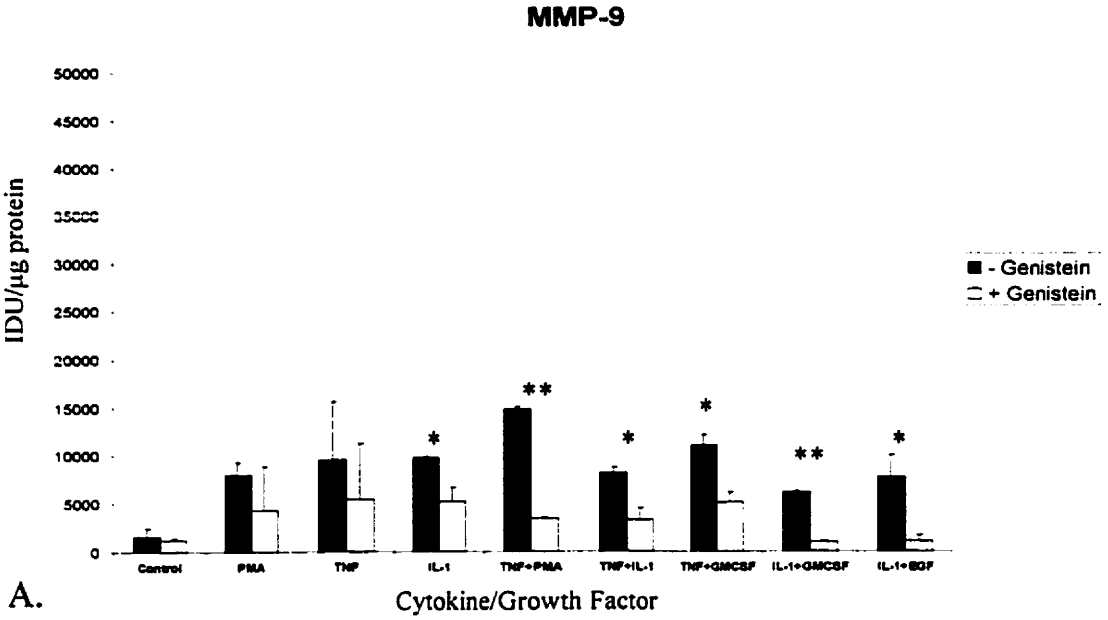


Figure 26: MMP-2 and MMP-9 expression upon stimulation with cytokines and growth factors in the presence or absence of Genistein.
Stromal cells were established in culture by seeding 1 x 10^8 bone marrow cells in T150 flask in Myelocult media supplemented with 10% l-glutamine, 1% hydrocortisone, and 10% antibiotic/antimicrobial. After two hours the non-adherent cells were removed and the adherent cells washed twice with PBS. The cultures were replaced with fresh media and grown at 5% CO_2 and 37°C for 21 days. At days 7 and 14 half the media was replaced with fresh media to sustain growth. Once the stromal layer was grown to confluence, the cells were removed from the flask by treatment with trypsin and plated in 48-well plate at a concentration of 2 x 10^5 cells/ml. The cells were left to grow to 80% confluence (~12hrs) and then exposed to serum-free media with Genistein (100μM) for 30 minutes. The cells were then treated with TNF-α (10ng/ml), PMA (5ng/ml), IL-1β(10ng/ml), TNF-α (10ng/ml) + PMA (5ng/ml), TNF-α (10ng/ml) + GM CSF (10ng/ml), TNF-α (10ng/ml) + IL-1β (10ng/ml), IL-1β (10ng/ml) + GM CSF (10ng/ml), and IL-1β (10ng/ml) + EGF (10ng/ml) and incubated for 24 hrs. The conditioned media was then collected and used for analysis by zymography.

Figure 27: Quantitative analysis of MMP-2 and MMP-9 expression upon stimulation with cytokines and growth factors in the presence or absence of Genistein.

Stromal cells were established in culture by seeding 1 x 10^8 bone marrow cells in T150 flask in Myelocult media supplemented with 10% l-glutamine, 1% hydrocortisone, and 10% antibiotic/antimicrobial. After two hours the non-adherent cells were removed and the adherent cells washed twice with PBS. The cultures were replaced with fresh media and grown at 5% CO_2 and 37°C for 21 days. At days 7 and 14 half the media was replaced with fresh media to sustain growth. Once the stromal layer was grown to confluence, the cells were removed from the flask by treatment with trypsin and plated in 48-well plate at a concentration of 2 x 10^5 cells/ml. The cells were left to grow to 80% confluence (~12hrs) and then exposed to serum-free media with Genistein (100μM) for 30 minutes. The cells were then treated with TNF-α (10ng/ml), PMA (5ng/ml), IL-1β(10ng/ml), TNF-α (10ng/ml) + PMA (5ng/ml), TNF-α (10ng/ml) + GM CSF (10ng/ml), TNF-α (10ng/ml) + IL-1β (10ng/ml), IL-1β (10ng/ml) + GM CSF (10ng/ml), and IL-1β (10ng/ml) + EGF (10ng/ml) and incubated for 24 hrs. The conditioned media was then collected and used for analysis by zymography and analyzed by densitometry. MMP-9 expression is graphed in (A) and MMP-2 expression is graphed in (B).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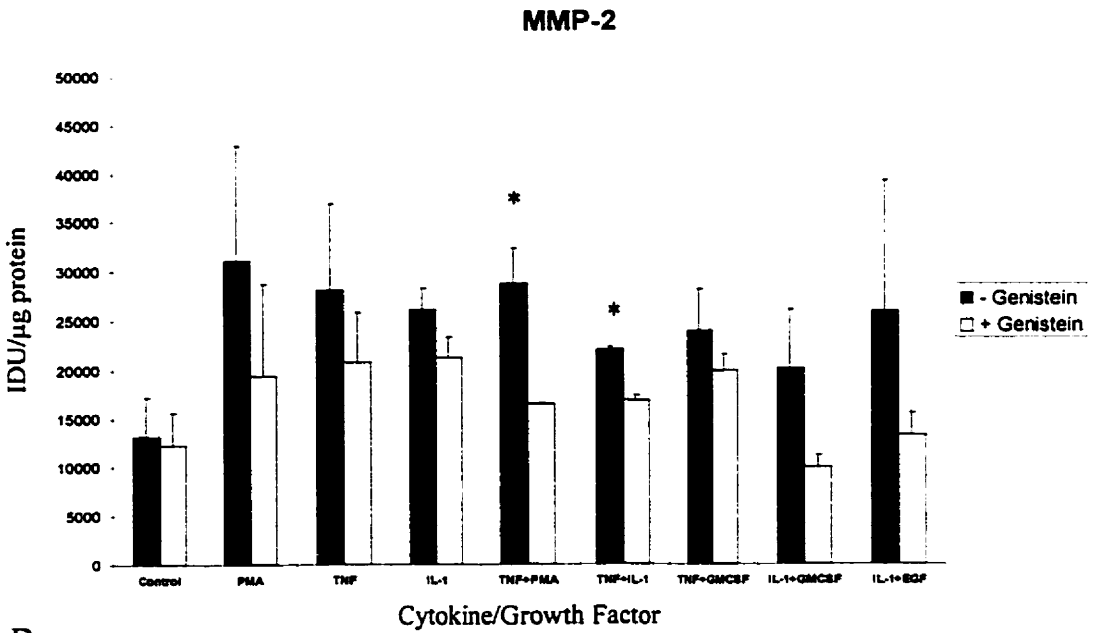
IDU/µg protein
50000
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
Control
PMA
TNF
IL-1
TNF+PMA
TNF+IL-1
TNF+GMCSF
IL-1+GMCSF
IL-1+EGF
- Genistein
+ Genistein
Cytokine/Growth Factor

A.


MMP-2
IDU/µg protein
50000
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
Control
PMA
TNF
IL-1
TNF+PMA
TNF+IL-1
TNF+GMCSF
IL-1+GMCSF
IL-1+EGF
- Genistein
+ Genistein
Cytokine/Growth Factor

B.

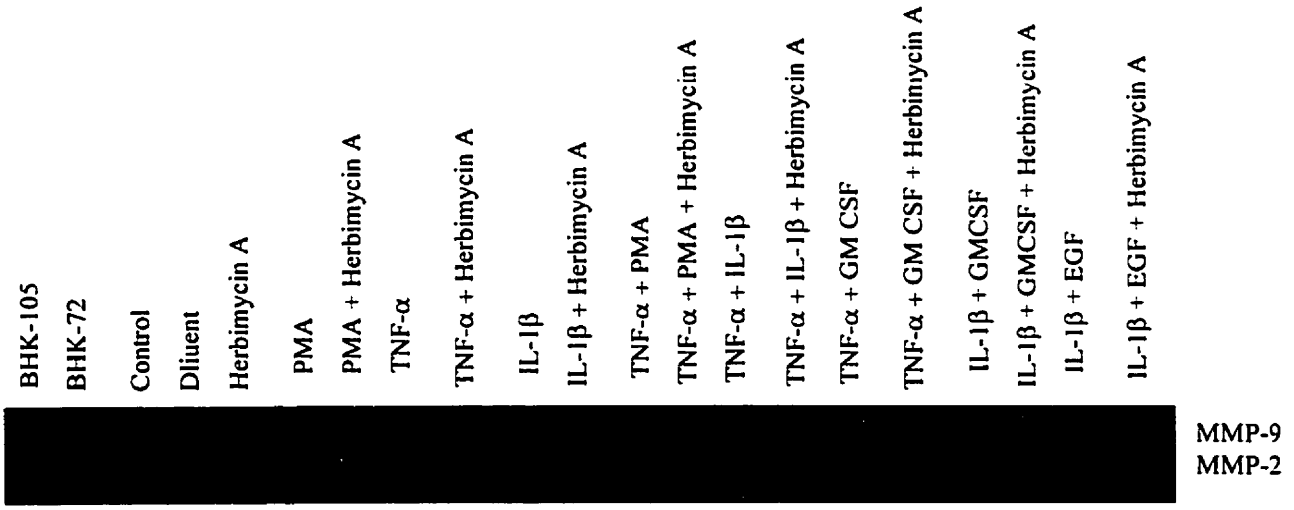
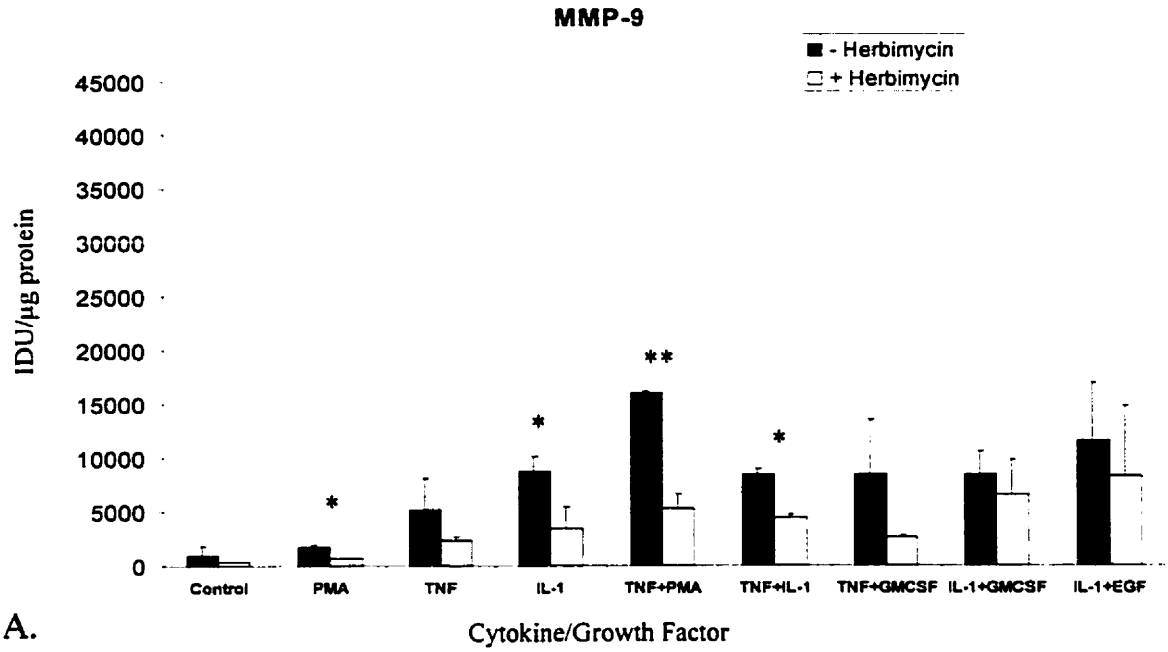


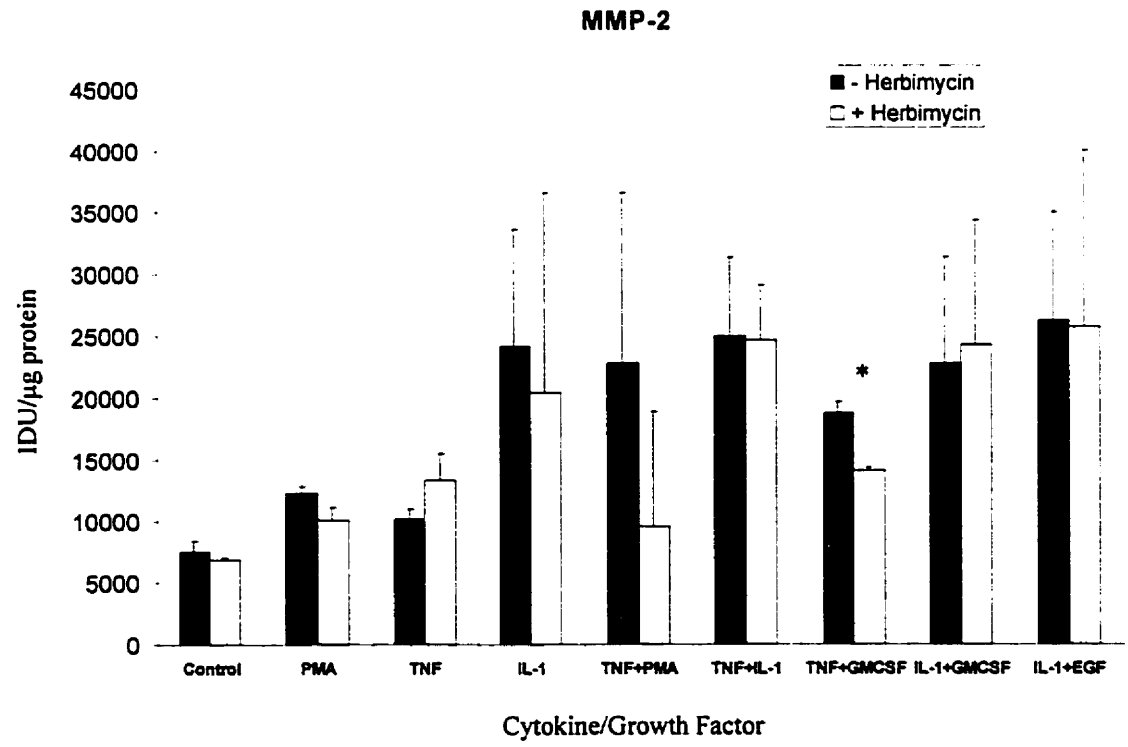
Figure 28: MMP-2 and MMP-9 expression upon stimulation with cytokines and growth factors in the presence or absence of Herbimycin A.
Stromal cells were established in culture by seeding 1 x 10^8 bone marrow cells in T150 flask in Myelocult media supplemented with 10% l-glutamine, 1% hydrocortisone, and 10% antibiotic/antimicrobial. After two hours the non-adherent cells were removed and the adherent cells washed twice with PBS. The cultures were replaced with fresh media and grown at 5% CO_2 and 37°C for 21 days. At days 7 and 14 half the media was replaced with fresh media to sustain growth. Once the stromal layer was grown to confluence, the cells were removed from the flask by treatment with trypsin and plated in 48-well plate at a concentration of 2 x 10^5 cells/ml. The cells were left to grow to 80% confluence (~12hrs) and then exposed to serum-free media with Herbimycin A (15μM) for 30 minutes. The cells were then treated with TNF-α (10ng/ml), PMA (5ng/ml), IL-1β(10ng/ml), TNF-α (10ng/ml) + PMA (5ng/ml), TNF-α (10ng/ml) + GM CSF (10ng/ml), TNF-α (10ng/ml) + IL-1β (10ng/ml), IL-1β (10ng/ml) + GM CSF (10ng/ml), and IL-1β (10ng/ml) + EGF (10ng/ml) and incubated for 24 hrs. The conditioned media was then collected and used for analysis by zymography.

Figure 29: Quantitative analysis of MMP-2 and MMP-9 expression upon stimulation with cytokines and growth factors in the presence or absence of Herbimycin A.

Stromal cells were established in culture by seeding 1 x 10^8 bone marrow cells in T150 flask in Myelocult media supplemented with 10% l-glutamine, 1% hydrocortisone, and 10% antibiotic/antimicrobial. After two hours the non-adherent cells were removed and the adherent cells washed twice with PBS. The cultures were replaced with fresh media and grown at 5% CO_2 and 37°C for 21 days. At days 7 and 14 half the media was replaced with fresh media to sustain growth. Once the stromal layer was grown to confluence, the cells were removed from the flask by treatment with trypsin and plated in 48-well plate at a concentration of 2 x 10^5 cells/ml. The cells were left to grow to 80% confluence (~12hrs) and then exposed to serum-free media with Herbimycin A (15μM) for 30 minutes. The cells were then treated with TNF-α (10ng/ml), PMA (5ng/ml), IL-1β(10ng/ml), TNF-α (10ng/ml) + PMA (5ng/ml), TNF-α (10ng/ml) + GM CSF (10ng/ml), TNF-α (10ng/ml) + IL-1β (10ng/ml), IL-1β (10ng/ml) + GM CSF (10ng/ml), and IL-1β (10ng/ml) + EGF (10ng/ml) and incubated for 24 hrs. The conditioned media was then collected and used for analysis by zymography. MMP-9 expression is graphed in (A) and MMP-2 expression is graphed in (B).


MMP-9
- Herbimycin
+ Herbimycin
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
IDU/µg protein
*
*
*
**
Control
PMA
TNF
IL-1
TNF+PMA
TNF+IL-1
TNF+GMCSF
IL-1+GMCSF
IL-1+EGF
Cytokine/Growth Factor

A.


MMP-2
- Herbimycin
+ Herbimycin
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
IDU/µg protein
*
Control
PMA
TNF
IL-1
TNF+PMA
TNF+IL-1
TNF+GMCSF
IL-1+GMCSF
IL-1+EGF
Cytokine/Growth Factor

B.

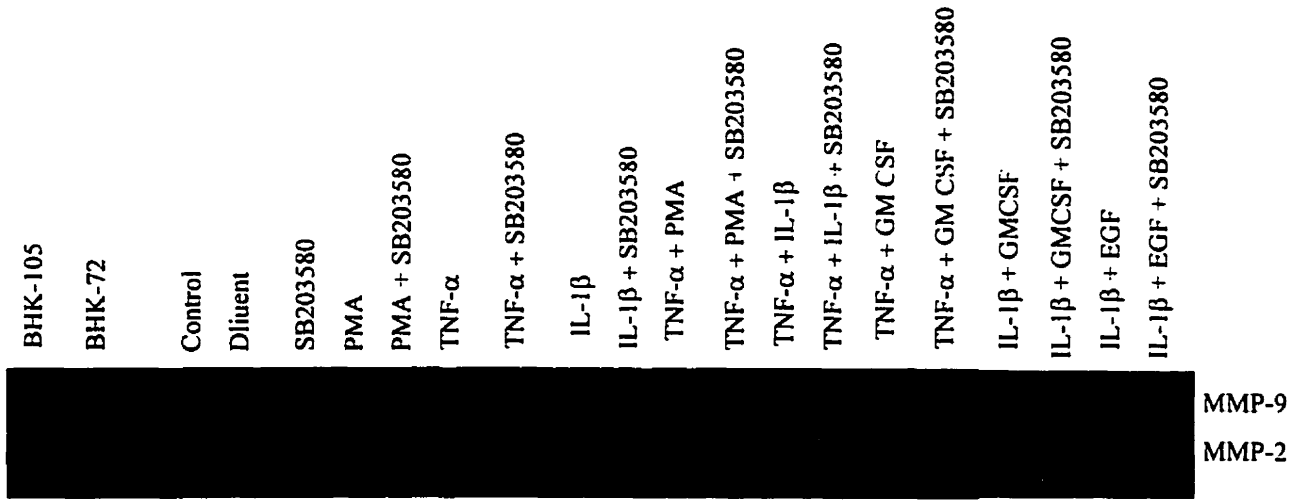
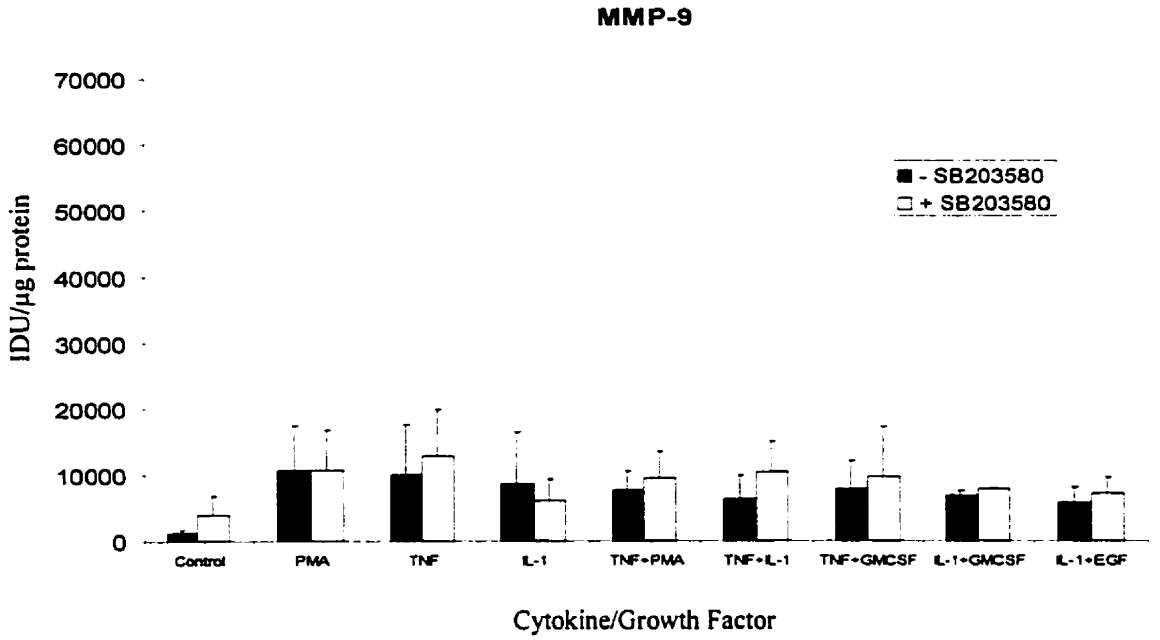


Figure 30: MMP-2 and MMP-9 expression upon stimulation with cytokines and growth factors in the presence or absence of SB203580.
Stromal cells were established in culture by seeding 1 x 10^8 bone marrow cells in T150 flask in Myelocult media supplemented with 10% l-glutamine, 1% hydrocortisone, and 10% antibiotic/antimicrobial. After two hours the non-adherent cells were removed and the adherent cells washed twice with PBS. The cultures were replaced with fresh media and grown at 5% CO_2 and 37°C for 21 days. At days 7 and 14 half the media was replaced with fresh media to sustain growth. Once the stromal layer was grown to confluence, the cells were removed from the flask by treatment with trypsin and plated in 48-well plate at a concentration of 2 x 10^5 cells/ml. The cells were left to grow to 80% confluence (~12hrs) and then exposed to serum-free media with SB203580 (50μM) for 30 minutes. The cells were then treated with TNF-α (10ng/ml), PMA (5ng/ml), IL-1β(10ng/ml), TNF-α (10ng/ml) + PMA (5ng/ml), TNF-α (10ng/ml) + GM CSF (10ng/ml), TNF-α (10ng/ml) + IL-1β (10ng/ml), IL-1β (10ng/ml) + GM CSF (10ng/ml), and IL-1β (10ng/ml) + EGF (10ng/ml) and incubated for 24 hrs. The conditioned media was then collected and used for analysis by zymography.

Figure 31: Quantitative analysis of MMP-2 and MMP-9 expression upon stimulation with cytokines and growth factors in the presence or absence of SB203580.

Stromal cells were established in culture by seeding 1 x 10^8 bone marrow cells in T150 flask in Myelocult media supplemented with 10% l-glutamine, 1% hydrocortisone, and 10% antibiotic/antimicrobial. After two hours the non-adherent cells were removed and the adherent cells washed twice with PBS. The cultures were replaced with fresh media and grown at 5% CO_2 and 37°C for 21 days. At days 7 and 14 half the media was replaced with fresh media to sustain growth. Once the stromal layer was grown to confluence, the cells were removed from the flask by treatment with trypsin and plated in 48-well plate at a concentration of 2 x 10^5 cells/ml. The cells were left to grow to 80% confluence (~12hrs) and then exposed to serum-free media with SB203580 (50μM) for 30 minutes. The cells were then treated with TNF-α (10ng/ml), PMA (5ng/ml), IL-1β(10ng/ml), TNF-α (10ng/ml) + PMA (5ng/ml), TNF-α (10ng/ml) + GM CSF (10ng/ml), TNF-α (10ng/ml) + IL-1β (10ng/ml), IL-1β (10ng/ml) + GM CSF (10ng/ml), and IL-1β (10ng/ml) + EGF (10ng/ml) and incubated for 24 hrs. The conditioned media was then collected and used for analysis by zymography. The conditioned media was then collected and used for analysis by zymography. MMP-9 expression is graphed in (A) and MMP-2 expression is graphed in (B).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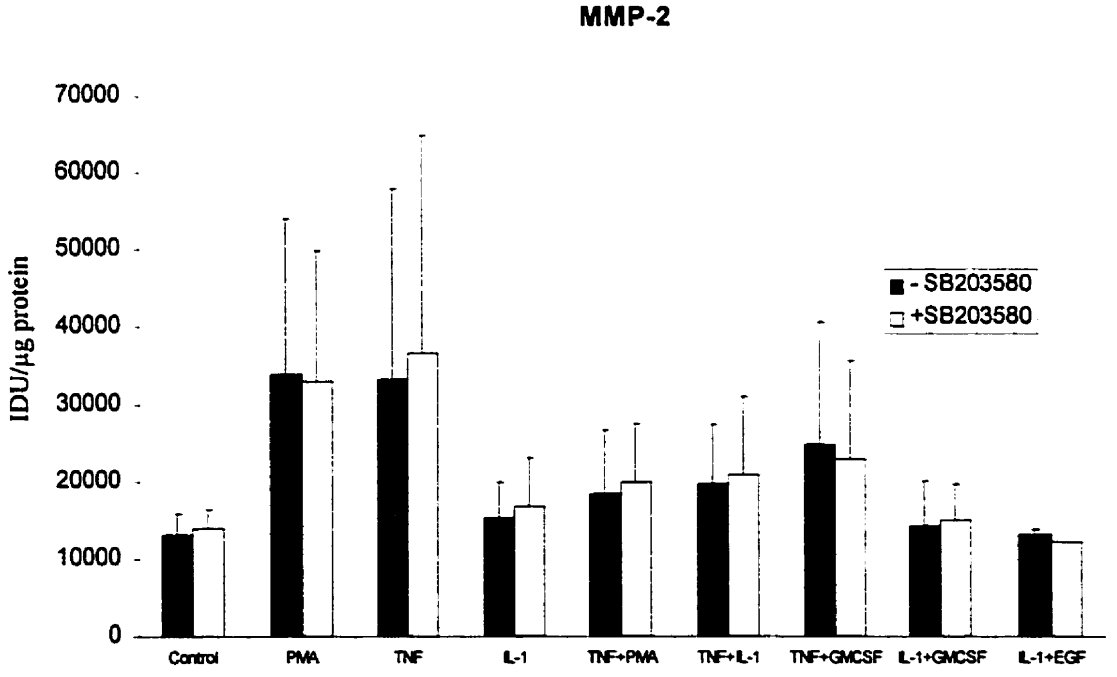
70000
60000
50000
40000
30000
20000
10000
0
IDU/µg protein
■ - SB203580
□ + SB203580
Control
PMA
TNF
IL-1
TNF+PMA
TNF+IL-1
TNF+GMCSF
IL-1+GMCSF
IL-1+EGF
Cytokine/Growth Factor

A.


MMP-2
70000
60000
50000
40000
30000
20000
10000
0
IDU/µg protein
■ - SB203580
□ +SB203580
Control
PMA
TNF
IL-1
TNF+PMA
TNF+IL-1
TNF+GMCSF
IL-1+GMCSF
IL-1+EGF
Cytokine/Growth Factor

B.

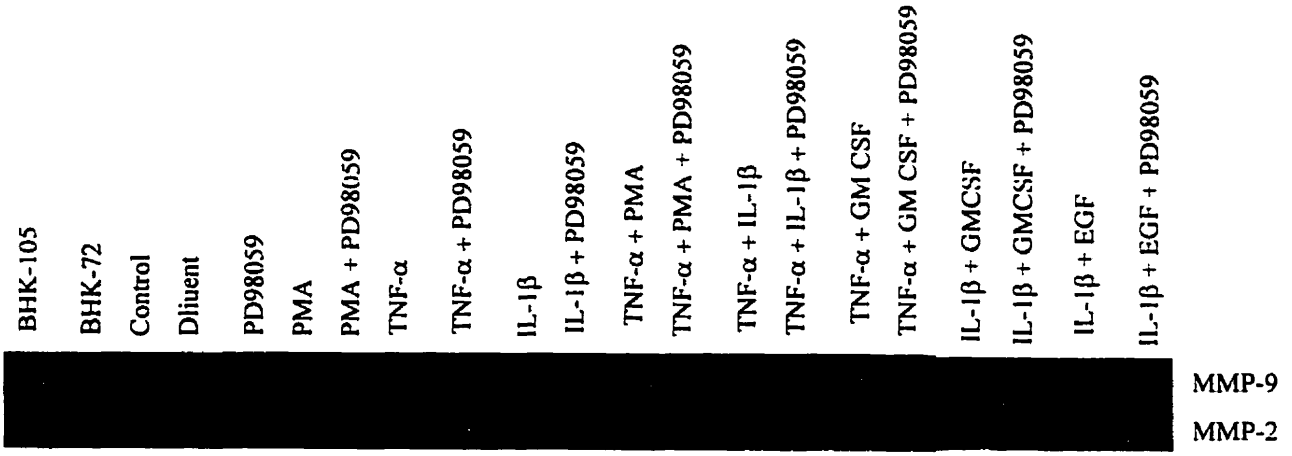
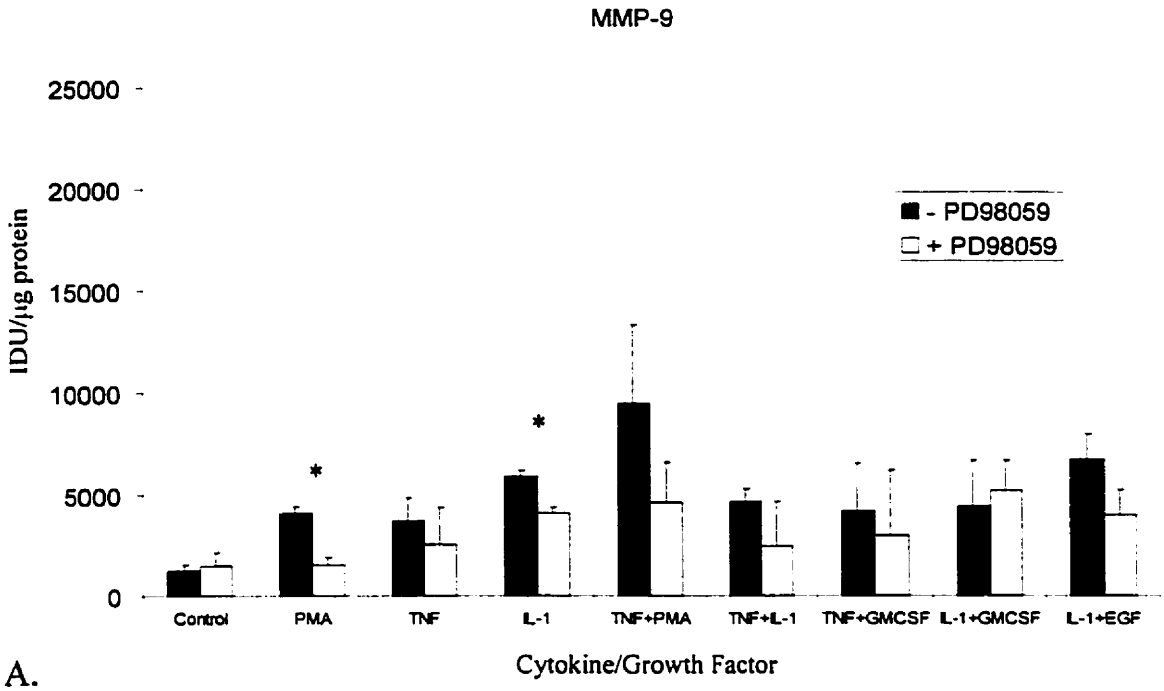


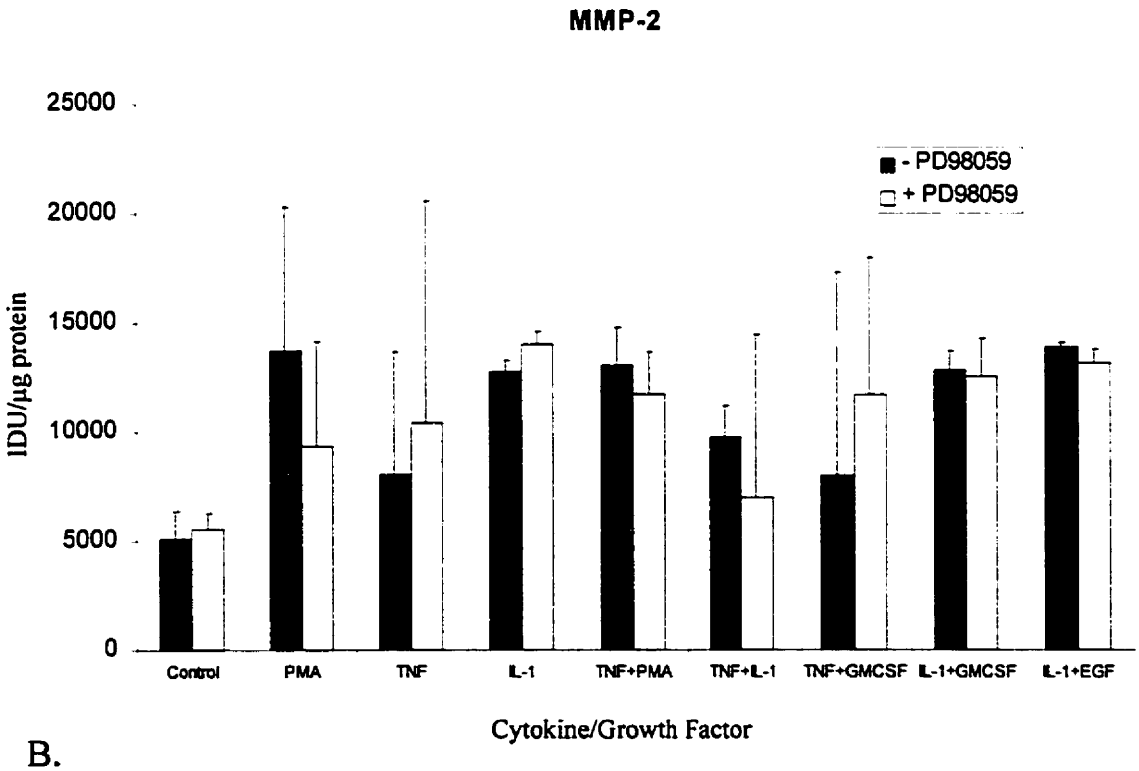
Figure 32: MMP-2 and MMP-9 expression upon stimulation with cytokines and growth factors in the presence or absence of PD98059.
Stromal cells were established in culture by seeding 1 x 10^8 bone marrow cells in T150 flask in Myelocult media supplemented with 10% l-glutamine, 1% hydrocortisone, and 10% antibiotic/antimicrobial. After two hours the non-adherent cells were removed and the adherent cells washed twice with PBS. The cultures were replaced with fresh media and grown at 5% CO_2 and 37°C for 21 days. At days 7 and 14 half the media was replaced with fresh media to sustain growth. Once the stromal layer was grown to confluence, the cells were removed from the flask by treatment with trypsin and plated in 48-well plate at a concentration of 2 x 10^5 cells/ml. The cells were left to grow to 80% confluence (~12hrs) and then exposed to serum-free media with PD98059 (100μM) for 30 minutes. The cells were then treated with TNF-α (10ng/ml), PMA (5ng/ml), IL-1β(10ng/ml), TNF-α (10ng/ml) + PMA (5ng/ml), TNF-α (10ng/ml) + GM CSF (10ng/ml), TNF-α (10ng/ml) + IL-1β (10ng/ml), IL-1β (10ng/ml) + GM CSF (10ng/ml), and IL-1β (10ng/ml) + EGF (10ng/ml) and incubated for 24 hrs. The conditioned media was then collected and used for analysis by zymography.

Figure 33: Quantitative analysis of MMP-2 and MMP-9 expression upon stimulation with cytokines and growth factors in the presence or absence of PD98059.

Stromal cells were established in culture by seeding 1 x 10^8 bone marrow cells in T150 flask in Myelocult media supplemented with 10% l-glutamine, 1% hydrocortisone, and 10% antibiotic/antimicrobial. After two hours the non-adherent cells were removed and the adherent cells washed twice with PBS. The cultures were replaced with fresh media and grown at 5% CO_2 and 37°C for 21 days. At days 7 and 14 half the media was replaced with fresh media to sustain growth. Once the stromal layer was grown to confluence, the cells were removed from the flask by treatment with trypsin and plated in 48-well plate at a concentration of 2 x 10^5 cells/ml. The cells were left to grow to 80% confluence (~12hrs) and then exposed to serum-free media with PD98059 (100μM) for 30 minutes. The cells were then treated with TNF-α (10ng/ml), PMA (5ng/ml), IL-1β(10ng/ml), TNF-α (10ng/ml) + PMA (5ng/ml), TNF-α (10ng/ml) + GM CSF (10ng/ml), TNF-α (10ng/ml) + IL-1β (10ng/ml), IL-1β (10ng/ml) + GM CSF (10ng/ml), and IL-1β (10ng/ml) + EGF (10ng/ml) and incubated for 24 hrs. The conditioned media was then collected and used for analysis by zymography. The conditioned media was then collected and used for analysis by zymography. MMP-9 expression is graphed in (A) and MMP-2 expression is graphed in (B).


MMP-9
25000
20000
15000
10000
5000
0
IDU/µg protein
- PD98059
+ PD98059
*
*
Control
PMA
TNF
IL-1
TNF+PMA
TNF+IL-1
TNF+GMCSF
IL-1+GMCSF
IL-1+EGF
Cytokine/Growth Factor

A.

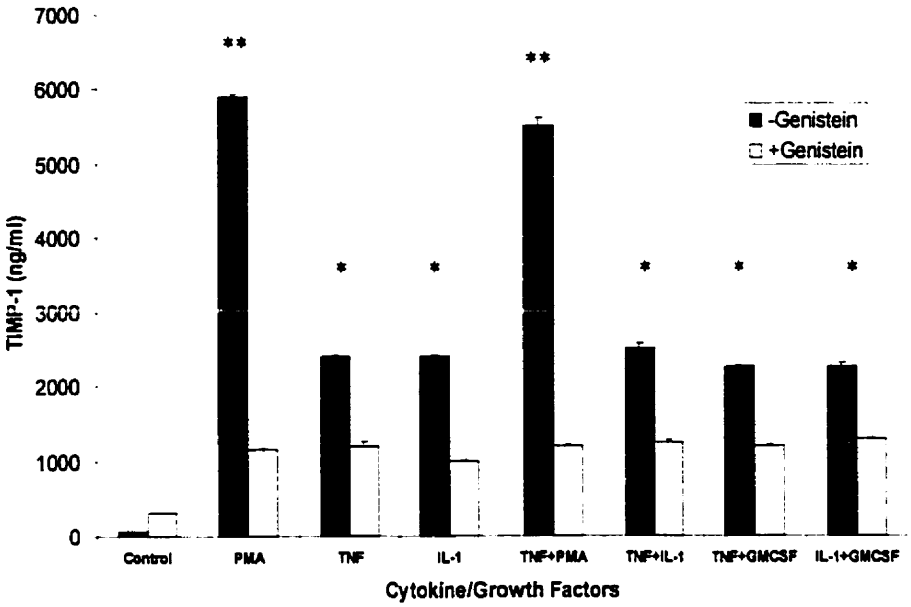

MMP-2
25000
20000
15000
10000
5000
0
IDU/µg protein
- PD98059
+ PD98059
Control
PMA
TNF
IL-1
TNF+PMA
TNF+IL-1
TNF+GMCSF
IL-1+GMCSF
IL-1+EGF
Cytokine/Growth Factor

B.

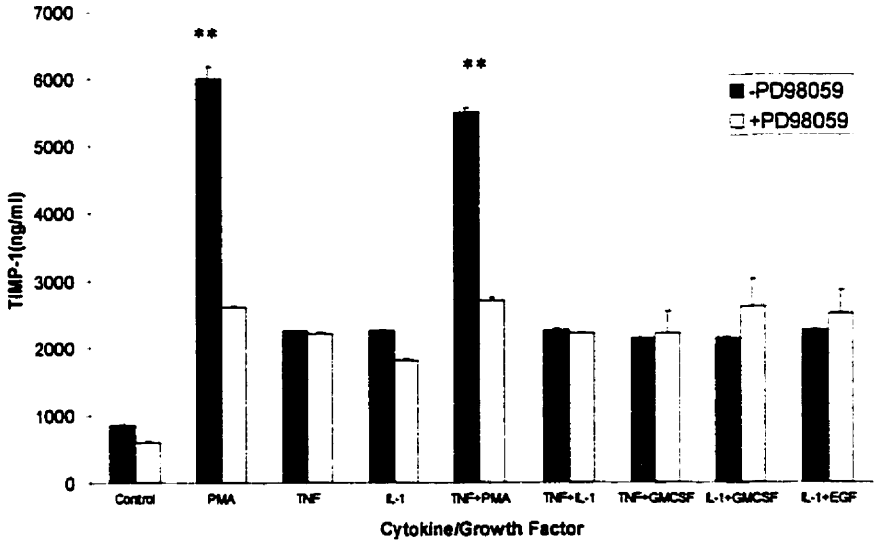
Figure 34: Quantitative analysis of TIMP-1 expression upon stimulation with cytokines and growth factors in the presence or absence of PD98059 or Genistein by ELISA.

Stromal cells were established in culture by seeding 1 x 10^8 bone marrow cells in T150 flask in Myelocult media supplemented with 10% l-glutamine, 1% hydrocortisone, and 10% antibiotic/antimicrobial. After two hours the non-adherent cells were removed and the adherent cells washed twice with PBS. The cultures were replaced with fresh media and grown at 5% CO_2 and 37°C for 21 days. At days 7 and 14 half the media was replaced with fresh media to sustain growth. Once the stromal layer was grown to confluency, the cells were removed from the flask by treatment with trypsin and plated in 48-well plate at a concentration of 2 x 10^5 cells/ml. The cells were left to grow to 80% confluency (~12hrs) and then exposed to serum-free media with Genistein (100μM) (A) or PD98059 (100μM) (B) for 30 minutes. The cells were then treated with TNF-α (10ng/ml), PMA (5ng/ml), IL-1β(10ng/ml), TNF-α (10ng/ml) + PMA (5ng/ml), TNF-α (10ng/ml) + GM CSF (10ng/ml), TNF-α (10ng/ml) + IL-1β (10ng/ml), IL-1β (10ng/ml) + GM CSF (10ng/ml), and IL-1β (10ng/ml) + EGF (10ng/ml) and incubated for 24 hrs. The conditioned media was then collected and used for analysis by ELISA assay.

(*represents $p<0.05$)(** represents $p<0.005$)


7000
6000
5000
4000
3000
2000
1000
0
TIMP-1 (ng/ml)
**
**
*
*
*
*
*
■ -Genistein
□ +Genistein
Control
PMA
TNF
IL-1
TNF+PMA
TNF+IL-1
TNF+GMCSF
IL-1+GMCSF
Cytokine/Growth Factors

A.


7000
6000
5000
4000
3000
2000
1000
0
TIMP-1(ng/ml)
**
**
■ -PD98059
□ +PD98059
Control
PMA
TNF
IL-1
TNF+PMA
TNF+IL-1
TNF+GMCSF
IL-1+GMCSF
IL-1+EGF
Cytokine/Growth Factor

B.

Chapter 4: Discussion

4.1 Expression of MMPs and TIMPs in LTBMC and Isolated Stromal Cell Cultures

Bone marrow progenitor cells and stromal cells have not been comprehensively analyzed for expression of MMPs and TIMPs. This study represents the first comprehensive analysis of MMP and TIMP expression in the *in vivo* LTBMC system. Although MMPs and TIMPs have been studied in other systems such as the lymphoid, thymic and in various cancer cell lines and tumors, studies involving hematopoiesis and the bone marrow system are limited. Bone marrow explants are difficult to study. Bone marrow stroma *in vivo* has very dense cell packing, which makes it difficult to determine cellular interactions. Since LTBMCs are representative of the *in vivo* hematopoietic microenvironment, it provides an excellent *in vitro* system to study the interactions and mechanisms involved in hematopoiesis. The LTBMC is claimed to most closely reproduce the *in vivo* conditions of hematopoiesis. The stromal cell layer of the LTBMC is very similar to bone marrow stroma. Fibroblasts are the major component of the adherent layer and compose 45-55% of the cells. This is followed by macrophages that comprise 25-35% of the adherent layer. Immunohistochemical studies of LTBMC revealed that macrophages have two different morphologies, large round phagocytic and elongated non-phagocytic cells, in bone marrow stromal cell cultures (Wilkins B and Jones D, 1996). Endothelial cells comprise 5-20% and adipocytes are also seen in these cultures (reviewed in Mayani H et al., 1992).

4.1.1 Morphology of LTBMC

The morphology of the LTBMC was photomicrographed at various time-points. On day 1 the bone marrow cells are suspended in the culture media. As the LTBMC progresses some bone marrow cells begin to adhere to the culture flask and spread out (as seen in days 3-11). The adherent/stromal layer becomes confluent as the LTBMC progresses and as seen on days 16 and 21 there is the formation of hematopoietic islands. The photomicrographs demonstrate the complexity and ever changing morphology of the LTBMC. This dynamic process would require various proteins to be expressed at specific intervals and at specific concentrations to induce morphological changes.

4.1.2 Expression of MMPs

Examination of gelatinase expression in LTBMC revealed variable expression of MMP-2 and MMP-9 over the duration of the culture. Initially, there was a high level of MMP-9 expression that was uniform through the culture. In contrast, MMP-2 expression began at a very low level and increased with the progression of the culture. One recent study analyzed the expression of MMPs and TIMPs of mononuclear cells (MNC) from bone marrow (Ries C et al., 1999). In this study, the MNC were separated by a Ficoll density gradient and were then cultured in serum-free media for 96 hrs. Through zymographic analysis, it was found that MNC of the bone marrow secreted MMP-9 but not MMP-2. This is consistent with our zymographic results, which showed strong MMP-9 activity and very little MMP-2 activity on day 3, which is very early in the LTBMC. The data from our study showed that progression of the

LTBMC is associated with the increased expression of MMP-2. This phenomenon occurs around day 11 of the LTBMC. The disadvantage of MNC studies is that there is a heterogeneous mixture of cells and one cannot accurately determine the source of MMP and TIMP expression (i.e. bone marrow mononuclear cells are composed of stromal cells, lymphocytes, etc.).

In our studies, EDTA and Western blot analysis helped confirm the identity of MMPs and TIMPs. Five bone marrow samples were analyzed. The data obtained demonstrates constitutive MMP-9 expression throughout the culture in all samples although absolute values varied from sample to sample. In contrast, there was an increase in the expression of MMP-2 during the LTBMC. The increase in activity of MMP-2 correlated with the increasing confluency of the stromal layer. This observation was further emphasized by the expression of MMP-2 without expression of MMP-9 (by Western Blot) by the isolated stromal cells. One study which isolated bone marrow stromal cells in the same manner as we did found that there was secretion of MMP-2 but not MMP-9 (Barille S et al., 1997). In addition, another study using microvascular endothelial cells from normal bone marrow reported the secretion of MMP-2 only as well (Janowska-Wieczorek A et al., 1999). Logarithmic transformation analysis of our data indicated a large difference in the slope of MMP-2 expression as compared to MMP-9 expression between days 3 to 11 of the LTBMC. This is consistent with our hypothesis that there is differential expression of MMPs within the LTBMC. This is indicative of differential MMP-2 and MMP-9 expression in the human bone marrow cells. The potential biological significance of these findings is that increased MMP-2 expression may contribute to the release of mature

blood cells into the circulation. In related studies, increased MMP-2 and MMP-9 expression has been correlated with acute myelogenous leukemia (AML) (Janowska-Wieczorek A et al., 1999; Ries C et al., 1999). AML is characterized by the premature egress of leukemic cells from the bone marrow into peripheral blood. In addition, increase in MMP-2 expression has been associated with invasive phenotype of cancer cells. Therefore, the increase in MMP-2 expression may aid in the migration of mature cells out of the hematopoietic microenvironment within the bone marrow and into circulation.

In addition, increased MMP-2 expression may have an effect on proliferation and differentiation of the cells in the hematopoietic microenvironment. A recent study investigated the relationship between MMP production and atherosclerosis. They found that proliferation and migration of cultured vascular smooth muscle cells was closely related to the stimulation of MMP-2 expression (Uzui H et al., 2000).

To further investigate the results of increased MMP-2 expression in hematopoiesis one could perform colony-forming unit (CFU) assays to assess cellular differentiation. Matrigel invasion assays could assess the migratory effect of increased MMP-2 expression on hematopoietic stem cells. Finally, removal of secreted MMP-2 from the microenvironment by passage of the supernatant through an anti-MMP-2 monoclonal antibody-sepharose affinity column would enable one to assess the growth promoting ability of MMP-2 within the LTBMC.

4.1.3 Expression of TIMPs

Our data on the expression of TIMPs in LTBMC are novel. The expression of TIMPs by cells of the LTBMC was analyzed using reverse zymography. Recent studies of normal bone marrow cells did not report the presence of TIMP activity (Janowska-Wieczorek A et al., 1999; Ries C et al., 1999). Our studies have demonstrated that there is a constant level of expression of TIMP-2 throughout the LTBMC whereas TIMP-1 levels increased in the latter stages of the LTBMC. Reverse zymography and western blot analysis have demonstrated this. There are some correlations that can be made regarding MMP and TIMP expression in the LTBMC. Firstly, MMP-2 and TIMP-1 both have low activity in early stages of the LTBMC that increases with LTBMC progression. Secondly, the increase in TIMP-1 and MMP-2 expression correlates with stromal layer development. Thirdly, MMP-9 and TIMP-2 expression remains constant during the LTBMC. The increase in TIMP-1 expression may affect cell function on many levels. It may play a role in proliferation of the hematopoietic stem cells due to its growth promoting ability. TIMP-1 could also aid in the release of matured blood cells, as with MMP-2 since it has been shown in some cases to promote tumor progression (Kossakowska AE et al., 1991; Stetler-Stevenson WG, 1997). TIMP-1 can also be released in response to the increases in MMP (MMP-2 and constant MMP-9) expression. The findings of increased expression of TIMP-1 and constant level of expression of MMP-9 was surprising since expression of these molecules usually occurs together. As previously alluded to, TIMP-2 expression was constitutive in the LTBMC. Again, it would be thought that MMP-2 and TIMP-2 expression would be simultaneously increased. The

discordant expression of MMP-9 and TIMP-1 and MMP-2 and TIMP-2 may be due to the heterogeneous mixture of cells found in the LTBMC.

TIMP-4 expression, as determined by RT-PCR, was variable throughout the LTBMC. There was an increase in expression during days 16 and 21. This expression pattern is similar to that of MMP-2 and TIMP-1. Therefore, it can be speculated that the expression of TIMP-4 correlates with the establishment of the stromal layer. This observation needs further confirmation.

4.1.4 Expression of MT1-MMP and TACE

Through preliminary analysis using RT-PCR analysis, it can be seen that TIMP-3 and TACE are expressed at all stages of the LTBMC at relatively constant levels. As TIMP-3 is an inhibitor of TACE (Amour A et al., 1998) the co-expression of these molecules is an expected finding. TACE analyses at the protein level (western blot analysis) and at the transcriptional level are similar. The exception to this is the decrease in TACE mRNA transcript on day 6. More investigation is required to delineate whether the lack of TACE seen on day 6 is indeed real or represents an artifact. To further characterize and quantify the expression pattern of TACE and TIMP-3 additional investigation is required.

The increase in MMP-2 expression leads one to the question of whether there is a simultaneous increase in MT1-MMP and TIMP-2 expression since these molecules are closely involved in the activation of MMP-2. MT1-MMP has been shown to associate and complex with TIMP-2 (Imai K et al., 1996) leading to activation of proMMP-2 (Kinoshita T et al., 1996). MT1-MMP is expressed by

stromal cells of colon, breast and head and neck carcinomas (Okada A et al., 1995). In addition, increased MT1-MMP expression is associated with invasiveness of tumor cells (Sato H et al., 1994), head and neck carcinoma (Yoshizaki T et al., 1997) and cervical cancer cells (Gilles C et al., 1996). MT1-MMP transcripts are only weakly expressed on day 16 although it has been reported that transcripts are expressed in bone marrow MNC (Ries C et al., 1999). In contrast, protein analysis shows steady expression of MT1-MMP. The absence of MT1-MMP PCR products in our assay could be attributed to ineffective primers or lack of full optimization of the PCR reaction.

In summary, this is the first study to comprehensively analyze MMP and TIMP expression in normal LTBMC.

4.2 Cytokines Regulate the Expression of MMPs and TIMPs in Bone Marrow Stromal Cells

4.2.1 Cytokines and MMP Expression

MMPs and TIMPs are inducible and can be regulated at the transcriptional and post-transcriptional level. As indicated earlier, unstimulated stromal cells of the LTBMC expressed only MMP-2. Upon stimulation with PMA, however, they expressed MMP-9 and activated forms of MMP-2 as demonstrated by zymographic analysis. MMP-9 activity increased 2-fold by PMA and 1.6-fold by TNF-α and IL-1β over control unstimulated stromal cells. In addition to the induction of MMP-2 (2-fold) stimulation with TNF-α and IL-1β resulted in the appearance of the activated form of MMP-2. Our results are distinct from previous reports that showed that bone

marrow stromal cell MMP-2 is not regulated by IL-6, IL-1β, TNF-α or TGF-β (Barille S et al., 1997). This discrepancy can be attributed to different culturing techniques of the bone marrow stromal cells since the concentration of cytokines added was the same. Combinations of the cytokines proved to have additive effects on the expression of MMP-9. The induction of MMP-9 ranged from 2-4-fold compared to control cells. MMP-2 was slightly induced by the cytokine combinations.

Many cytokines and growth factors such as TNF-α, TNF-β, IL-1, IL-6 and EGF have been demonstrated to up-regulate MMP expression (Goetzl EJ et al., 1996; Ries C and Petrides, 1995). In fact, TNF-α and IL-1β induce MMP-9 expression in CD3+, CD4+ T cells (Johnatty RN et al., 1997) and macrophages (Saren P et al., 1996). In addition, another study investigating cytokine regulation of MMPs and TIMPs found that TNF-α, IL-1β and GM CSF stimulated MMP-9 and TIMP-1 only (Zhang Y et al., 1998). These findings support our studies since the stromal cell layer is comprised of 25-35% macrophages.

The cytokine induction of MMP and TIMP expression in the bone marrow stromal cells is not surprising. Others have shown that tumor cells from breast and other cancer cell lines can produce factors that stimulate the stromal layer to produce MMPs and TIMPs. It does provide support for the hypothesis that cytokines may modulate processes such as myelofibrosis and leukemic cell motility via their regulation of MMPs and TIMPs. Studies comparing normal versus leukemic bone marrow cells would help in answering this question.

4.2.2 Cytokines and TIMP Expression

Preliminary studies using reverse zymography demonstrated that PMA increased stromal cell TIMP-1 expression by more than 2-fold, which is similar to its effect on MMP-9. IL-1β and EGF also induced TIMP-1 by approximately 1.5-fold. There was no evident effect on TIMP-2 expression upon stimulation by cytokines alone. The combined effects of the cytokines on TIMP-1 expression were large with greater than 2-fold increases by all combinations. The most pronounced induction of TIMP-1 was produced by the combination of TNF-α and PMA (2.5-fold increase).

To aid in the assessment of TIMP-1 induction , a commercial TIMP-1 ELISA system was used. ELISA analysis demonstrated that PMA alone or PMA+ TNF-α treatment induced a statistically significant amount of TIMP-1 expression (6-fold). Induction by other cytokines and combinations yielded a 2.5-fold increase in TIMP-1 expression ($p<0.05$).

The induction of TIMP-2 was not well visualized in the reverse zymogram analysis. To further investigate TIMP-2 induction, ELISA experiments can be performed on the conditioned media collected from the cultures. Analysis of the bone marrow stromal cell expression of MMPs and TIMPs by cytokine stimulation is not reported in the literature. The findings of cytokine induction of gelatinase and TIMP-1 are novel.

4.3 Mechanism of Cytokine Regulation of Bone Marrow Stromal Cell Expression of MMPs and TIMPs

To investigate the mechanism by which the cytokines induce MMP and TIMP expression in stromal cells, tyrosine kinase and MAPK inhibitors were utilized. Two protein tyrosine kinase inhibitors, Genistein and Herbimycin A, and two MAPK inhibitors, PD98059 (MEK inhibitor) and SB203580 (p38 specific inhibitor) were utilized. It has been demonstrated that MMP-13 induced expression in human fibroblasts is mediated through a p38 MAPK pathway (Ravanti L et al., 1999). In addition, induction of MMPs in mammary tumor cells (Aguirre-Ghiso J et al., 1998), breast epithelial cells (Reddy KB et al., 1999), HaCaT Keratinocytes (Sudbeck B et al., 1999), articular chondrocytes (Li L et al., 1998), and squamous cell carcinoma cell lines (Simon C et al., 1998) have been shown to involve MAPK and/or tyrosine kinase pathways. **In our study, Genistein and Herbimycin A had inhibitory effects on the induction of MMP-9 expression.** Incubation with Genistein greatly decreased MMP-9 expression, specifically with the PMA and TNF-α combination ($p<0.005$). Cytokine induced MMP-2 expression was decreased slightly upon incubation with Genistein. Herbimycin A reduced TNF-α+PMA mediated MMP-9 expression by approximately 3-fold ($p<0.005$). SB203580 and PD98059 had little effect on the cytokine-induced expression of MMP-2 or MMP-9. Therefore, it can be concluded that the mechanism of MMP induction in bone marrow stromal cells is through a tyrosine kinase pathway and not the MAPK pathway.

The regulatory mechanism controlling TIMP-1 induction was investigated using tyrosine kinase inhibitor, Genistein and MAPK inhibitor PD98059. PMA and

TNF-α+PMA-mediated expression of TIMP-1 was decreased 6-fold upon treatment with genistein ($p<0.005$). Genistein inhibited all other cytokine-mediated TIMP-1 expression 2-fold ($p<0.05$). In contrast, MAPK inhibitor, PD98059 inhibited PMA and TNF-α+PMA-mediated expression of TIMP-1 by 2-fold ($p<0.005$) but had no effect on the other cytokine-stimulated expression.

4.4 Future Directions

The beauty of these investigations is that they open the door and provide a basis for many new studies. With the knowledge of the endogenous expression of MMPs and TIMPs in both the LTBMC and isolated stromal cells, one can investigate pathological states for comparison.

Another interesting area of investigation is MMP and TIMP expression in bone marrow following or during drug therapy. Genistein is one of many drugs used in the treatment of cancer. We have shown here that genistein has inhibitory affects on the expression of gelatinases and TIMP-1 in normal bone marrow. The potential effects on bone marrow and MMP and TIMP expression during drug therapy is an area that has not yet been investigated.

Tumors have been shown to exhibit aberrant expression of tyrosine kinases. Thus, tumors cells may alter bone marrow function by modulating cytokine induced signal transduction pathways.

In addition, the investigation of cytokine-induction of TIMP-3, TIMP-4, MT1-MMP and TACE expression would shed some much-needed light on their control. Essentially, one would speculate that TIMP-3 would be regulated in a similar fashion

as TIMP-1 and that TIMP-4 would have constitutive expression as with TIMP-2. A very good method of investigating TIMP function in hematopoiesis is through the use of TIMP knockout mice. The effects of TIMP knockouts on hematopoiesis can be studied using bone marrow cultures of knockout mice and normal mice for direct comparisons in areas such as morphology, motility, proliferation and differentiation.

4.5 Conclusions

In this study, we demonstrate by zymography, reverse zymography, Western blot analysis, ELISA and RT-PCR that MMPs and TIMPs are expressed in cells of the LTBMC. LTBMC are an *in vitro* model of the hematopoietic microenvironment. Using isolated bone marrow stromal cell cultures we also demonstrate that cytokines IL-1β, TNF-α and PMA, as well as combinations of these, regulate MMP and TIMP expression. Cytokines induced a dramatic increase in MMP-9 expression compared to MMP-2. In addition, MMP-9 was much more susceptible to inhibition by tyrosine kinase inhibitors. This may account for the variability in MMP-9 expression that was observed from patient to patient. We also demonstrated that a tyrosine kinase pathway regulates cytokine-induced TIMP-1 expression. These finding are significant, as they have not been previously reported in the literature. These findings will further enhance the field of MMP and TIMP studies by providing the information of their expression in normal bone marrow cells and the signal transduction pathways by which cytokines regulate gelatinase and TIMP expression.

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