UNIVERSITY OF CALGARY

Tissue Inhibitors of Metalloproteinases
in Human Malignant Gliomas

by

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ABSTRACT

In this study, a detailed analysis of TIMP expression in human normal brain and malignant gliomas at the mRNA and protein levels was undertaken. RT-PCR analyses of total RNA from 42 surgical tumor specimens revealed unique expression patterns for individual TIMPs 1-4, with TIMP-1 and -4 showing positive and negative correlations, respectively, with glioma malignancy. Similar results were obtained on Western blots. *In situ* hybridization localized TIMP-1 to glial tumor cells and the surrounding tumor vasculature. TIMP-4 transcript was predominantly localized to tumor cells, a pattern further confirmed by immunohistochemistry. Reverse zymography showed inhibitory activity for all TIMPs in the various tumor grades and pointed to the existence of TIMP-protein complexes *in vivo*. Finally, both TIMP-4-overexpressing U87 clones and glioma cells treated with rTIMP-4 showed reduced invasive capacity *in vitro*. These data suggest that TIMPs are differentially expressed in human malignant gliomas, and the relative contribution of these to malignancy, particularly TIMP-1 and -4, needs to be evaluated.

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For my uncle, William Kelly

"In this world you will have trouble. But take heart! I have overcome the world."

John 16:33

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List of Abbreviations

General

AP-1	activator protein-1
PEA-3	polyoma virus enhancer-activator protein-3
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
MMP	matrix metalloproteinase
TIMP	tissue inhibitor of metalloproteinase
ECM	extracellular matrix
ec	endothelial cell
IL	interleukin
EGF	epidermal growth factor
FGF	fibroblast growth factor
TNFα	tumor necrosis factor alpha
RT	reverse transcription
PCR	polymerase chain reaction
RZ	reverse zymography
CNS	central nervous system
WHO	world health organization
N	normal brain
A	astrocytoma
AA	anaplastic astrocytoma
MA	malignant astrocytoma
MO	malignant oligodendroglioma
ENM	extraneural metastasis
GBM	glioblastoma multiforme
LG	low grade
MG	middle grade
HG	high grade
TGF	transforming growth factor
FAK	focal adhesion kinase
IGF	insulin growth factor
SFD	Sorsby's fundus dystrophy
MT-MMP	membrane type-matrix metalloproteinase
MRNA	messenger RNA
VEGF	vascular endothelial growth factor
TACE	TNF-alpha converting enzyme
ADAM	A Disintegrin and Metalloproteinase
H & E	hematoxylin and eosin
NIH	National Institute of Health
MuLV	murine leukemia virus

Units of Measurement

cpm	counts per minute
kDa	kilodalton
kb	kilobase
°C	degrees celcius
M	molar
mM	millimolar
μM	micromolar
g	gram
mg	milligram
μg	microgram
ng	nanogram
pg	picogram
L	litre
ml	millilitre
μl	microlitre
μCi	microcurie
bp	basepair
kb	kilobase
w/v	weight per unit volume
ELISA	enzyme-linked immunoabsorbant assay

Chemicals and Solutions

FCS	fetal calf serum
PBS	phosphate-buffered saline
TBS	TRIS-buffered saline
TRIS	tris-(hydroxymethyl)aminomethane
EDTA	ethylenediaminetetraacetic acid
SDS	sodium dodecyl sulfate
EtBr	ethidium bromide
puro	puromycin
DTT	dithiothreitol
NBT	nitroblue tetrazolium chloride
BCIP	5-bromo-4-chloro-3-idoyl-phosphate, 4-toluidine salt
SSC	standard saline-citrate
Ab	antibody
TN	tween
MTT	methylthioltetrazole
PMA	phorbol ester
DAB	3 3'-diaminbenzidine
BSA	bovine serum albumin

Nucleic Acids

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

1. INTRODUCTION

This is my letter to the world
that never wrote to me----
The simple news that Nature told
with tender majesty.

Her message is committed
to hands I cannot see,
For love of her, sweet countrymen,
judge tenderly of me!

Emily Dickinson

1.1 Gliomas

Glioblastoma multiforme is one of the most dramatic forms of human illness. Patients suffer from severe mental and physical disabilities while faced with the terrifying prognosis of death within 12-24 months. Although considered an uncommon malignancy (1), gliomas are the second leading cause of cancer death in children and account for almost 7% of years of life lost to cancer before the age of 70 (2). The treatment of brain tumors has expanded rapidly over the past few years, but the prognosis of the disease has yet to change. For these reasons, it is imperative that novel and effective therapies for gliomas be investigated.

Gliomas originate from glial cells, which are composed of both astrocytes and oligodendrocytes. Normally, these cells function to provide cellular support, maintain ion homeostasis, uptake neurotransmitters, and to preserve tissue integrity following injury (3). In order to become malignant, glia are thought to undergo a number of genetic "hits", and the degree of cytogenetic aberrancy generally relates to histological grade. Thus, a high grade glioma would likely have the greatest degree of genetic atypia, while a low grade tumor would have few, if any, genetic abnormalities (4).

Chromosomal alterations found in gliomas include gains on chromosome 7 (80% of cases), losses on chromosome 10 (60%) and rearrangements on chromosome 9 (35%) (5). Interestingly, chromosome 7

houses two genes of importance in tumorogenesis: epidermal growth factor receptor (EGFR) and platelet derived growth factor alpha chain (PDGF-alpha) (6). Oncogenes in the human central nervous system (CNS) which are frequently mutated include MDM2, c-myc, N-myc, and N-ras (6, 7). Mutated tumor suppressors include p53, p16, and Rb (8-11). A summary of some of the genetic alterations found in primary tumors of the CNS is shown in Table 1.

The method of grading gliomas varies slightly between laboratories. According to the World Health Organization (WHO), tumors with atypical nuclei alone are considered grade II (astrocytomas) and those which also demonstrate mitotic activity are grade III (anaplastic astrocytomas). Neoplasms showing atypical nuclei, mitosis, angiogenesis, and necrosis are considered grade IV (glioblastoma multiforme or GBM), and represent approximately 50% of gliomas in adults (2). Most GBMs result from the malignant progression of grade II and III astrocytomas, although a portion arise *de novo* (unaccompanied by low grade lesions).

1.2 Invasion

The lethality of gliomas is largely related to their extensive infiltration into surrounding normal brain tissue. Although gliomas may appear to be well demarcated, glial cells typically migrate along the blood vessels and myelinated fiber tracts of the brain (12). The latter process

Table 1. Genetic Alterations in Primary Human CNS Tumors

Tumor type	Probe	Aberration
Astrocytoma	c-erb B (EGFR)	A
	c-myc	EE
	v-fos	EE
	v-sis	EE
	p53	mutation
Anaplastic Astrocytoma	c-erb B (EGFR)	A, RA
	N-myc	A
	c-sis (PDGF)	EE
	p53	mutation
Glioblastoma multiforme	c-erb B (EGFR)	A, RA, EE
	gli	A, EE
	N-myc	A, EE
	v-myc	EE
	v-sis	A, EE
	c-sis (PDGF)	EE
	v-fos	EE
	N-ras	A, EE
	Ha-ras	rare alleles
	c-mos	rare alleles
	Rb gene	deletion
	p53	point mutation
Oligodendroglioma	c-erb B (EGFR)	A, RA
	Ha-ras	rare alleles
	c-mos	rare alleles
Medulloblastoma	c-erb B (EGFR)	A
	N-myc	A, RA
	c-myc	A, EE
	v-myc	EE
	N-ras	point mutation
	p53 gene	mutation
Meningioma	c-sis (PDGF)	EE
	c-myc	A
	c-fos	EE
	N-myc	A, RA
	Ha-ras	rare alleles
	c-mos	rare alleles

Abbreviations: A = gene amplification; RA = gene rearrangement; EE = enhanced gene expression

Modified from (5)

likely involves myelin-associated ligands, while invasion along blood vessels is receptor-mediated (13).

The basement membranes of the central nervous system are restricted to the glia limitans externa and the wall of the large blood vessels, and remain largely undisrupted in most glial tumors (14). Thus, glial invasion depends not only on the extracellular matrix (ECM) that confronts the tumor cell, but also on the capacity of the cell to make it's own ECM (13). For example, an inverse relationship was found between a cell's expression of the ECM component fibronectin, and its invasive capacity. With abundant fibronectin expression, such as in U87 cells, migration of gliomas is hindered and anchorage is promoted. This possibly promotes tumor growth by exposing the U87 cells to growth factors within the ECM, and by facilitating neovascularization. However, when fibronectin expression is low, such as in U251 cells, gliomas are able to migrate along the fibronectin produced by the host and show an increased invasive capacity.

1.3 Angiogenesis

Angiogenesis, or the formation of new capillaries from pre-existing vessels, is another factor contributing to the pathology of malignant gliomas (15). This process is elicited by angiogenic stimuli such as aFGF, bFGF, and TGF-α, whereby the endothelial cell (EC) lining in the existing blood vessel dissolves the underlying basement membrane

to form capillary sprouts (16-19). These sprouts elongate toward the stimuli by way of endothelial cell migration and proliferation (20). Final maturation occurs when the basement membrane surrounding the EC is reconstituted (21).

Normally, angiogenesis is restricted to specific developmental stages such as embryogenesis, placental formation and uterine changes that occur during the female reproductive cycle. However, this process is also induced by solid tumors, which recruit ECs from pre-existing vasculature to provide increased nourishment for their growing metabolic needs (22). Malignant gliomas are among the most vascularized human neoplasms and the extent of angiogenesis is a major determinant of histological grade, as well as patient prognosis (15).

1.4 Matrix metalloproteinases

Two essential aspects of glioma pathophysiology, invasion and neovascularization, are emerging as primary targets of intervention. Both processes require degradation of the ECM by proteolytic enzymes and subsequent migration of the cells through the degraded structures (23a). ECM breakdown and tissue remodeling are principally mediated by a family of degradative enzymes known as matrix metalloproteinases (MMPs). There are currently 19 known members of the MMP family, which together degrade a variety of matrix components (Table 2). These proteases are grouped according to their structures and substrate

Table 2. The MMP Family

Group	Members	MMP Number	Main Substrates
Collagenases	Interstitial collagenase	MMP-1	Fibrillar Collagens
	Neutrophil collagenase	MMP-8	"
	Collagenase-3	MMP-13	"
	Collagenase-4	MMP-?	?
Gelatinases	Gelatinase A	MMP-2	Gelatin Type IV, V collagen Fibronectin
	Gelatinase B	MMP-9	"
Stromelysins	Stromelysin-1	MMP-3	Laminin Non-fibrillar collagen Fibrinectin
	Stromelysin-2	MMP-10	"
	Matrilysin	MMP-7	"
MT-MMPs	MT1-MMP	MMP-14	pro-MMP-2, Collagen, gelatin
	MT2-MMP	MMP-15	"
	MT3-MMP	MMP-16	"
	MT4-MMP	MMP-17	"
Others	Stromelysin-3	MMP-11	proteinase inhibitor (serpin)
	Metalloelastase	MMP-12	Elastin
	Enamelysin	MMP-?	?
	Xenopus MMP	MMP-?	?
	?	MMP-19	Aggrecan

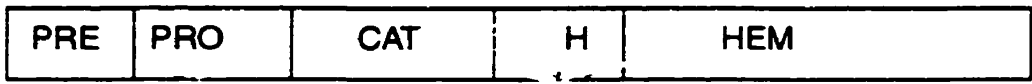
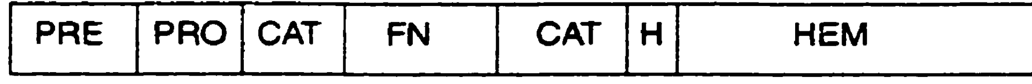
Note: There is no MMP-4, -5, or –6.
Modified from 25 and 25b.

specificity's, and can be divided into four main sub-classes including membrane type matrix metalloproteinases (MT-MMPs), collagenases, stromelysins and gelatinases.

MMPs are characterized by several common structural features: all share a catalytic domain that requires a zinc ion at the active site. The N-terminus of MMPs consists of a hydrophobic signal peptide followed by a pro-peptide domain, and an N-terminal catalytic domain (Figure 1). Activation of the MMP requires cleavage of the pro-peptide region, disrupting the "cysteine-switch"; a zinc-cysteine bond which holds the enzyme in a latent conformation (24, 25). The hemopexin-like C-terminal is preceded by a proline-rich hinge region. Truncations of this region do not affect proteolytic activity, but rather seem to alter substrate specificity (26) and affect the binding of MMPs such as gelatinases A and B to their natural inhibitors (27).

Controlled MMP activity is essential to numerous physiological processes such as ovulation, wound healing, and bone growth. However, when MMP activity goes unchecked, excessive tissue destruction occurs, leading to a myriad of pathological states such as atherosclerosis, arthritis and tumor invasion and metastasis. Numerous studies have assessed the role of MMPs in human cancers, and it appears that MMP-2 (gelatinase-A) and MMP-9 (gelatinase B) act as principal mediators of invasiveness (23).

Figure 1. Domain structure of the matrix metalloproteinases (MMPs). The 19 members of the MMP family of proteases share a common five domain modular structure, characteristic of the collagenases and the stromelysins. Three tandem 58 amino acid fibronectin II like domains exist which distinguish the gelatinases, while the trans-membrane domain is unique to the MT-MMPs (modified from 23b). MT = membrane type; PRE = leader sequence; PRO = prodomain; CAT = catalytic domain; H = hinge; HEM = hemopexin-like domain; F = furin consensus site; FN = fibronectin like domain; C = collagen-like domain; TM = transmenbrane domain.

MMP	DOMAIN STRUCTURE
Matrilysin	
Interstitial Collagenase Neutrophil Collagenase Collagenase-3 Stromelysin-1 Stromelysin-2 Metalloelastase MMP-18 MMP-19	
Stromelysin-3	
MT1-MMP MT2-MMP MT3-MMP MT4-MMP	
Gelatinase A	
Gelatinase B	

Specifically, MMPs have been implicated in the invasiveness of gliomas. Both gelatinases A and B are present and over-expressed in glioma cell lines (28-34). Their distribution within the tumor is different, however, suggesting unique pathological roles. Localization studies have shown gelatinase A is constitutively expressed, and is produced by many cell types within the tumor (i.e. astrocytes, neurons, blood vessels) and also by host stromal cells at the invasion front. In contrast, gelatinase B expression is more localized, particularly around the vasculature at the proliferation margin (28). This points to gelatinase B as a potential marker of neovascularization. Interestingly, gelatinase A was activated only in extra-neural metastatic cases of GBM (28). Metastasis of glioma cells outside of the nervous system is extremely rare, and it is possible that the activity of gelatinases, as opposed to their expression levels, is a more accurate indicator of glioma malignancy.

MT1-MMP has also been implicated in glioma malignancy. mRNA levels of this MMP were reported to increase dramatically in higher grade gliomas although protein levels did not concur, suggesting that MT1-MMP was regulated at the level of transcript stability or post-transcription. Immunohistochemistry localized MT1-MMP to the neoplastic glial cells, whereas normal white brain matter was negative for this protein (33). Finally, a correlation was noted between levels of MT1-MMP and levels of activated gelatinase A, the latter being associated with facilitated tumor invasion.

1.5 MMP activation and regulation

Biologically, MMPs are tightly regulated at several levels. First, they are synthesized as inactive zymogens, requiring proteolysis for exposure of their active site. This proteolysis is autocatalytic and relies on disruption of the cysteine switch, which holds the molecule in its pro-enzyme conformation (25).

Regulation also occurs at the level of gene transcription, where MMP mRNA is synthesized in response to a variety of growth factors and cytokines. In the case of MMP-9, this usually involves the binding of enhancer elements such as AP-1 and PEA3 to the gene promoter region. The lack of AP-1 and PEA3 sites in the promoter region of an MMP, such as MMP-2, suggests constitutive gene expression. However, MMP-2 is thought to lie downstream of p53 and is therefore subject to regulation by this tumor suppressor (35).

Further methods of regulating MMP activity also exist. For example, enhanced secretion of MMP-9 was noted in response to the treatment of ovarian cancer cells by fibronectin, which involved an integrin signaling pathway and activated focal adhesion kinase (FAK) and c-ras (36). Activation of MMP-2 is uniquely regulated by its cell surface association with a subfamily of membrane-type MMPs. Thus, calcium influx into a cell, which disrupts MT-MMP processing, also regulates MMP-2 activation (37). Insulin growth factor also seems involved in MMP-

2 regulation, as IGF overexpression in cells results in increased levels of the protease (38).

A most intriguing strategy for controlling tissue destruction by MMPs is through a family of proteins known as tissue inhibitors of metalloproteinases (TIMPs). TIMPs form tight 1:1 complexes with MMPs, and ECM integrity is maintained through a fine balance of protease/inhibitor activity. Shifting this balance in favor of MMPs results in net ECM destruction, potentially driving the malignant phenotype of many cancers.

1.6 Tissue inhibitors of metalloproteinases

The TIMP family is comprised of four gene products (TIMPs 1-4) that display distinct biochemical properties and functions (Table 3). Some features common to all TIMPs include the characteristic 6 loop structure, resulting from 12 conserved cysteine residues forming intrachain disulphide bonds (Figure 2) (39). TIMPs also possess two domains; a highly conserved N-terminus that is critical for binding to and inhibiting MMP activity and a C-terminus, which governs TIMP-pro-MMP interactions (Figure 3)(40-42).

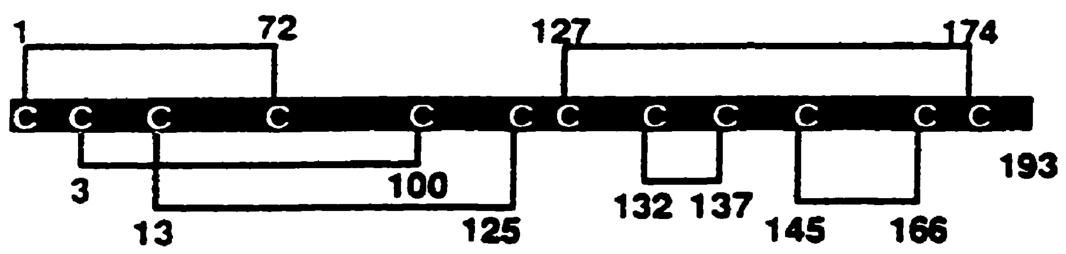
Although TIMPs are clearly homologous in amino acid sequence and structure, there are important differences in the regulation of expression and tissue localization of these proteins (43). For example, TIMPs 1, 2, and 4 are freely diffusable, whereas TIMP-3 is ECM-

Table 3. Properties of TIMPs

	TIMP-1	TIMP-2	TIMP-3	TIMP-4
Gene (human)	Xp11.23-11.4	17q2.3-2.5	22q12.1-13.2	?
Protein (kDa)	28	21	24	22
RNA (kb)	0.9	3.5, 1.0	4.5 (2.8, 2.4)	1.2
Major sites	ovary, bone	lung, brain	kidney, decidua Brain	brain heart
Expression	inducible	constitutive	inducible	?
Pro-MMP complex	MMP-9	MMP-2	MMP-2	MMP-2
Inhibits MT1-MMP?	No	Yes	Yes	?
Induces Apoptisis?	No	No	Yes	?
Inhibits TACE?	No	No	Yes	No
Stimulates Proliferation?	Yes	Yes	?	?

Modified from 25.

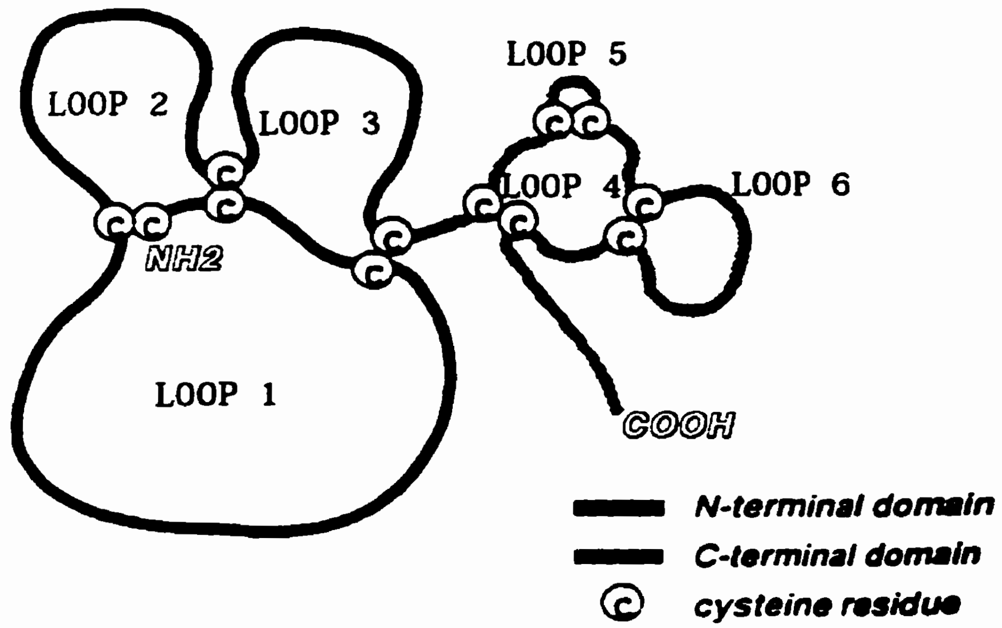
Figure 2. Domain structure of the tissue inhibitors of metalloproteinases (TIMPs). The disulphide bridge pattern within TIMPs is dictated by the 12 cysteine (C) residues conserved among all of the TIMP family members. Modified from 39b.



BINDS TO AVAILABLE ACTIVE SITE OF ENZYME

BINDS TO CARBOXY TERMINUS OF PROENZYME

Figure 3. TIMP tertiary structure. The pattern of disulphide bonds gives rise to a functional, modular structure which distinguishes the N- and C- terminal domains of the mature 6 loop TIMP protein (modified from 40b).


LOOP 5
LOOP 2
LOOP 3
LOOP 4
LOOP 6
NH2
LOOP 1
COOH
N-terminal domain
C-terminal domain
cysteine residue

associated. TIMP-1 was also found in the nuclei of human gingival fibroblasts, and it is thought that an active transport mechanism exists for the passage of cytosolic TIMP-1 into the nucleus against the concentration gradient (44).

In terms of tissue specific expression, high levels of TIMP-1 mRNA were detected in tissues undergoing osteogenesis in the mouse embryo, while in the female adult mouse, the highest level of TIMP-1 expression was seen in the uterus and corpus luteum of the ovary (45). TIMP-2 was also highly expressed in placenta just prior to birth concomitant with increased gelatinase-A expression (46). High levels of TIMP-3 were detected in kidney, lung and brain but only low levels in bone, a site of high TIMP-1 transcription (47). Finally, TIMP-4 was predominantly found in the heart (48). The latter is noteworthy, as the heart is an extremely rare site of malignant disease.

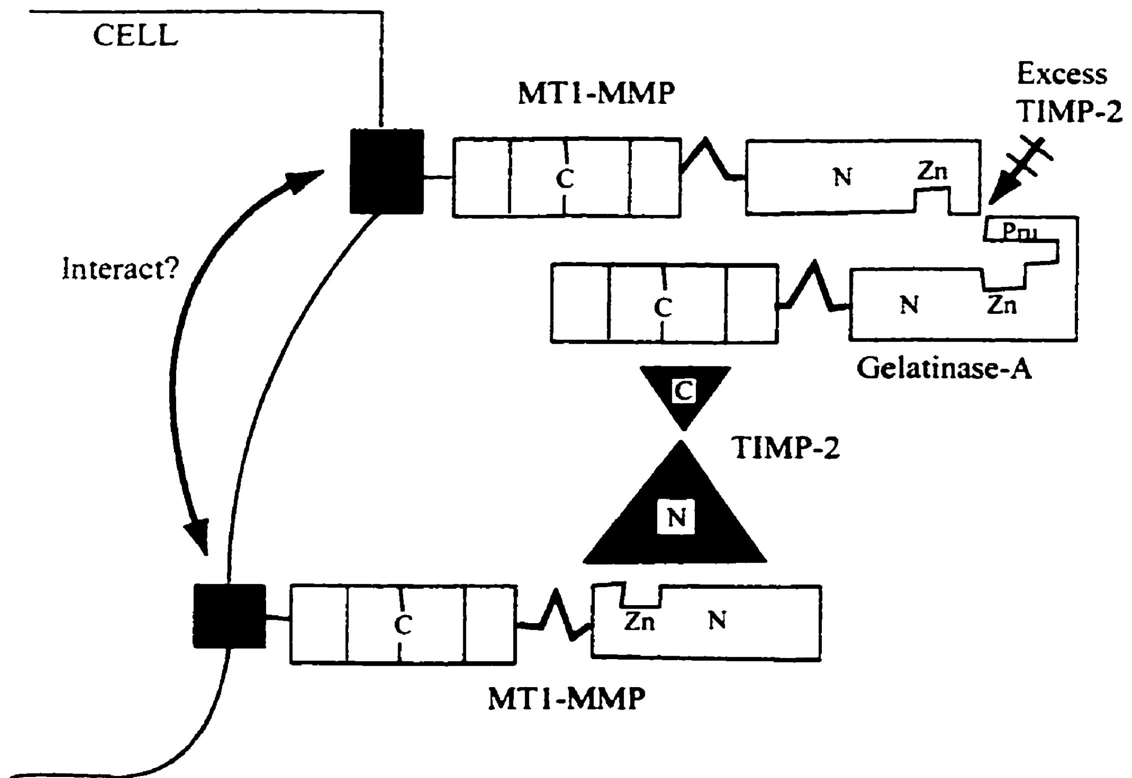
Regulation of TIMP transcription is also variable. TIMP-1 and TIMP-3 contain AP-1 binding sites in their promoter regions, rendering them inducible by phorbol esters and cytokines such as epidermal growth factor (EGF) and transforming growth factor-ß1 (TGF-ß1) (47). In contrast, the TIMP-2 promoter region is lacking this element and its expression is largely constitutive (39). It was reported that TIMP-4 was induced by dexamethasone in endothelial cells (49), but the promoter region and the inducibility of this inhibitor are in need of further investigation.

Finally, structural differences among TIMP proteins include the presence of N-linked glycosylation sites on TIMP-1, 3, and 4 but not TIMP-2. Both TIMP-1 and TIMP-2 cDNA code for proteins that are 21 kDa in length, but TIMP-1 can either be singly glycosylated (24 kDa) or doubly glycosylated (28 kDa) (39, 47). Since both glycosylated and unglycosylated forms of the TIMPs have been shown to inhibit MMPs, the functional significance of glycosylation remains unclear (49).

1.7 TIMPs and differential MMP inhibition

All TIMPs are able to inhibit all forms of active MMPs, however, TIMPs differ in their specificity for binding pro-MMP forms. Studies using truncated versions of TIMP revealed that the C-terminal region, distinct from the region responsible for inhibitory activity, is necessary for pro-MMP binding. Only TIMP-3 appears not to favor a TIMP:pro-MMP complex. TIMP-1 preferentially binds pro-MMP-9, where as TIMP-2 binds the latent form of MMP-2. The latter interaction is unique in that a third protein, known as membrane-type matrix metalloproteinase (MT-MMP) is required (Figure 4). MT1-MMP is bound to the cytoplasmic membrane and acts as a docking site for TIMP-2. MMP-2 then binds the anchored TIMP-2 molecule, and is activated by an adjacent MT-MMP molecule. In this case, the concentration of TIMP-2 in the immediate vicinity can either promote or inhibit MMP-2 activation. Low concentrations of TIMP-2 bind some MT1-MMP molecules while leaving others free for protease

Figure 4. Regulation of gelatinase-A activation through MT1-MMP. Gelatinase-A is activated by binding to a TIMP-2/MT1-MMP complex on the cell surface. In the case of low TIMP-2 concentrations, adjacent MT-MMP molecules are free to activate bound gelatinase-A. However, when TIMP-2 concentrations are high, all MT1-MMP molecules are bound by inhibitor, leaving no free MT1-MMP to activate pro-gelatinase-A (50b).


CELL
MT1-MMP
Excess
TIMP-2
C
N
Zn
Pro
Interact?
C
N
Zn
Gelatinase-A
C
TIMP-2
N
C
Zn
N
MT1-MMP

activation, whereas high concentrations of TIMP-2 completely saturate MT1-MMP sites.

TIMP-4 has only recently been discovered and little is known about its physiological role. This molecule shares 37% sequence homology with TIMP-1 and 51% with TIMP-2 (51). Studies suggest that TIMP-4 also binds pro-gelatinase A in the C-terminal hemopexin like domain (K_d $=1.7 \times 10^{-7}$ M) with slightly less affinity than does TIMP-2 ($K_d = 6.6 \times 10^{-8}$ M) (53). This binding is extremely tight as it is undisruptable by NaCl or DMSO. This interaction may also involve a trimeric complex with an MT1-MMP molecule, offering an alternative pathway to MMP regulation. Competition between TIMP-2 and TIMP-4 for common overlapping sites on MMP-2 may occur, modulating the cell surface activation of this protease.

1.8 TIMPs as multifunctional proteins

The classical notion of TIMPs as MMP inhibitors in tumor biology is rather narrow, and there is a growing appreciation that these proteins may affect a broad spectrum of cellular behaviors. For example, TIMP-1 stimulates the proliferation of erythroid precursors (53, 54), and both TIMP-1 and TIMP-2 affect cellular proliferation of numerous cell types (55-59). Additionally, TIMP-2 inhibits *in vitro* proliferation of human microvascular endothelial cells stimulated with bFGF (60), an effect that is not seen using BB94, a synthetic MMP inhibitor. This suggests TIMP-2

is acting in a way other than MMP-2 inhibition, and implies that the absence of TIMP-2 may be critical for initiating tumor neovascularization.

TIMP-2 not only regulates MMP-2 activation, but also plays a role in protecting the enzyme from degradation. Farina et al. (61) propose that TIMP-2 regulates MMP longevity in plasmin containing environments, where plasmin degrades the N-terminus of free MMP-2, thus destroying it's enzymatic activity. Protection by TIMP-2 was disrupted in the presence of chelators and divalent cations. This presents a novel mechanism for reversing TIMP protection of MMP-2 activity in situations where control and repression of MMP activity is necessary.

TIMP-3 is one of the most unique proteins in this inhibitor family. Along with MMP inhibition, TIMP-3 has been implicated in cellular apoptosis, angiogenesis and proliferation. TIMP-3 is the only TIMP whose mutation is known to cause a specific disease, Sorsby's fundus dystrophy (SFD). SFD is characterized by the development of choroidal neovascular membranes and subretinal hemorrhages (62) which lead to studies of the role of TIMP-3 in the regulation of angiogenesis. *In vitro*, TIMP-3 was an effective inhibitor of endothelial cell migration in the presence of bFGF and VEGF (63). Furthermore, TIMP-3 inhibited bFGF induced angiogenesis in the CAM assay *in vivo* (63).

TIMP-3 transfected DLD-1 cells had a decreased growth potential and formed large aggregates in suspension, followed by cell death, an effect that was not mimicked by BB94 (64). Further studies showed that

TIMP-3 induced cell death by stabilizing TNF-α receptors on human colon carcinoma cells (65). TIMP-3 also influenced pro-TNF-α processing by inhibiting tumor necrosis factor alpha converting enzyme (TACE, also called a disintegrin and metalloproteinase-17 or ADAM-17) (66). Both of these actions implicate TIMP-3 as a regulatory molecule of p55 signaling and thus apoptosis.

The functions of TIMP-4 are still to be elucidated. Its specific expression in the heart may implicate this protein in the process of angiogenesis (48, 51). *In vitro* assays show that TIMP-4 also inhibits migration and invasion of endothelial cells through fibrin treated Boyden chambers (67). The effects of TIMP-4 on proliferation and apoptosis have not been investigated. As TIMP-4 shares many of the structural and binding characteristics of TIMP-2, it may mimic some of its functions. Functional redundancy between TIMP-2 and –4 may be avoided by differential expression and regulation of these two proteins.

1.9 TIMPs and tumor invasion

Given the establishment of increased MMP activity in tumors, numerous studies have sought to define the role of TIMPs in the malignant process (43). The classical notion of TIMPs controlling overzealous MMP activity suggests that increased malignancy would correlate with decreased inhibitor levels. This trend has been reported in cell culture models, where levels of TIMP-1 were inversely correlated with

increasing metastatic potential (68). Also, exogenous or forced overexpression of TIMP-1 or TIMP-2 reduced invasion and proliferation of cell lines both *in vitro* and *in vivo* (69, 70).

The effects of TIMP-3 and TIMP-4 in cancers are much less characterized. A lack of TIMP-3 mRNA expression at the invasive edge of highly aggressive colorectal carcinomas suggests a correlation between inhibitor absence and increased invasiveness (71). Also, breast carcinoma and malignant melanoma cell lines with adenoviral delivery of TIMP-3 showed significant suppression of tumor growth *in vivo* (72). Only one report of TIMP-4 exists, where TIMP-4 transfected breast carcinoma cell lines showed decreased invasive potential, proliferation and microvascular density *in vivo* (73).

1.10 TIMPs in gliomas

Currently, limited information is available on a putative link between TIMPs and brain tumors. The literature data are often conflicting, and most studies are based on a small number of surgical samples. For example, Mohanam et al. (74) found that TIMP-1 and TIMP-2 mRNA were downregulated in glioblastoma multiforme (GBM) compared to lower grade brain tumors. In contrast, Nakano et al. (75) found a positive correlation between TIMP-1 and TIMP-2 expression in glioma malignancy. Increases in TIMP-2 were also reported by Saxena et al. (76), but this survey involved mRNA analysis of only 7 surgical

samples, and made no correlation between TIMP-2 expression and tumor grade. Finally, Rutka et al (77) reported no correlation between TIMP-1 and TIMP-2 expression and glioma cell invasiveness *in vitro*. No studies to date have addressed the roles of TIMPs 3 and 4 in malignant brain tumors.

1.11 Hypotheses

On the basis that TIMPs show altered expression in numerous cancers, and that manipulation of TIMPs may provide a novel glioma therapy, a detailed analysis of TIMP expression and localization in human malignant gliomas was undertaken. Results from these studies prompted *in vitro* investigations into the effects of excess TIMP-4 (both endogenous and recombinant) on the malignant behavior of glioma cells. The specific aims of this thesis were:

1. **To establish a relationship between TIMP expression in glioma cells and *in vivo* aggressiveness by characterizing TIMP expression patterns in glioma surgical samples.**

2. **To determine whether TIMP-4 overexpression or excess addition of rTIMP-4 to glioma cells would act as a negative regulator of *in vitro* malignant behavior.**

2. METHODS

2.1 Tissue collection

Procedures were performed by Dr. Garnet Sutherland at the Foothills hospital. Induction consisted of a combination of pentothal, fentanyl, and vecuronium with maintenance achieved using isoflurane and intermittent fentanyl as needed. During the craniotomy, mannitol (1 g/kg) was administered for cerebral decompression. Tumor specimens were obtained prior to thermal coagulation and placed immediately in liquid nitrogen and stored at -80°C in the neuro-specimen Tumor Bank. Our institutional ethics board has approved the study. All patients gave signed, informed consent for their tissue to be used. The following tissues were studied from the tumor bank: 19 glioblastoma multiforme (GBMs), 7 anaplastic astrocytomas (AA), 5 malignant oligodendrogliomas (MO), 8 low grade gliomas (LG), 1 clivail chordoma and 1 spinal epedymoma: these were compared to 3 controls (1 obtained at autopsy and two frozen sections).

2.2 RNA and protein preparation

Total RNA was extracted using the acid guanidinum isothiocyanate method (78). The final RNA concentrations were determined by absorption using a GeneQuant spectrophotometer (Pharmacia). Protein extraction involved homogenization of tissue in extraction buffer (0.5 M Tris-HCl, pH 7.6, containing 0.2M NaCl, 10 mM $CaCl_2$, and 1% Triton X-

100). The homogenate was centrifuged at 4°C for 15 minutes at 15000 g and supernatant was stored at -70°C for western blot and reverse zymography assays.

2.3 Reverse transcription (RT) reactions

Each 20 µl cDNA synthesis reaction contained 1 µg of total RNA, 1X PCR buffer (10mM Tris-HCl, pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$), 1 mM of each deoxyribonucleotide triphosphate, 20U placental ribonuclease inhibitor, (RNAguard, Pharmacia), 160 units of MuLV-reverse transcriptase (Bethesda Research Laboratories) and 100 pmol of random hexamer oligodeoxynucleotides (Pharmacia). Reaction mixtures were pre-incubated 10 min at 21°C prior to cDNA synthesis. Reverse transcription was carried out for 50 min at 42°C and then heated to 95°C for 5 min to terminate the reaction. Samples were stored at 4 °C or - 20 °C until use.

2.4 Polymerase chain reactions

Multiplex PCRs were performed in 50 µl reaction volumes. Each reaction contained 2 µl of RT reaction product, 1X PCR buffer, 80 µM of each deoxynucleotide, and 20 pmol of each 5' and 3' starter primer pair (Table 4). Two units of Taq DNA polymerase (Gibco-BRL) were added to each tube during the first denaturation step ("hot start") and equal aliquots (20 pmol) of GAPDH primer sets were added at the appropriate

Table 4. Primer Sequences

GAPDH

5'-primer= 5'-CGGAGTCAACGGATTTGGTCGTAT

3'-primer= 5'-AGCCTTCTCCATGGTGGTGAAGAC

TIMP-1

5'-primer= 5'-GATCCAGCGCCCAGAGAGACACC

3'-primer= 5'-TTCCACTCCGGGCAGGATT

TIMP-2

5'-primer= 5'-GGCGTTTTGCAATGCAGATGTAG

3'-primer= 5'-CACAGGAGCCGTCACTTCTCTTG

TIMP-3

5'-primer= 5'-CTTCTGCAACTCCGACATCGTG

3'-primer= 5'-TGCCGGATGGAGGCGTAGTGTTT

TIMP-4

5'-primer= 5'-AATCTCCAGTGAGAAGGTAGTTCC

3'-primer= 5'-CGATGTCAACAAAACTCCTTCCTGA

cycle number by the primer dropping method (78). Each PCR cycle consisted of a heat denaturation step (94°C for 1 min), a primer-annealing step at (55°C for 30 sec) and a polymerization step (72 °C for 1 min). Reactions were performed in a Temp-Tronic Thermal Cycler (Barnstead/Thermolyne). PCR products were electrophoresed through a 2% agarose gel containing 0.2 µg ethidium bromide. Band intensities were calculated by scanning densitometry (NIH imaging program), and TIMP to GAPDH ratios for each sample were plotted as a function of tumor grade.

2.5 Western blotting

Twenty µg of protein from each brain tumor sample were mixed with 3X SDS gel-loading buffer (0.188 mM Tris-HCl (pH 6.8), 3% SDS (w/v), 0.0075% bromophenol blue, 30 % glycerol and 3% β-mercaptoethanol) and separated on a 12 % polyacrylamide gel. After transfer to a nitrocellulose membrane, blots were blocked in 5% nonfat milk overnight at room temperature, and then incubated with 600 ng/ml anti-TIMP-1 (monoclonal, Calbiochem) or 1 ng/ml anti-TIMP-4 antibody (polyclonal, Chemicon) antibody. After incubation with peroxidase labeled secondary antibody, signals were detected using an enhanced chemiluminescence detection system (Amersham).

2.6 cRNA probe preparation

TIMP-1: A 185 bp HindIII / BamHI fragment of human TIMP-1 in plasmid pBS KS was used to generate antisense and sense riboprobes using T3 polymerase/Bam HI and T7 polymerase/Xho I digested plasmid respectively.

TIMP-4: A 400 bp HindIII / BamHI fragment of human TIMP-4 in plasmid pBS KS was used to generate antisense and sense riboprobes using T3 polymerase/Bam HI and T7 polymerase/Hind III digested plasmid respectively.

2.7 *In situ* hybridization

Tissue sections were deparaffinized using xylene and rehydrated through a graded ethanol series (100 %, 95%, 80%, 70% respectively). After proteinase K treatment at 37°C for 25 min and acetylation using a solution of 0.5 % acetic anhyride in 0.1 M triethanolamine, pH 8.0, tissues were prehybridized for 2 hours at 50°C (50 % formamide, 5X SSPE, 1X Denhart's solution) and 20 ng of probe (diluted in prehybridization buffer with 8 µg/ml *E coli* tRNA) was added. Sections were incubated at 60°C overnight. Sections were washed in 2X SSC and were incubated in 20 mg/ml RNAse A at 37°C for 45 min. After washing in a graded SSC series (2X, 1X, 0.5X, and 0.1 X (60 °C)) sections were incubated in blocking buffer containing lamb serum for 2 hours. Anti-dig antibody was added at a concentration of 3/500 and slides were left for 4 hours at room temperature. NBT/BCIP chromogens were applied to sections and color was developed in dark. When desired intensity was reached, color reaction was terminated by placing sections in 20 mM Tris-HCl pH 7.5, 10 mM EDTA. Sections were mounted with Flo-texx medium (Lerner Laboratories). Photographs were taken using Kodak Royal Gold 35 mm film in a Ziess photomicroscope II under bright field illumination.

2.8 Reverse zymography

Reverse gelatin zymography was used to determine the presence of functional TIMP inhibitory activity. Brain tumor protein extracts (20 μg) were loaded onto a 12% polyacrylamide gel, and reverse zymography was carried out as previously described (79). Enzymatically active TIMP appeared as a dark band against a clear background.

2.9 Immunohistochemistry

Formalin-fixed paraffin embedded specimens (sectioned at a thickness of 4μm) were used. Slides were deparaffinized in xylene, and rehydrated in graded concentrations of ethanol. Endogenous peroxidase activity was blocked by incubation in 1% peroxide/methanol for 30 min. Slides were rehydrated in PBS and non-specific binding by the primary antibody was blocked by incubation with 10% normal goat serum plus 2% BSA in PBS for 1 hour.

Sections were incubated for 30 minutes with rabbit anti-TIMP-4 polyclonal antibody at a concentration of 2 ng/ml. Slides were rinsed 5X (5 min) in PBS and antibody was localized using goat anti-rabbit horse radish peroxidase detection. Antibody was visualized using a DAB (3 3' diaminbenzidine) Substrate Kit for Peroxidase (Vector Laboratories, Brulingame, CA). After counterstaining for 2 min in hematoxylin, sections were dehydrated and mounted using Flo-texx media.

2.10 Cell culture

U87 and U251 cell lines were obtained from ATCC (American Tissue Culture Collection, Rockville, MD, U.S.A). These cells were grown in DMEM containing F-12/10% fetal calf serum. Cells were passaged after reaching approximately 80% confluency, harvested by trypsin treatment and replated in DMEM-F12/10% FCS.

2.11 Transfections

TIMP-4 tranfections were conducted by cloning cDNA for mouse heart (675 bp) into the EcoRI site of the pBABE-puromycin vector. Vector was amplified by transformation with E coli and purified using Maxi Prep protocol. Purified vector was introduced into glioma cells using a Sigma Calcium Phosphate Transfection Kit and cells were then grown in DMEM F12/10% FCS selection media with 20 μg/ml puromycin. Cloning rings were used to isolate viable clones and functional expression of the TIMP-4 insert was confirmed using reverse zymography and western blot assays.

2.12 ^{3}H-thymidine assays

As a measure of DNA synthesis in transfected and parental cells, ^{3}H-thymidine uptake was analyzed. All experiments were performed in replicates of three. Following passage, transfected and parent cell lines were seeded at a density of 1×10^4 cells per 13 mm glass coverslip in a

24 well culture plate and flooded with 1 ml of DMEM-F12/10%/ well. Cells were pulsed with 1 μCi of ^{3}H thymidine for a period of 16 hours before being harvested at 24, 28, or 72 hours after initial seeding. Coverslips were washed 3 times in phosphate-buffered saline and analyzed with a Betascan-counter.

To measure the effect of recombinant TIMP protein on DNA synthesis of glioma cells, U87 and U251 cells were seeded as above only in serum free medium and starved for 24 hours. Medium was then exchanged for serum free DMEM-F12 containing pre-determined concentrations of TIMP protein. Cells were then pulsed and collected as above.

2.13 Methylthioltetrazole (MTT) viability assays

A modified colorimetric assay based on the selective ability of living cells to reduce MTT to formazan was used to quantify TIMP-mediated cytotoxicity in both transfected cell lines and parent cells exposed to recombinant TIMP protein. Following passage, cells were seeded at a density of 2.5×10^{5} cells / ml into 24 well culture plates. After 24, 48 and 72 hours, 5mg/ml MTT (Sigma Chemical Co. USA) was added to each well for 60 minutes. Culture medium was then removed and formazan crystals were dissolved in 200 μl/well dimethyl sulphoxide. Well contents were transferred to a 96-well plate and absorbence was measured at 600 nm using an ELISA counter. In some experiments, viability was also

assayed using the trypan blue counting method. Cells were seeded as above and typsinized after 24, 48, and 72 hours. Addition of Trypan blue allowed detection of the number of viable cells.

Parent cells exposed to recombinant TIMP were serum starved for 24 hours before the addition of various concentrations of rTIMP-2/4.

2.14 Invasion assay

Matrigel was thawed at 4°C on ice overnight. Using cooled pipette tips, matrigel and 0.5% FBS were mixed at a ratio of 1:5 and 30 μl of the mixture was spread evenly over the bottom of Boyden Chambers in a 24 well plate. Matrigel was allowed to solidify for 30 min at 37°C and then an additional 20μl of the matrigel mixture was applied to the Boyden Chambers. After 30 min at 37 °C, cells were seeded at a density of 2.0 $x10^5$ /ml into each chamber and covered with 200 μl of media (0.5% FBS) containing the desired concentration of inhibitor. Chambers were set into 700 μl of fibroblast media (ensuring no bubbles were caught under the chamber) and for 24 hrs at 37°C. Chambers were then rinsed in PBS and cells were scraped off the top of the membrane using a cotton-tipped swab. Cells are fixed to the bottom of the chamber using cold methanol for 10 min and then stained in hematoxylin before mounting on slides.

Note: When using the pre-made chambers, Matrigel was hydrated with medium for 2hrs prior to seeding cells and the assay was carried out as above.

2.15 Immunocytochemistry

Glioma cells were grown on coverslips in 6-well plates to approximately 50% confluency. Cells were washed 2X with PBS and fixed in 2% paraformaldehyde for 10 min at room temperature. After gentle rinsing in PBS, cells were treated with 1% H2O2 in methanol for 30 min at room temperature. Cells were washed again 3X in PBS and were permeabilized with 0.2% Triton-X 100 in PBS for 5 min at room temperature. Cells were washed 3X 5min in PBS and blocked for 1 hour in 3% BSA/10% goat serum in PBS at room temperature. After 5X 5 min washes in PBS, anti-rabbit TIMP-4 Ab was added at a concentration of 1 ng/ml in blocking buffer for 1 hour. The 5X 5 min PBS washings were repeated and the cells were incubated and goat anti rabbit secondary Ab was added for 30 min. Signal was visualized using DAB staining for 45 min at room temperature and treatment with hematoxylin for 1.5 min. Coverslips were washed in water, PBS, and then mounted using Kaiser's mounting medium.

2.16 Subcellular Fractionation

Cells were washed 2X in ice-cold PBS without Ca^{2+} and Mg^{2+} and then suspended in 1 ml of hypotonic buffer (10 mM Tris-HCl (pH 8.0), 0.2 mM KCl, 1mM PMSF, and 10 μg/ml of aprotinin and leupeptin (all inhibitors freshly added)). Cells were allowed to swell for 20 min on ice,

and then were lysed by dounce homogenization. Samples were adjusted to a final concentration of 0.25 M sucrose and 1 mM EDTA and nuclei and unlysed cells were removed by low-speed centrifugation (10 min at 1 000 g). Membrane fractions were isolated from the post nuclear supernatant by centrifugation at 100 000 g for 1 hour at 4°C. The supernatant (cytosol fraction) was removed and the remaining pellet (membrane fraction) was solubilized in SDS sample buffer. Both cytosolic and membrane fractions were then run out on western blots.

3. RESULTS

Part 1. Expression and localization of TIMPs in glioma surgical specimens

3.1 mRNA quantification of TIMPs 1-4 by RT-PCR.

To determine the relationship between TIMP expression and glioma aggressiveness, specimens from normal brain and tumor were analyzed using RT-PCR. Total RNA was extracted from surgically obtained specimens, and each sample was amplified by PCR in three separate assays to ensure consistent results. GAPDH was used as an internal control in individual PCR reactions to monitor input levels of cDNA.

A representative profile for each of the four TIMPs is shown in Figure 5. When comparing the expression patterns of the four transcripts, both TIMPs -1 and -4 showed a correlation with glioma malignancy over the range of tumor grades assayed. For TIMP-1, transcript was barely detectable in RT-PCR specimens of the normal brain tissue and lower grade tumors, but increased dramatically for GBM tumors. In contrast, TIMP-4 mRNA expression showed a negative correlation with glioma grade, but was higher in tumor samples than in normal brain. Relative to the GAPDH signal in each PCR reaction, the most intense TIMP-4 bands appeared for lower and middle grade samples, while the ratio diminished for GBM specimens; some GBMs showed only a barely detectable TIMP-4 band. Whereas TIMP-1 and -4 expression varied with glioma malignancy, those for TIMP- 2 and TIMP-3 remained fairly constant over all tumor grades examined. The relationships between expression of TIMPs and glioma grade from the series of specimens analyzed are summarized in Figure 6.

Figure 5. mRNA expression of TIMP-1 to –4 in glioma surgical specimens. As seen in the top panel, TIMP-1 transcript levels increase dramatically in higher grade compared to lower grade glioma samples. In the bottom panel the opposite in true, where the ratio of TIMP-4 to GAPDH is the lowest in the higher grade tumors. **Abbreviations: LG = low grade, MG = middle grade, HG = high grade.**

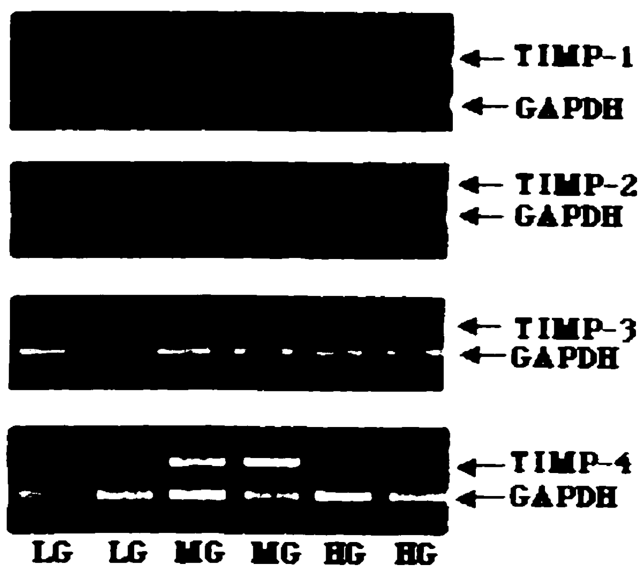
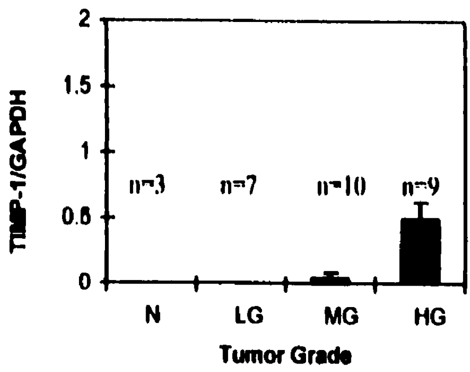
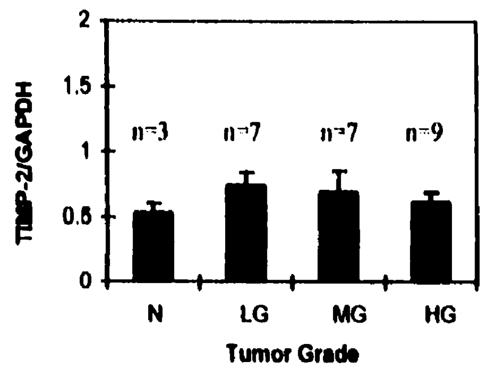
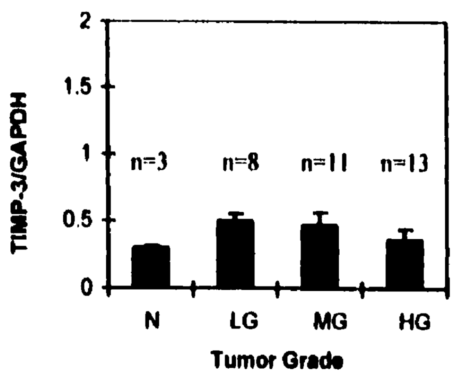
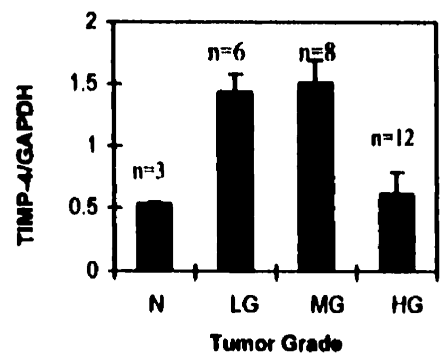

TIMP-1
GAPDH
TIMP-2
GAPDH
TIMP-3
GAPDH
TIMP-4
GAPDH
LG
LG
MG
MG
HG
HG

Figure 6. Scanning densitometry of TIMP to GAPDH ratios. RT-PCR analyses resulted in the following correlations for each of the TIMPs in glioma surgical specimens. **Abbreviations : N = normal brain, LG = low grade, MG = middle grade, HG = high grade.**


TIMP-1/GAPDH
2
1.5
1
0.5
0
n=3
n=7
n=10
n=9
N
LG
MG
HG
Tumor Grade


TIMP-2/GAPDH
2
1.5
1
0.5
0
n=3
n=7
n=7
n=9
N
LG
MG
HG
Tumor Grade


TIMP-3/GAPDH
2
1.5
1
0.5
0
n=3
n=8
n=11
n=13
N
LG
MG
HG
Tumor Grade


TIMP-4/GAPDH
2
1.5
1
0.5
0
n=3
n=6
n=8
n=12
N
LG
MG
HG
Tumor Grade

3.2 Western blot analyses of TIMP-1 and TIMP-4 protein expression

To characterize the protein expression of TIMP-1 in brain tumors, we performed Western blot analyses using tissue homogenates from normal brain and glioma surgical specimens and probed these with anti-TIMP-1 monoclonal antibody (Figure 7A). A 28 kDa band corresponding with the TIMP-1 standard was present in GBM samples. In contrast, TIMP–1 levels were considerably lower in normal brain tissue and lower grade tumors. These data are in agreement with our RT-PCR analyses, supporting the notion that TIMP-1 expression positively correlates with glioma malignancy. In contrast to TIMP-1, TIMP-4 is down regulated in the higher grade tumors compared to the normal and low grade tissue (Figure 7B). Similar results were obtained through the RT-PCR analysis except that TIMP-4 protein levels in normal tissue were high relative to the tumor samples.

The predominant product of our western blot analyses for TIMP-1 was the 28 kDa protein, but this was not the only band detected. Several proteins of higher molecular weight (approximately 60 kDa) were also found in each of the samples. These bands were further detected using immunoprecipitation studies of TIMP-1 on brain tumor extracts (data not shown). Several other investigators have reported high molecular weight structures in TIMP western assays, and we suspect these structures may represent high affinity complexes of TIMP-1 and other proteins, such as gelatinase A or B. In the case of TIMP-4, these high molecular weight

Figure 7. Western blot analyses of (A) TIMP-1 and (B) TIMP-4 protein in glioma samples Ten μg of total protein was loaded in each lane. Note the positive and negative correlations of respective TIMP-1 and –4 protein levels with glioma grade. **Abbreviations: N = normal brain, LG = low grade, MG = middle grade, and HG = high grade**.

N LG MG MG HG HG HG

a) 28 kDa TIMP-1

N LG MG MG HG HG

b) 23 kDa TIMP-4

structures also exist, possibly representing a complex with either gelatinase A and/or MT1-MMP (24). Further studies are required to characterized the precise identity of these TIMP-protein complexes.

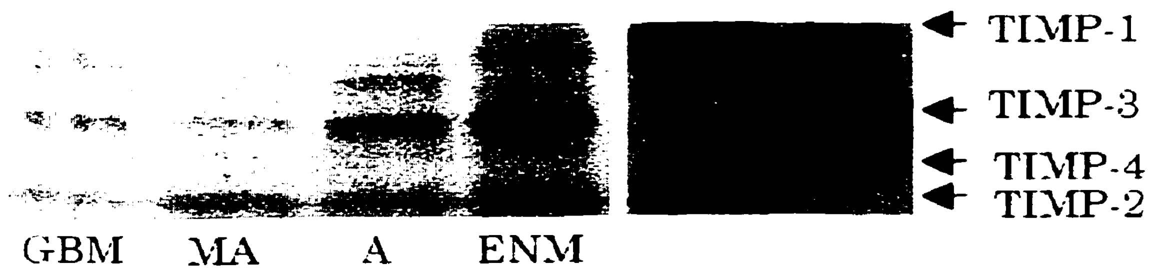
3.3 Inhibitory activity shown by reverse zymography

Reverse zymography was used to determine the inhibitory activity of TIMPs (Figure 8). All TIMPs inhibited MMPs in brain tumors, but with little variation in MMP inhibition among tumor grades. As reverse zymography is significantly more sensitive than Western blotting, it was surprising that inhibitor levels appeared so low (as equivalent protein quantities were loaded in both western blot and reverse zymography gels) and showed no correlation with glioma grade. In particular, TIMP-4 was nearly undetectable which may be due to low levels of free TIMP protein *in vivo* resulting from the formation of complexes with other peptides.

3.4 Localization of TIMP using *in situ* hybridization

To determine the cellular origin and distribution of TIMPs-1 and -4, *in situ* hybridization was carried out on paraffin-embedded glioma and normal brain specimens. Hybridized sections were compared to identical

Figure 8. Reverse Zymography of glioma surgical specimens. 10 µg of protein from each sample was assayed for TIMP activity. In all samples, TIMP-1, -2, and –3 were detected. TIMP-4 activity was not visible in this assay. **Abbreviations : GBM = glioblastoma, MA = malignant astrocytoma, A = astrocytoma, ENM = extraneural metastasis.**


TIMP-1
TIMP-3
TIMP-4
TIMP-2
GBM
MA
A
ENM

hematoxylin and eosin (H and E) stained sections to facilitate pathological interpretation.

TIMP-1 mRNA was detected in all GBM tumors analyzed. Strong localization of transcript was seen in the cytoplasm of malignant cells (Figure 9), with little or no staining seen in the cells of the surrounding host stroma. There was also intense staining for TIMP-1 transcript around vascular structures and within endothelial cells in GBM specimens (Figure 9). This feature was noted in all high grade tumor sections analyzed, and was absent in lower grade astrocytomas. Normal brain showed low levels of TIMP-1 mRNA staining that were confined to neurons (data not shown).

TIMP-4 mRNA was also detected in all GBM sections studied. Similar to TIMP-1, TIMP-4 transcripts were localized to malignant cells, but only low levels were observed in endothelial cells (Figure 11). TIMP-4 was also found in low grade astrocytomas, and within the neurons of normal brain tissue (data not shown).

Earlier *in situ* hybridization studies had been focused on the differential localization of TIMP-1 and TIMP-2 transcripts. As seen in Figure 12, TIMP-2 appeared to be expressly absent around vascular structures. Because of technical difficulties, further analysis of TIMP-2 expression was discontinued.

Figure 9. Localization of the TIMP-1 transcript. TIMP-1 mRNA was localized to the glioma cell cytoplasm and also showed strong staining in the neovasculature of the tumor.

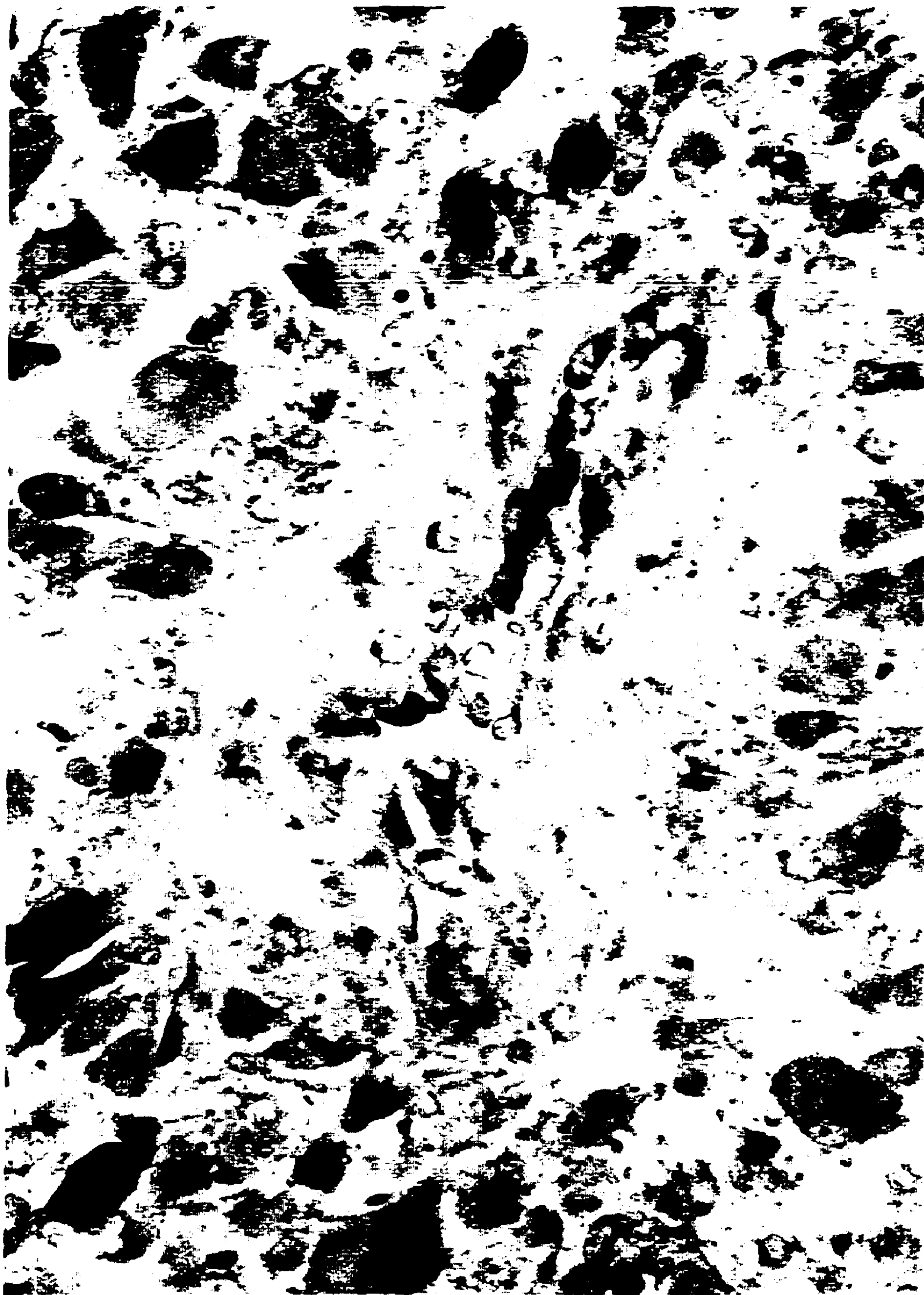


Figure 10. TIMP-4 localization in GBM. Like TIMP-1, TIMP-4 also localizes to the tumor cells of GBM samples, yet lacks the strong association within the capillaries.

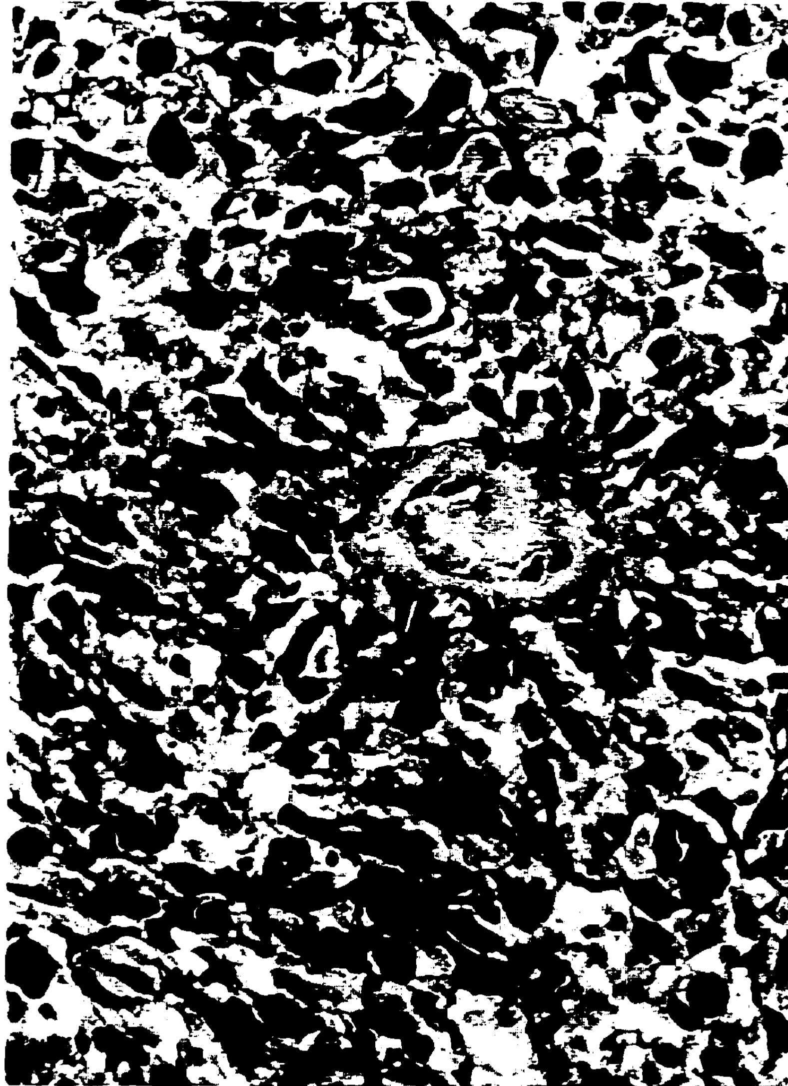
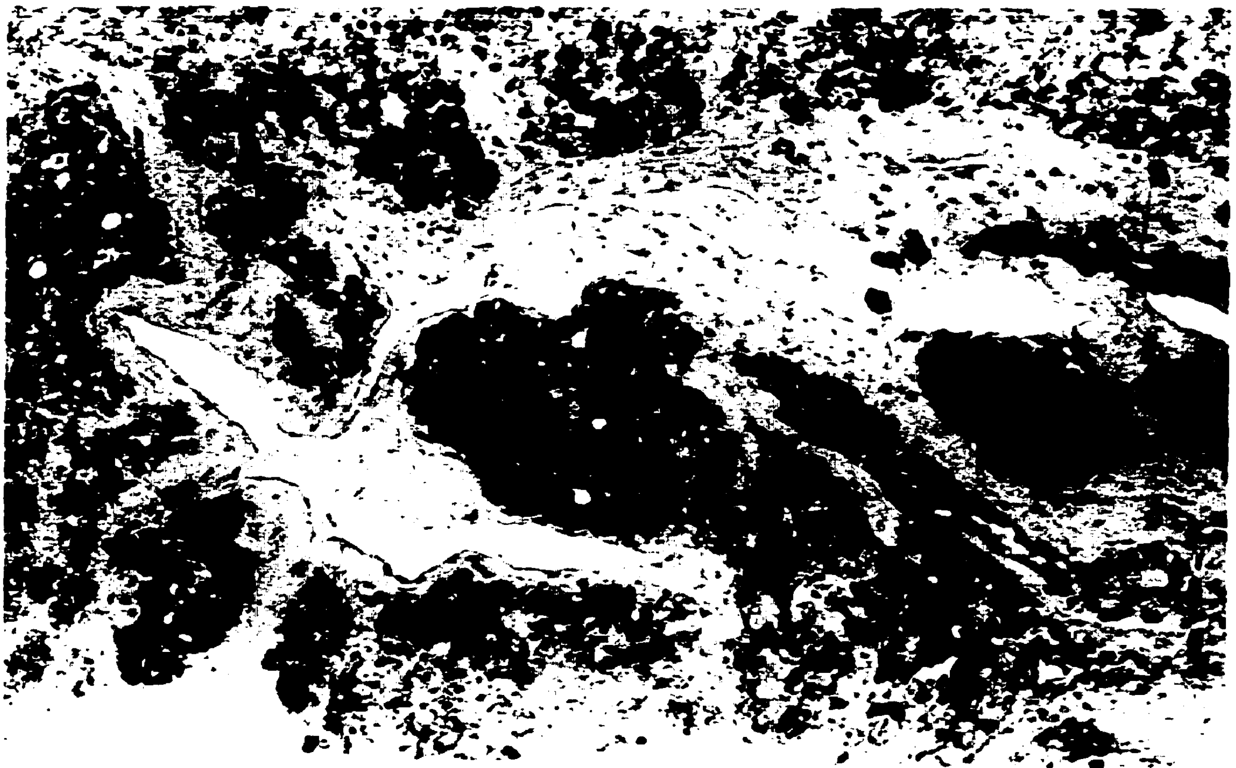


Figure 11. TIMP -2 localization in GBM tumors. B) TIMP-2 assays showed no detectable transcript around vascular structures.



3.5 Immunohistochemical analysis of TIMP-4

The cellular distribution of TIMP-4 was further investigated by immunohistochemistry (Figure 12) where positive signal (brown) was seen within the tumor cells. In some tumors, low levels of TIMP-4 protein staining were also detected within the vasculature of the tumor, while others showed no vascular staining at all. Low grade astrocytomas and normal brain tissue were also analyzed by immunohistochemistry and showed TIMP-4 expression patterns consistent with the results from our transcript localization. Based on the distinct localization patterns for TIMP-1 and -4, along with their differential mRNA and protein expression patterns, it is possible that these inhibitors play unique roles in the progression of malignant gliomas.

Figure 12. Immunohistochemical analysis of TIMP-4 in GBM. A). Glioma cells stained positive (brown) within their cytoplasm for TIMP-4 protein. Blood vessels in the region are relatively negative for TIMP-4. (100X) **B)** Cytoplasmic staining is seen again, with some staining in the neovasculature of the tumor (400X). **C)** Control sections in which primary antibody was omitted show no staining.

Results Part II

In vitro studies of TIMP and glioma malignancy

3.6 TIMP-4 overexpression had no effect on glioma viability

To determine the effects of increased TIMP-4 expression in glioma cells, pBABE TIMP-4 transfected U87 clones were assayed for proliferation, invasive capacity and viability. These results were compared to the empty vector control and parental U87 cells in each experiment. As seen in Figure 13, TIMP-4 overexpression had no significant effect on the viability of U87 cells as determined by MTT and trypan blue assays. Cells were monitored for viability at 24, 48, and 72 hours after initial seeding.

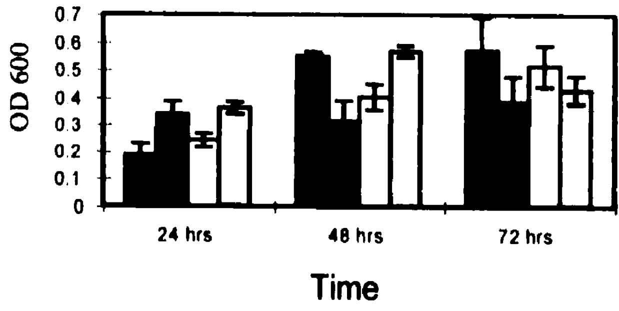
To see if exogenous TIMP-4 had differential effects on the cells, parental U87 cells were seeded in 24-well plates and treated with rTIMP-4 (at 10ng/ml, 100 ng/ml), then counted trypan blue and MTT assays after 24, 48, and 72 hours. rTIMP-4 also showed no effect on glioma cell viability in the given concentration range (Figure 14).

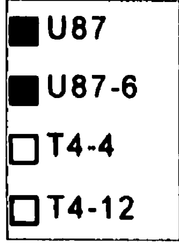
3.7. TIMP-4 overexpression had no effect on glioma proliferation

To determine whether TIMP-4 affected the proliferative ability of gliomas, U87 cells transfected with pBABE TIMP-4 were seeded in 24 well plates and treated with ^{3}H thymidine to monitor DNA synthesis as a measure of proliferation. These cells were monitored for 24, 48 and 72 hours and thymidine incorporation was compared to the empty vector control and the parental cell line. Figure 15 shows that TIMP-4 overexpression had no effect on proliferation *in vitro*.

Figure 13. The effects of TIMP-4 overexpression on U87 viability. **(A)** Both MTT assays and (B) trypan blue showed that TIMP-4 overexpression had no effect on the viability of glioma cells at 24, 48, or 72 hours. The lack of cell growth that appears in both assays over the three day period is due to the confluency level of the cells at the start of the experiment.

A)





B)

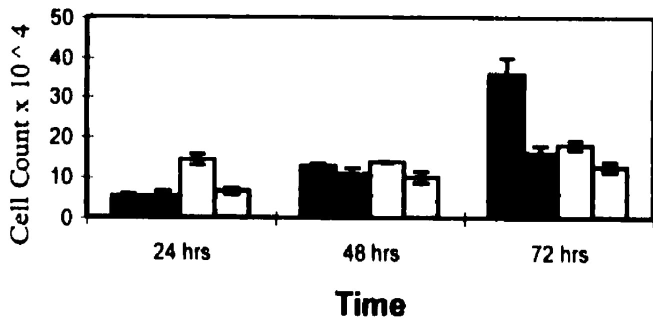
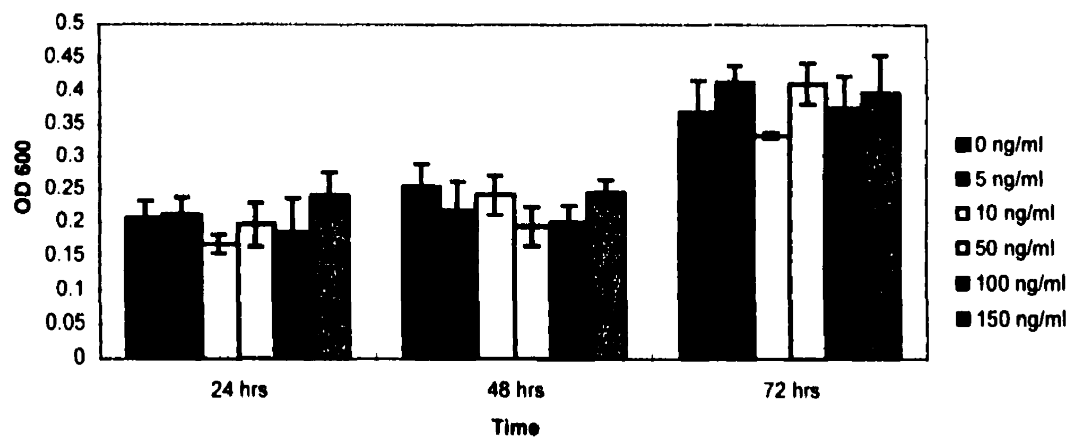
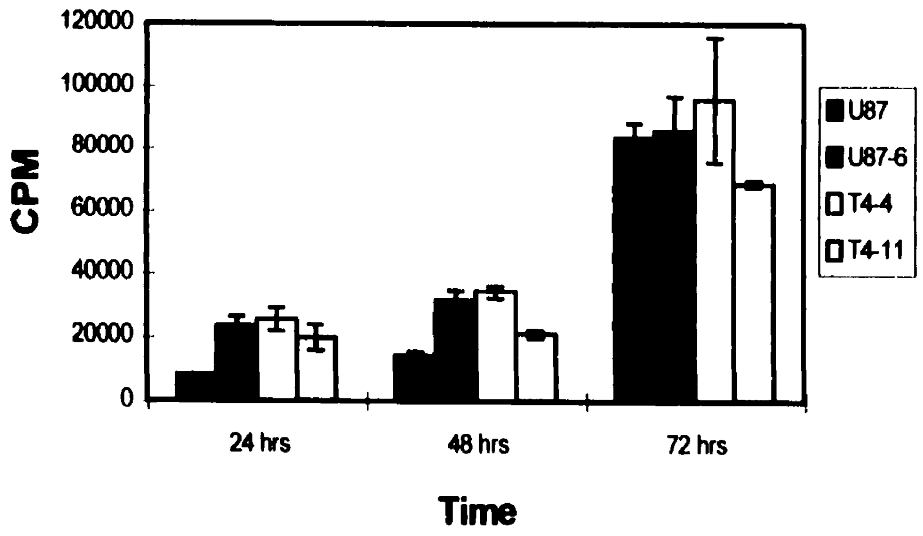


Figure 14. The effects of rTIMP-4 on U87 viability. Viability of U87 glioma cells was tested in the presence of 5-150 ng/ml rTIMP-4. No difference was seen between treated and control cells at 24, 48, or 72 hours as assessed by MTT assay.



rTIMP-4 and U87 Viability (MTT Assay)
0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
OD 600
24 hrs
48 hrs
72 hrs
Time
0 ng/ml
5 ng/ml
10 ng/ml
50 ng/ml
100 ng/ml
150 ng/ml

Figure 15. The effects of TIMP-4 overexpression on U87 proliferation. Proliferative ability of TIMP-4 transfectants was assessed by ^{3}H-thymidine incorporation at 24, 48, and 72 hours. TIMP-4 overexpression was shown not to affect cell growth compared to the vector control in these assays.


CPM
120000
100000
80000
60000
40000
20000
0
24 hrs
48 hrs
72 hrs
Time
U87
U87-6
T4-4
T4-11

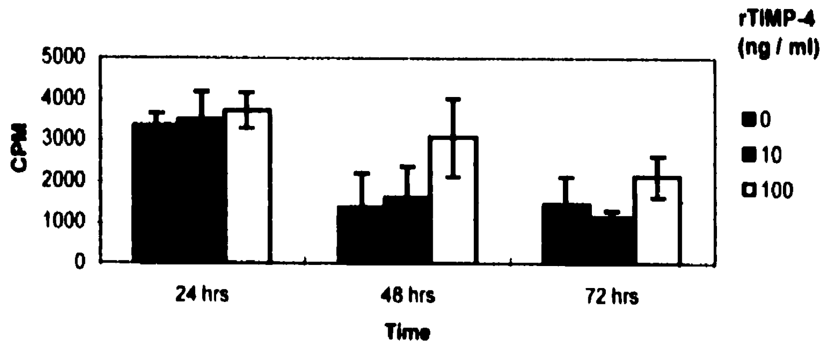
Parental U87 cells were treated in a similar manner in the presence of varying amounts of rTIMP-4 (10 and 100 ng/ml). The results in Figure 16 indicate that exogenous rTIMP-4 also has no effect on proliferative ability of U87 glioma cells compared to cells treated with no rTIMP-4.

3.8 **TIMP-4 overexpression inhibited glioma cell invasion**

To test the effects of TIMP-4 overexpression on the invasiveness of glioma cells, U87 clones transfected with pBABE TIMP-4 were seeded onto a 60 μl layer of Matrigel in a Boyden chamber and were allowed to invade for 36 hours (Figure 17). Cells transfected with TIMP-4 showed a 75% decrease in invasion compared to the vector control (which was assigned a value of 100% invasion).

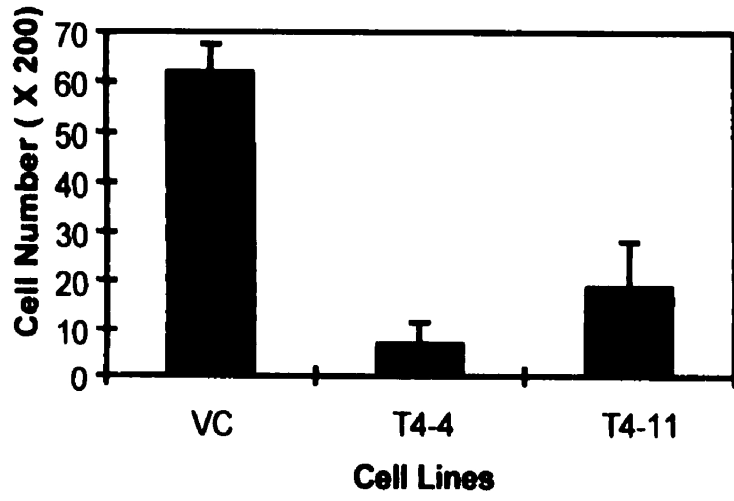
U87 parent cells were assayed in an identical fashion and were treated with 10, 100 and 1000 ng/ml of rTIMP-4, which was applied both into the Matrigel and in the chamber supernatant. After 36 hours of invasion, cells exposed to rTIMP-4 showed no decrease in invasive capacity relative to U87s in chambers with no rTIMP-4 (Figure 18).

Figure 16. The effects of rTIMP-4 on U87 proliferation. U87 cells in the presence of 10 and 100 ng/ml rTIMP-4 were examined for changes in ^{3}H-thymidine incorporation compared to the vector control cells. rTIMP-4 did not affect proliferation in these assays.



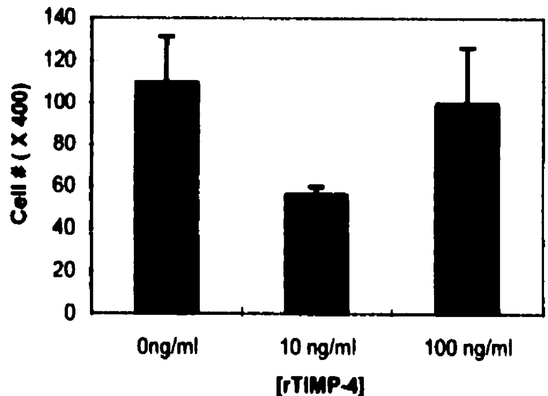
Effect of rTIMP-4 on U87
rTIMP-4
(ng / ml)
0
10
100
5000
4000
3000
2000
1000
0
CPM
24 hrs
48 hrs
72 hrs
Time

Figure 17. Invasive capacity of TIMP-4 overexpressing U87 cells. PBABE TIMP-4 transfected cells lines (T4-4 and T4-11) showed reduced invasion through matrigel chambers compared to the vector control (VC).



Cell Number (X 200)
0
10
20
30
40
50
60
70
VC
T4-4
T4-11
Cell Lines

Figure 18. The invasive capacity of U87 cells treated with rTIMP-4. U 87 cells treated with 10 and 100 ng/ml rTIMP-4 were tested for their invasive capacity. Though a decrease in invasion was seen initially at 10 μg/ml, results at 100 ng/ml were not significantly different than those for the control cells. The reduced invasion at 10 μg/ml was not repeatable and it was assumed rTIMP-4 had no effect on the invasion of U87 cells.



Effect of rTIMP-4 on U87 Invasion (48 hrs)
140
120
100
80
60
40
20
0
Cell # (X 400)
0ng/ml
10 ng/ml
100 ng/ml
[rTIMP-4]

3.9 Invasion Assay Optimization

The discrepancy in results between TIMP-4 overexpressing U87 clones (with a decreased invasive capacity) and the U87 parent cells treated with rTIMP-4 (no change in invasiveness) led to a series of experiments with Matrigel Boyden chambers. Much of my time in Norwich was spent optimizing these assays using various cell lines and conditions. Table 5 lists the invasion experiments performed using pre-coated matrigel chambers. All of these assays were performed using 10%FBS in the top chamber. Except for the U87 TIMP-4 transfected cells, which showed a 75% decrease in invasion compared to the parent cells, no change was noted comparing treated (transfected) and control (parent) cells.

The lack of inhibitor effect when adding recombinant TIMP was thought to result from an inability of the inhibitor molecule to reach the matrigel surface where cells were invading. Thus, control inserts were coated with matrigel that had inhibitor added at a desired concentration. The results of the assays using self-coated chambers can be seen in Table 6.

The results of these experiments were largely disappointing. It was found that both U87 and U251 cells needed some FBS (0.5%) in the upper chamber or cell viability and invasive capacity was completely diminished. Enriched fibroblast medium was somewhat useful in the bottom as a chemoattractant compared to regular DMEM-F12/FBS 10%. It was necessary to embed TIMP-4 in the Matrigel (which was poured at

Table 5. Invasion Assays performed using Precoated Matrigel Chambers

Cell Line	Inhibitor Or Cytokine	[Inhib.] ng/ml	Time (hrs)	Ratio of media to matrigel	Volume Of Matrigel	Inhib. Added to top Matrigel Bottom chamber	Chemo-attract?	Trypsin Or EDTA EGTA?	Change in Invasion
U87 parent + TIMP-4 clones	--	--	24 48 72	--	--	--	yes	tryp	Yes --clones showed reduced Invasion
U87 parent + GelB Clones	--	--	24 48 72	--	--	--	yes	tryp	No
U87	rTIMP-4	10 100 500 1000	24 48 36 72	--	--	T T and B	No	Tryp	No
U251	rTIMP-4	10 100	24 48 72	--	--	T T and B	No	Tryp	No
U87	aprotinin	100	24 48 72	--	--	T T and B	No	Tryp	No
U87	leupeptin	100	24 48 72	--	--	T T and B	No	Tryp	No
U87	Agouron	1000	24 48 72	--	--	T and B	No	Tryp	No
U87	torimefen	1000	24 48 72	--	--	T and B	No	Tryp	No
U87	rTIMP-2	10 100	24 48 72		--	T T and B	No		

Table 6. Matrigel Invasion Assays using Self-Coated Matrigel Chambers

Cell Line	Inhibitor Or Cytokine	[Inhib.] ng/ml	Time (hrs)	Ratio of media to matrigel	Matrigel (ul)	Inhib. Added Top, Matrigel Bottom	FBS in Top Chamber (%)	Chemo-attract?	Trypsin Or EDTA EGTA?	Change in Invasion?
U87	rTIMP-4	500	24 48	2:1	180	M	0.5	No	tryp	No
U87	rTIMP-4	500	24 48	2:1	180	T, M, B	0.5	No	tryp	No
U251	rTIMP-1	500	24 48	2:1	180	T, M, B	0.5	No	Tryp	No
U251	EGF EGF + rTIMP-4	1000	24 48	2:1	180	T, M, B	0.5	No	Tryp	No
U87 U251	--	--	24 48	2:1	180	--	10	No	Tryp	No
U87 U251	--	--	24 48	2:1	180	--	10	No	Tryp	No
U87 U251	--	--	24 48	5:1	180	--	10	No	Tryp	No
U87 U251	--	--	24 48	5:1	15, 30, 60, 120, 150, 200	--	10	No	Tryp	Lower Volume of Matrigel Improves Invasion
pascales	--		24	5:1	60		10	No	Tryp EDTA/ EGTA	No
U87 U251	--	--	36	5:1	60	--	0, 0.5, 1, 2, 5, 10	No	Tryp	Yes—no invasion with no serum; must seed in 10%
U87 U251	--	--	24	5:1	60		10	No	Tryp	No
U87	rTIMP-4	1000	36	5:1	60	T, M	10 (6hrs) then 0.5	Yes	Tryp	No
U251	rTIMP-4	1000	36	5:1	60	T, M	10 (6hrs) then 0.5	Yes	Tryp	Yes rTIMP-4 decreased invasion

It was necessary to embed TIMP-4 in the Matrigel (which was poured at 60 µl thickness in two different aliquots to eliminate a concave surface) and also in the top chambers. U87 and U251 cells were seeded in 10% FBS, and the media was changed after six hours to 0.5% FBS containing 1µg/ml rTIMP-4. Invasion was allowed to proceed for only 36 hours. No effect was noted on the invasion of the U87 cells. However, invasive capacity of the U251 cells was reduced by approximately 80% in the presence of 1 µg/ml TIMP-4. The results of this assay are seen in Figure 19. U251 viability assayed at 1 µg/ml and proliferation, assayed at 10 and 100 µg/ml were not affected by rTIMP-4 (Figure 20).

3.10 **Expression of TIMP-4 in human glioma cell lines**

To determine a possible relationship between TIMP-4 expression and glioma invasiveness, 8 human malignant glioma cell lines plus 4 rat glioma cell lines were analyzed for TIMP-4 expression. RT-PCR was performed to look for TIMP-4 transcript expression, and western blots and reverse zymography were performed on both the conditioned medium and lysate portions of the cell lines. Conditioned medium was collected from cells after treatment with serum-free media for 24 hours.

The results of the RT-PCR assay are seen in Figure 22. TIMP-4 was present in all cell lines, although the band appears faint. Both A172, and U251 Lac Z showed the highest TIMP-4 expression. All samples were run with GAPDH as an internal control.

Figure 19. Reduced invasion of U251 cells in the presence of 1000 ng/ml rTIMP-4. U251 cells treated with 1000 ng/ml recombinant TIMP-4 showed 75% reduced invasion compared to the control cells.

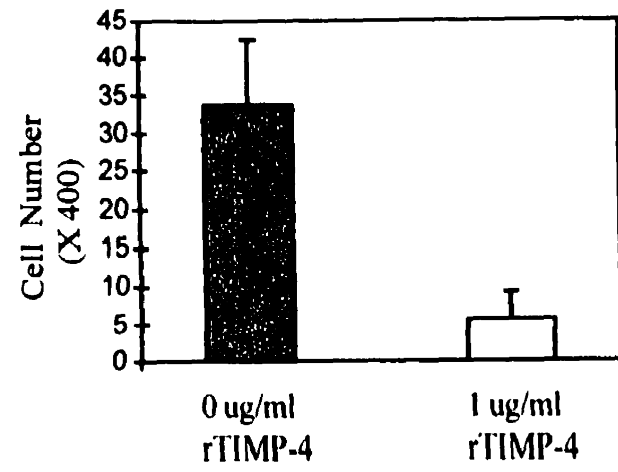
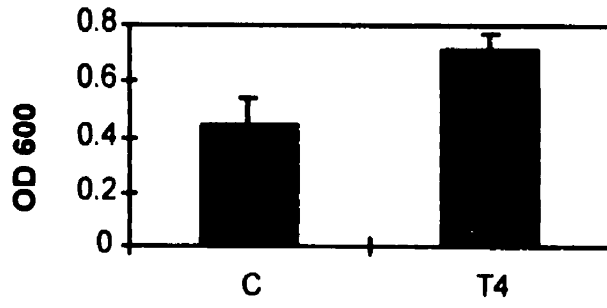

Cell Number
(X 400)
45
40
35
30
25
20
15
10
5
0
0 ug/ml
rTIMP-4
1 ug/ml
rTIMP-4

Figure 20. rTIMP-4 and U251 viability and proliferation. Neither (A) viability of U251 cells (assayed at 1 μg/ml rTIMP-4) nor (B) proliferation (assayed at 10 and 100 ng/ml TIMP-4) were significantly different than that measured for untreated U251 cells.


MTT Viability Assay of U251 Cells
(TIMP-4 1ug/ml)
OD 600
0.8
0.6
0.4
0.2
0
C
T4

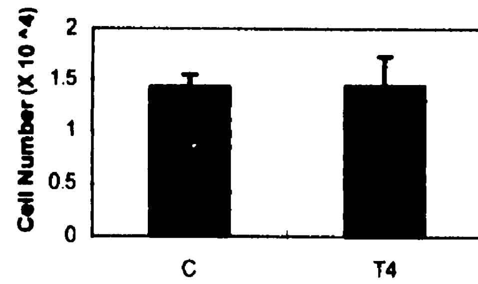

Trypan Blue Viability Assay of U251
Cells (1ug/ml TIMP-4)
Cell Number (X 10 ^4)
2
1.5
1
0.5
0
C
T4

Figure 21. RT-PCR detection of TIMP-4 transcript in gliomas. TIMP-4 mRNA expression was highest in A172 and U251 LacZ cell lines, although weak expression is seen in all the samples. GAPDH was run as an internal control.

U251
U251 LacZ
SNB19
A172
TE671
HS683
U87
C6
C6 LacZ
9L
9L LacZ
TIMP-4 -->
GAPDH -->

Protein detection by western blot is seen in Figure 22, where 30 μg of protein sample were run out for each cell line. In all samples, a 23 kDa band representing TIMP-4 was found in the lysate portion. Also found were high molecular weight complexes of unknown identity. In the lysate portions of the cells, HSB683 and U87 showed the highest TIMP-4 expression. U87 also showed the highest expression of TIMP-4 in the supernatant. Both SNB19 (a human cell line) and C6 (a rat cell line) were lacking the 23 kDa band in the conditioned medium portions of the western.

TIMP-4 activity was also analyzed using reverse zymography (Figure 23). In this case, no TIMP-4 was detectable in any of the conditioned medium of the cell lines examined. This was excepted, due to the possibility of TIMP-4/protein complexes discussed earlier. In the lysate portions, both U87 and HSB683 showed faint TIMP-4 activity. A summary of TIMP-4 expression in these cell lines is found in Table 7.

3.11 Invasion of human glioma cell lines.

To determine a possible correlation between glioma invasiveness and TIMP-4 expression, several of the above human glioma cell lines were tested for invasive capacity using pre-coated Matrigel chambers. Cells were seeded into DMEM F12/10% FCS and were allowed to invade for 48 hours. The number of cells that invaded through the membrane

Figure 22. Western Blot detection of TIMP-4 in human and rat glioma cell lines. The top panel shows the lysate portion of the cells, where the 23kDa band is present in each sample, although is shows the highest expression in HSB683 and U87 cells. Notice the high molecular weight bands in each sample. The lower panel shows TIMP-4 expression in the conditioned media of the cells. In this case, TIMP-4 expression is highest in U87 cells, and is expressly absent in SNB19 and C6 cells.

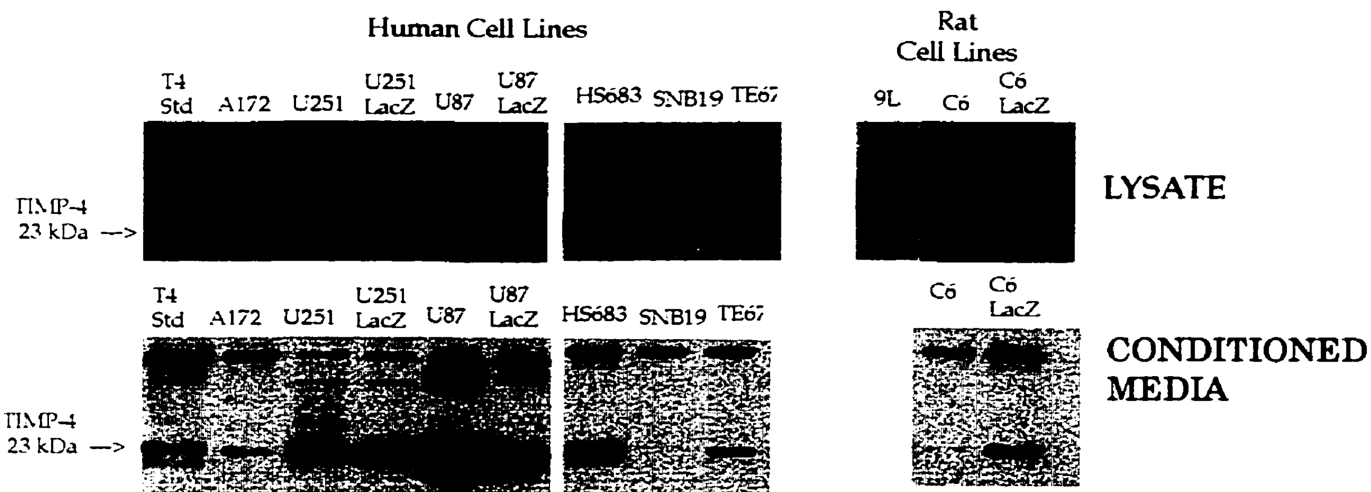
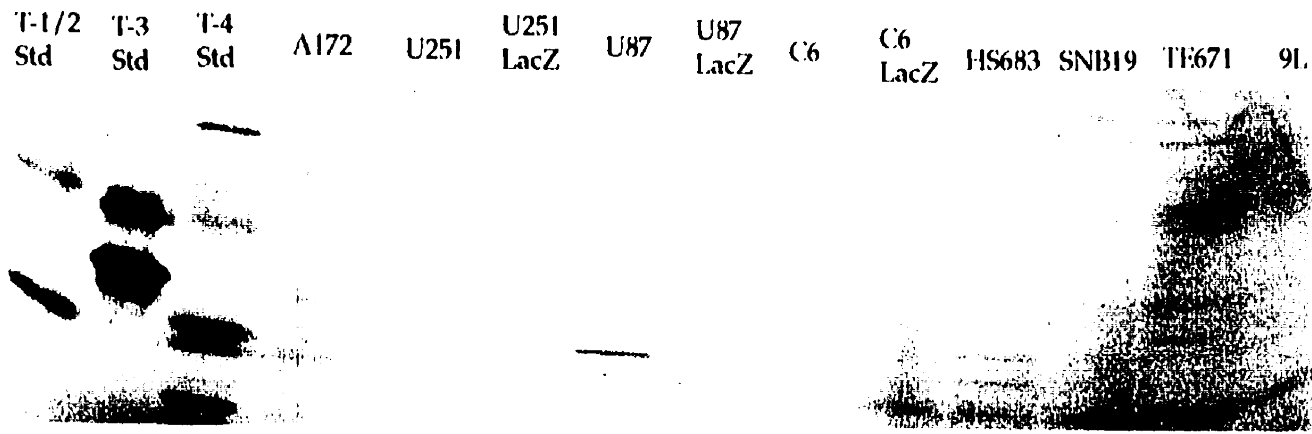

Human Cell Lines
Rat
Cell Lines
T4
Std
A172
U251
U251
LacZ
U87
U87
LacZ
HS683
SNB19
TE67
9L
C6
C6
LacZ
TIMP-4
23 kDa —>
LYSATE
CONDITIONED
MEDIA

Figure 23. Reverse zymography of TIMP activity in glioma cell lines. The top panel shows TIMP-4 activity in the cell lysate. TIMP-4 is visible only in U87, HSB683, and slightly in U87 lacZ. The bottom panel shows TIMP-4 activity in the conditioned media. No bands are visible in any of the samples.


T-1/2 Std
T-3 Std
T-4 Std
A172
U251
U251 LacZ
U87
U87 LacZ
C6
C6 LacZ
HS683
SNB19
TE671
9L
LYSATE

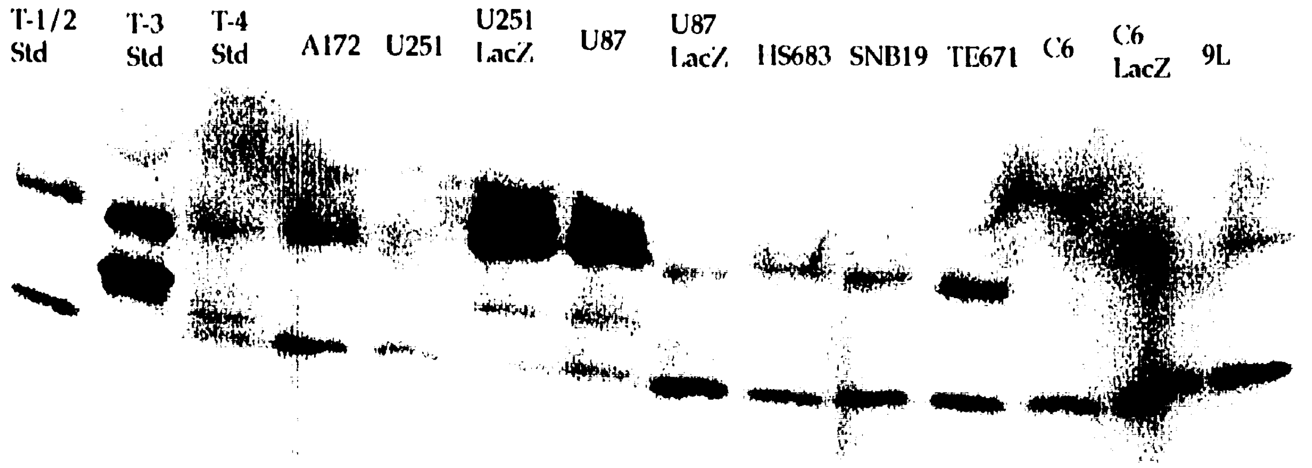

T-1/2 Std
T-3 Std
T-4 Std
A172
U251
U251 LacZ
U87
U87 LacZ
HS683
SNB19
TE671
C6
C6 LacZ
9L
CONDITIONED MEDIUM

Table 7. TIMP-4 Expression in glioma cell lines

Cell Line	RT-PCR		R. Z.*		Western Blot**	
	Lysate	Super	Lysate	Super	Lysate	Super
HUMAN						
U251	++	—	—	—	+	+++
U251LacZ	++	—	—	—	++	++
SNB19	++	—	—	—	½+	—
A172	+++	—	—	—	+	+
TE671	+	—	—	—	+	+
HSB	+	—	+	—	++++	++
U87	++	—	++	—	+++	(+)+++
U87LacZ		—		—	++	++
RAT						
C6	++	—	—	—	++	—
C6LacZ	—	—	—	—	++	+
9L	—	—	—	—	+	+
9LLacZ	—	—	—	—	—	—

*RZ: lysate: 10 ug ; super :5 ug
**Western: lysate and super : 3 ug

was then compared to TIMP-4 expression to see if increased TIMP-4 correlated with decreased invasive capacity.

The results of the invasion study can be seen in Table X. It should be noted that cells were not counted in this assay. Rather, a visual assignment of a number on a scale of 1 to 4 (representing low to high invasiveness respectively) was utilized. Both U87 and HSB683, which showed TIMP-4 activity by reverse zymography, were the least invasive cell lines. The most invasive cell line was U251.

3.12 **Subcellular fractionation of glioma cell lines**

To further investigate the high molecular complexes found on TIMP-4 Western blots, and to determine the association of TIMP-4 with the cell membrane, subcellular fractionation was performed on several human glioma cell lines as well as the TIMP-4 overexpressing clones discussed previously in this thesis. Figure 24 shows western blot detection of TIMP-4 in both the membrane and cytosolic portions of each of the cell lines. No TIMP-4 was found in any of the membrane fractions, while TIMP-4 was present in most cytosolic portions of the cells.

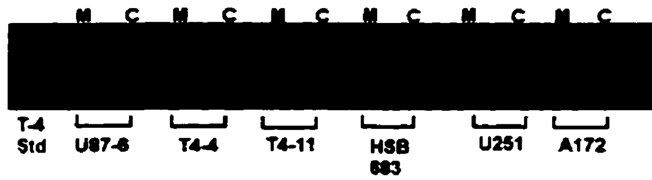
Table 8. Invasive Capacity of Human Glioma Cell lines

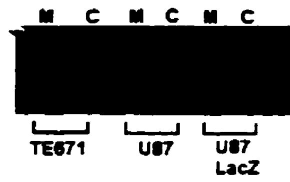
Cell line	Invasiveness
U87	+
HSB683	+1/2
U87 LacZ	++
A172	+++
TE671	+++
U251	++++

- + = relatively low invasive capacity

  ++++ = relatively high invasive capacity

Figure 24. Western Blot detection of TIMP-4 in membrane and cytosol of glioma cell lines. In all samples, TIMP-4 was absent from the membrane fraction, while most cell lines showed expression in the cytosol. **Abbreviations: C = cytosolic fraction, M = membrane fraction.**


M C M C M C M C M C M C
T-4 Std
U87-6
T4-4
T4-11
HSB 683
U251
A172


M C M C M C
TE671
U87
U87 LacZ

4. DISCUSSION

We reach great heights by a winding staircase.

-Francis Bacon

4.1 TIMP quantification in gliomas

The current study is the most extensive survey of the TIMP family in human gliomas to date. Based on expression and localization studies, the data in this thesis suggest that TIMPs are involved in the progression of gliomas and that their roles differ significantly from each other in this process. Furthermore, TIMPs may provide a novel therapeutic approach in the treatment of this currently fatal disease.

According to the traditional notion of TIMPs in tumor biology, excessive MMP activity is promoted by a lack of available TIMP for binding and halting proteases. This suggests that in highly malignant tumors, TIMP should be absent or downregulated, promoting increased invasiveness. However, when mRNAs from several gliomas were analyzed, a paradox was observed (panel A, Figure 5).

Rather than being down-regulated in gliomas, TIMP-1 mRNA levels increased with increasing glioma grade, a trend that was also seen using Western blot analysis (Figure 7A). It is possible that TIMP-1 expression increases in highly malignant tumors in response to increased gelatinase activity (28), yet is somehow unable or ineffective against the proteolysis and invasion. Alternatively, TIMP-1 may act in a manner other than MMP inhibition to promote tumor growth. For example, TIMP-1 has erythroid potentiating activity (EPA) which stimulates the proliferation of erythroid precursors (53, 54). Other studies have shown TIMP-1 to stimulate proliferation of schleroderma, gingival fibroblasts, and

keratinocytes (55-57). Furthermore, TIMP-1 expression has recently been localized to the nucleus of gingival cells, implicating it as a possible transcription factor (44). Given these potential growth promoting abilities, TIMP-1 may actually contribute to glioma malignancy when upregulated.

Another interesting panel in Figure 5 is that of TIMP-4, which shows a negative correlation of inhibitor expression with increasing tumor grade. Although the change in TIMP-4 expression is not as striking as the expression pattern for TIMP-1, there is a consistent decrease in the ratio of TIMP-4 to GAPDH in the high grade tumors compared to those of lower grade. Curiously, TIMP-4 mRNA in normal brain specimens was relatively low. The reasons for this discrepancy are unknown.

Differential TIMP-4 mRNA expression prompted several Western blot assays, where a negative correlation between glioma grade and TIMP-4 expression was also found (Figure 7B). This time the normal brain samples showed the highest relative TIMP-4 expression. These results suggested that TIMP-4 might act as a negative regulator of glioma malignancy. Whether TIMP-4 affects behaviors outside of MMP inhibition and thus invasion is unknown.

When performing reverse zymography to analyze TIMP activity in human glioma cell lines, TIMP-1 activity was not found to correlate with glioma grade as was noted in western blot detection, yet it appeared in

every tumor sample (Figure 8A). The reasons for these discrepancies may lie in the formation of complexes between TIMP and other proteins *in vivo*. As reverse zymography is significantly more sensitive than western blotting for detection of gelatinase inhibitors, we would expect that TIMP protein bands would be equally or more abundant in our reverse zymogram (Figure 8A) than in Western blot assays (Figure 7A and B). However, reverse zymography samples do not undergo denaturation and any complexes in the sample remain intact. Such bound TIMP would be unable to generate an active band on the gel.

The existence of TIMPs in complex was also supported by Western blot data. For both TIMP-1 and -4, the respective 28 kDa and 23 kDa bands were not the only proteins detected. In both cases, higher molecular weight complexes (approximately 60 kDa) appeared (data not shown). TIMP-1 immunoprecipitation assays were performed and these suggested that TIMP-1 was complexed with fragmented gelatinase A and B (data not shown). However, problems with antibody cross-reactivity required that this complex be further investigated. The TIMP-4 immunoprecipitation assays resulted in similar technical difficulties.

In breast cancer, TIMP-4 overexpression cell lines *in vitro* and *in vivo* resulted in decrease invasion and microvascular density (73). Other studies have localized TIMP-4 to endothelial cells and suggest that TIMP-4 is a possible player in angiogenesis (49, 80). Based on the literature data, and on the negative correlation of TIMP-4 expression and glioma

grade, the role of TIMP-4 was further pursued by generating TIMP-4 overexpressing glioma cell lines and analyzing their malignant behavior *in vitro*. Results from these assays are discussed in a later section.

4.2 TIMP localization in gliomas

The localization data of TIMPs in gliomas suggested unique roles for these inhibitors in glioma pathophysiology. TIMP-1 was expressed not only in the tumor cells, but also in the endothelial cells of the surrounding blood vessels (Figure 9). Since gelatinase B is a marker of neovascularization, it is possible that TIMP-1 is expressed here to control MMP activity.

In order for endothelial cells to migrate and invade during angiogenesis, they require a "scaffold" of enzymatically linked fibrinogen produced by the surrounding vascular bed. Degradation of this fibrin network allows ec's to form endothelial lined tunnels, which rely on the integrity of the fibrin for support (81). Overdegradation of the surrounding environment by ec's would prevent the migration and formation of new vessels. Thus, endothelial cells may produce TIMP-1 to allow for proper neovascularization by controlling MMP activity in the area.

In contrast to TIMP-1, TIMP-2 was expressly absent from neovascular structures (Figure 11). This is intriguing, as previous reports have suggested that TIMP-2 inhibits cytokine induced endothelial cell

proliferation (60). The absence of TIMP-2 from newly forming capillaries may therefore encourage neovascularization of malignant tumors.

In a recent study by Brookes et al. (82), the TIMP-2/MMP-2 protease system was implicated in angiogenesis through a protein known as PEX. PEX represents the noncatalytic, hemopexin-like, C-terminal fragment of MMP-2, which is processed from the active protease in the absence of TIMP-2. PEX interferes with MMP-2 binding to integrin $\alpha v \beta 3$, which localizes MMP-2 to the cell surface and promotes neovascularization. When PEX binds this integrin, MMP-2 is prevented from binding and angiogenesis is reduced. As in the case of TIMP-1, TIMP-2 expression in the vicinity of vascular beds (in this case a lack of it) may contribute to the tight control of protease activity and angiogenesis through regulating the production of PEX, which has specific antiangiogenic properties.

TIMP-4 mRNA was also localized to the cytoplasm of tumor cells (Figure 12). Unlike TIMP-1, it lacked a strong localization to the blood vessels. These data were supported by immunohistochemical studies, where TIMP-4 expression was relatively negative in the capillaries compared to the tumor cells (Figure 12). Previous data have suggested TIMP-4 is a negative regulator of angiogenesis (49, 67). Furthermore, TIMP-4 overexpressing breast carcinoma cells resulted in tumors with decreased microvascular density (73). It is possible that TIMP-4 negatively regulates angiogenesis, and the lack of it in highly malignant

tumors results in excessive neovascularization, promoting tumor growth. Whether TIMP-4 acts in concert with MMP-2, PEX, integrins or other cellular proteins during the angiogenic process is unknown.

An important point in these initial investigations is that full surgical specimens were used for the study, not specific sections of the tumor. Each specimen may, therefore, contain different ratios of tumor, endothelial, and normal brain cells. Some specimens may have come from the tumor center, while others represented the invasive edge. Thus, the data collected from these tumors becomes semi-quantitative and must be interpreted in that light. Based on what we have learned to date, tumors currently collected are properly sectioned before entering the tumor bank for analysis.

4.3 *In vitro* studies of TIMP-4 on malignant glioma behavior

Both U251 and U87 glioma cells were transfected with full length TIMP-4 from mouse heart cDNA, however only U87 cells were used in this study. In the TIMP-4 overexpressing U87 cells, no difference was seen in proliferative ability (Figure 15) or viability (Figure 13) compared to the vector control over 24, 48, or 72 hours. However, when plated on pre-made matrigel coated chambers, the TIMP-4 overexpressing U87 cells showed 75% less invasion than the empty vector control during a 24-hour period (Figure 17). This suggested that increased TIMP-4 expression

might act to control invasion, although it failed to halt invasion completely.

Concerning the data for proliferation and viability, these charts were generated using confluent (and overly confluent, in the case of the 72 hour assays) cells which explains the lack of exponential growth over the three day period. It is possible that small effects on proliferation may be noted in the early stages, if the cells are analyzed at low levels of confluency (when they are in their growth stage). After caring for the cell lines for several weeks, no obvious difference in proliferative rate, as judged by microscopic examination, was noted between transfectants and controls.

U87 parent cells were also tested for proliferation and viability in the presence of 10 and 100 ng/ml of rTIMP-4. Again, no difference was seen in either of these factors between the treated and untreated cells (Figures 16 and 14 respectively). In contrast to the transfectants, excessive recombinant TIMP-4 was not successful at reducing the invasive capacity of U87s. This was disconcerting as other reports of rTIMP-1 and rTIMP-2 reducing invasion in Boyden chambers existed in the literature. We assayed rTIMP-1, -2, AG3340, tamoxifen, aprotinin, and leupeptin at various concentrations in Boyden chambers, none of which showed an effect on the invasive capacity of U87 cells (data not shown). It was concluded that the optimal conditions for *in vitro* invasion

assays needed to be determined, and this lead to a series of experiments that were conducted in the Edwards laboratory in Norwich, U.K.

4.4 The perils of invasion

To optimize the invasion assay for analysis of glioma cells treated with rTIMP-4, several conditions were tested. It was first proposed that rTIMP-4 needed to be inserted directly into the matrigel. If suspended only in the media of the top chamber, the size of the inhibitor molecule would likely prevent it from finding its way to the matrigel membrane surface where invasion occurs. To overcome this, control inserts were used and coated with matrigel containing rTIMP-4 at the desired concentration. To ensure the inhibitor did not diffuse out the matrigel after solidification, the media in the top chamber also contained rTIMP-4 at an equivalent concentration. The matrigel was poured in two layers, as the use of a single layer resulted in a concave surface that pooled all glioma cells to the centre of the membrane. Therefore, after pouring the first layer, a second aliquot of matrigel was added in the centre to ensure a flat, even surface for the cells to attach to. A series of matrigel volumes were assayed and it was decided that 60 μl, composed of 50 μl media and 10 μl matrigel, produced an optimal thickness for invasion.

When originally seeding glioma cells into these chambers, media containing rTIMP-4 without FBS was used. None of the cells invaded after 48 hours under these conditions and much of the cell population

died. To overcome this, the cells were seeded in media with 10% FBS for 6 hours, and the media was changed to 0.5% serum and rTIMP-4. Additionally, the bottom chamber was filled with the conditioned media from fibroblasts, which contained numerous growth factors and acted as a chemoattractant. Under these conditions, U251 glioma cells in the presence of 1μg/ml rTIMP-4 showed an 80% decrease in invasion over 36 hours, compared to the control cells treated with no rTIMP-4 (Figure 19). U87 cells showed no change in invasion, even under these conditions.

As a final component to the *in vitro* studies, several glioma cell lines were analyzed for their TIMP-4 mRNA and protein expression, then tested for invasion to see if any correlation existed between inhibitor levels and invasive capacity. mRNA levels varied little between each of the cell lines (Figure 21). Using western blots, the highest lysate expression was seen in HSB683 and U87, the latter also showing the highest expression into the conditioned medium (Figure 22). TIMP-4 activity in the conditioned medium was undetectable by reverse zymography, but the lysate assays showed TIMP-4 activity for U87 and HSB683 (Figure 23). Using pre-coated matrigel chambers, invasion for each of these cell lines was assayed over 36 hours. The lowest invasive capacity was seen for U87 and HSB683 cells while the highest was seen for U251 cells (Table 8). Although little can be concluded from such preliminary assays, it is interesting that the only cells lines showing TIMP-4 activity in the lysate also had the lowest invasive capacity. U87

and HSB683 also showed the highest TIMP-4 protein expression using Western blot. It could be that the high levels of inhibitor in these two cell lines contribute to their low invasive abilities. U251 cells, which were the most invasive cell line in this analysis, did not show a relative lack of TIMP-4 compared to the other cells lines outside of U87 and HSB683. Whether TIMP-4 overexpression in these cells would inhibit invasion needs to be further explored.

Though matrigel invasion assays provide a means of monitoring malignant behavior of cells *in vitro*, the data here suggest that these assays are not always reliable. Results were inconsistent on several occasions, and the data found using rTIMP-1 and-2 did not always correlate with observations reported in the literature.

There are several reasons why one should be cautious of *in vitro* invasion results. The ECM of the brain is limited to blood vessels and glia limitans, and glioma cells must migrate along myelinated fiber tracts and several non-matrix related structures (14). Thus, matrigel doesn't accurately reflect a true *in vivo* environment for glioma cells. To closer approach the *in vivo* situation, brain slices could potentially be cultured and grown in Boyden chambers (83). Even this would not be completely accurate, however, as numerous cytokines and growth factors that influence malignant behavior *in vivo* would be missing.

Upon counting the cells after fixation, one often sees patches of cells over the membrane, implying that cell distribution and thus,

invasive potential was not equal over the entire matrigel surface. Cell counts, therefore, can be very unreliable. Cells often invade heavily at the center, and heavily at the outside, leaving large portions of the membrane surface without cells. Counting cells at the invasive center was difficult with the microscope available in the Calgary lab, which is why a semi-quantitative approach was used in correlating TIMP-4 expression with invasion in glioma cell lines.

In summary, although several studies have been published presenting such *in vitro* invasion assays, I believe that few of these studies have been without the aforementioned problems. Therefore, I have determined that while invasion assays may provide us with some information, they must be followed by *in vivo* studies before conclusions can be made.

4.5 Future directions and implications of TIMPs in glioma therapy

Brain cancer is an extremely devastating disease. Gliomas, the most frequently encountered type of brain tumor, are highly aggressive and carry a median survival rate of less than a year. These tumors are unresponsive to available cancer treatments, and the development of more effective therapies for gliomas is desperately needed.

The most important aspect of the work discussed in this thesis is not the data within, but the future directions in which they will take us. In terms of the localization and characterization of TIMP expression in

gliomas, it would be valuable to compile a complete record of all TIMP mRNA expression in tumor samples through *in situ* hybridization. Of particular interest is the contrast discussed previously between TIMP-1 and -2 expression, where TIMP-1 localized to blood vessels and TIMP-2 appeared explicitly absent in this region. This being the case, TIMP-2 may act as an antagonist of angiogenesis, and increased expression of this inhibitor may provide a novel means of controlling the growth of gliomas by restricting the surrounding blood supply. As TIMP-2 and –4 both interact with MT1-MMP, it could be that these molecules work in concert to control vascularization in malignancy.

Since TIMP-3 overexpression has proved to cause apoptosis through protection of the TNFα receptor in several neoplastic cell lines, *in vitro* and *in vivo* assays of overexpressing TIMP-3 glioma transfectants would be worthwhile. The role of TIMP-4 in apoptosis has yet to be studied. FACS analysis of cells overexpressing TIMP-4 needs to be performed to see if mechanisms comparable to TIMP-3/TNF alpha interaction occur with this newest inhibitor.

Regarding the TIMP-4 overexpressing U87 clones, these cells should be introduced into mice to test their malignant behavior *in vivo*. It is possible that they will act in a completely different manner than was found *in vitro*, as they will be subject to the influence of numerous growth factors and cytokines in the *in vivo* situation. Such a phenomenon was noticed with Agouron, a synthetic MMP inhibitor that

showed no effect on cellular proliferation *in vitro*, but significantly reduced cell growth *in vivo* (86). The mechanisms behind this decreased growth *in vivo* are unknown and could relate to numerous signaling pathways. For example, insulin like growth factor (IGF) is a potent cytokine that is regulated by several binding proteins (insulin-like growth factor binding proteins or IGF-BP) which bind IGF and prevent its interaction with its receptor. IGF-BP3 is degraded by MMP-1, -2, and -3, which results in increased concentrations of IGF in the cell, altering cell growth and proliferation (87). By inhibiting MMP activity, natural or synthetic MMP inhibitors like Agouron may indirectly control proliferation by preventing degradation of IGF-BP3, thus reducing the bioavailability of IGF.

The effects of TIMP-4 in angiogenesis are of great interest, but to date an *in vitro* assay to examine this phenomenon has not been perfected. Thus, intracerebral implants into mice of these cells would hopefully show not only a decreased invasive capacity, but also some effect on neovascularity. Ms. Huong Muzik, in the Forsyth laboratory, is currently developing a TIMP-4 inducible system (using ecdysone) in various malignant glioma cell lines. This will allow the control of TIMP-4 expression so that the role of TIMP-4 in malignancy can be more accurately determined. It should also be noted that numerous U251 clones are available in our laboratory that overexpress TIMP-4. Since U251 cells are by nature much more invasive than U87s, the results of *in*

vitro assays may be quite unique from those of the U87 clones presented in this thesis. These clones may also be worth examining both *in vitro* and *in vivo* using the ecdysone inducible system

During the course of my stay in England, I did preliminary work with Marc Lafleur related to the effects of TIMP-4 and angiogenesis *in vitro*. We attempted to seed various endothelial cell lines with different concentrations of TIMP-4 (and other TIMPs) to see if endothelial cell migration and tube formation could be halted using the inhibitor. These assays could not be optimized during my stay in England due to time restraints but would be worthwhile pursuing to further define the role of TIMP-4 in angiogenesis without necessitating animal work.

TIMPs contribute only in part to the malignant nature of gliomas. It may be unrealistic to believe that using TIMPs will halt glioma cell aggressiveness and save a brain tumor patient's life, although it may prolong it with conventional therapies, which is no small feat in the management of this disease. Unfortunately, by the time the gliomas have been diagnosed, invasion is likely already underway.

We must keep in mind the multifunctionality of TIMPs. The data presented here indicate that TIMP expression is altered in malignant cells. We are also aware that TIMPs are involved in numerous cellular functions outside of MMP inhibition. So while invasion control may be a method of slowing the mortality of glioma patients, investigations into

TIMPs and angiogenesis or apoptosis are promising aspects of glioma therapy that may eventually allow us to eradicate the disease.

One area of interest is the involvement of the MMP/TIMP system in the availability and growth promoting activity of several cytokines. This system was previously mentioned in the case of IGF, where the MMP protease system affected the bioavailability and activity of this signaling molecule by affecting the degradation of it's binding protein.

Interleukin type II receptor is another protein that is proteolytically shed, generating a soluble decoy which captures IL-1 and prevents its interaction with a signaling receptor (88). Recent studies showed that levels of MMP-9 correlate with increased expression of IL-1, suggesting that IL-1 stimulates the production of this protease (89). BB94, a synthetic MMP inhibitor, inhibited decoy shedding and increased the bioavailability of IL-1, thus increasing its growth promoting activities. This suggests that MMPs contribute to a feedback control mechanism for the production of IL-1, and inhibitors of MMPs may actually promote growth and proliferation of cells by inhibiting this buffer system.

TNF-α is another transmembrane protein that mediates signaling, proliferation and adhesion. TIMP-3 was reported to inhibit shedding of this receptor by interacting with TNF-α converting enzyme (TACE or ADAM-17), leaving the receptor intact for signaling in apoptosis (90). TIMP-1, -2, and -4 were much less effective. TACE is a membrane bound

disintegrin metalloproteinase that processes pro- TNF-α to a soluble form. The possibility that TACE may be involved in the shedding of membrane proteins other than pro-TNF-α, implicates TIMP-3 and possible other TIMPs in the control of numerous signaling pathways.

4.6 Questions, questions, questions...

How can we relate the above scenarios to the story of TIMPs in gliomas? We could, for instance, look at levels of TACE in glioma cells. A lack of TACE expression or a lack of active TACE would result in increased levels of apoptosis. Cancer cells often lack proper apoptotic signaling. Does increased TACE correlate with increased glioma aggressiveness? Does TACE affect the processing of other signaling receptors such as EGFR, whose increased expression in glioma cells correlates with decreased patient survival (91)? Does TIMP-3 or -4 restore apoptosis in gliomas through its interactions with TACE?

What about the role of TIMP-4 in apoptosis? Does TIMP-4 interact with other cytokines involved in the programmed cell death of glioma or endothelial cells? Does TIMP-4 affect proliferation or apoptosis in the presence of certain cytokines? Remember that TIMP-2 was inhibitory to cell growth only in the presence of insulin (59). Perhaps TIMP-4 affects proliferation or apoptosis in a similar manner.

Is there a possible TIMP-4 receptor that is responsible for the lack of activity seen using reverse zymography? Previous studies in our lab

showed that TIMP-4 was localized to the membrane surface of stem cells using fluorescent immunocytochemistry (92). Is this evidence of a TIMP-4 receptor, or is TIMP-4 interacting with MT1-MMP to control gelatinase activation? Is there any connection between TIMP-4 and EGF or VEGF? Does TIMP-4 alter the adhesive properties of integrins on the surface of glioma cells like TIMP-2, controlling the interaction of MMP-2 and integrins through PEX?

Does the MMP/TIMP system interact with EGFR? EGFR is equipped with a tyrosine kinase domain that regulates the function of apoptotic proteins like Bcl-X_L and caspases in the cell (91). In gliomas with this EGFR mutation, survival is promoted through increased expression of Bcl-X_L and a lack of caspase activity. Such EGFR mutations, which result in a truncated version of the receptor, which becomes constitutively active, occur in 40-50% of de novo gliomas. Could the constitutively active mutant EGFR in gliomas result in increasingly activated MMPs and therefore aggressiveness? Are MMPs involved in processing this receptor at the cell surface? How does EGFR signaling affect TIMP expression?

4.7 Conclusion

In summary, both TIMP-1 and –4 expression levels were found to correlate with glioma malignancy in a positive and negative manner

respectively. Localization and expression data suggest that TIMP-1 and –4 play unique roles in malignant gliomas and these may not be completely dependent on their MMP inhibitory properties. The precise functions of each TIMP protein in malignant behavior need to be further investigated through *in vitro* and *in vivo* studies.

Our expanding knowledge in the area of metalloproteinase inhibitors has offered a range of new strategies in the battle against brain tumors. One area already showing promise as an anticancer strategy is that of synthetic inhibitors. Several MMP inhibitors such as Batimistaat and Marimastat are currently being tested in preclinical trials and are proving to be effective against many cancers. However, these inhibitors of MMPs are broad range inhibitors, and many of the long-term side effects are unknown (94).

Expanding our knowledge of how TIMPs are involved in brain tumor progression and specifically which TIMPs do what in the process will allow for the development of specific synthetic inhibitors. Such specificity may both facilitate the action of the drug, and eliminate negative long-term side effects of the broad range inhibitor treatment. Furthermore, this knowledge may be applied to molecular therapies, such as the delivery of TIMPs by gene therapy in order to control glioma invasion and angiogenesis. In the future, the combination of inhibitory strategies with conventional treatments will surely improve the prognosis for glioma patients.

4.8 Process

I would like to close with a word about process. Process transcends time. It teaches patience, requires sacrifice, involves frustration and repair, and embodies trust in our unfolding potential. Process transforms any journey into a series of small steps, taken one by one, to reach a worthwhile and challenging goal (95).

The cure towards malignant gliomas is a process. So let us continue with perseverance, the journey marked out for us. For a process that requires perseverance, builds character, and character itself builds hope.

And hope, will not disappoint us...

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