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MATRIX METALLOPROTEINASES IN TISSUE REMODELING, REGENERATION, AND PROSTATE CANCER

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Major professor

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MATRIX METALLOPROTEINASES IN TISSUE REMODELING, REGENERATION AND PROSTATE CANCER

By

William Winston Chu

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ABSTRACT

MATRIX METALLOPROTEINASES IN TISSUE REMODELING REGENERATION AND PROSTATE CANCER

By

William Winston Chu

The turnover of extracellular matrix (ECM) occurs during tissue remodeling, regeneration and cancer. Matrix metalloproteinases (MMPs) play a key role in ECM turnover because they have the ability to degrade the ECM, especially collagen, a major component of the extracellular matrix. MMPs are, therefore, involved in ECM turnover during normal development and wound healing as well as arthritis and cancer. The activity of MMPs is highly regulated at three levels: 1) synthesis and secretion; 2) activation and 3) extracellular proteolytic activity. The extracellular protease activity is regulated by specific, tissue inhibitors of matrix metalloproteinases or TIMPs. This tight regulation allows for the spatial temporal control of MMP activity required under normal conditions where homeostasis in ECM turnover is maintained by on a balance between active MMP and TIMP. A loss in this balance leads to pathological conditions. For example, a proportional increase in TIMP levels results in accumulation of ECM as seen in glomerular and arterial sclerosis. Similarly, a proportional increase in MMP levels results in increased degradation of ECM, as seen in arthritis and cancer. This study reviews the family of MMPs, their substrates and inhibitors, and examines their roles in both normal and pathological conditions.

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Figure 7.11. Matrix MMP-2 and MT-MMP expression in Caco-2 cells. (Top) Gelatin zymography of proteins secreted into the cell culture medium. Lane 3 demonstrates the result following treatment with sulindac sulfide (SS; 25 micro M) for 24 hr. A 68- and 62-kDa band are seen in the COX-2-expressing Caco-2 cells; whereas, only the inactive 68-kDa band is een in the control Caco-2 cells. (Middle) Immunoblotting results with an anti-MMP-2 antibody in an identical experiment to that shown in the Top panel. This result confirms the gelatin zymography data shown in cells which is inhibited in lane 3 by treatment with 25 microM sulindac sulfide for 6 hr. (Tsujii 1997).

LIST OF ABBREVIATIONS

4-HPR	N-(4-hydroxyphenyl)-retinamide
AP-1	activator protein transcription factor
APMP	amino phenyl mercuric acetate
ATRA	all *trans* retinoic acid
bFGF	basic fibroblast growth factor
BPH	benign prostatic hyperplasia
C	collagen-like domain
Cat	catalytic domain
cDNA	complementary DNA
CT	connective tissue
cys	cysteine
DNA	deoxyribonucleic acid
ECM	extracellular matrix
EGF	epidermal growth factor
F	furin consensus site
FN	fibronectin-like gelatin binding domain
glu	glutamic acid
GMC	glomerular mesangial cell
H	hinge sequence
HEM	hemopexin-like domain

his	histidine
hyp	hydroxyproline
MMP	matrix metalloproteinase
NGF-β	nerve growth factor beta
PA	plasminogen activator
PAI	plasminogen activator inhibitor
PDGF	platelet derived growth factor
PEA-3	polyomavirus enhancer A binding protein
PIN	prostatic interepithelial neoplasia
PKC	protein kinase C
Pre	pre-enzyme domain
Pro	pro-enzyme domain
pro	proline
RA	retinoic acid
RER	rough endoplasmic reticulium
RNA	ribonucleic acid
TGF-α	tumor growth factor alpha
TIMP	tissue inhibitor of matrix metalloproteinase
TNF-α	tumor necrosis factor alpha
TPA	12-o-teradecanoylphorbol-13-acetate
uPA	urokinase-type plasminogen activator
Xaa	any amino acid

CHAPTER 1

COLLAGEN CLASSIFICATION

ABSTRACT

Current research on collagens concentrates on the newly discovered types of collagen. Researchers scramble to uncover their molecular and supramolecular structure, metabolism, and biological functions. Several pathologies are associated with collagens in the extracellular matrix. Further elucidation of the structure and functions of the extracellular matrix will help to provide valuable tools for the management of many human diseases such as Alport's Syndrome (hereditary progressive glomerulopathy), Nail Pattella Syndrome (hereditary disease involving abnormal collagen structure in the basement membrane), Osteogenesis Imperfecta (brittle bone syndrome), atherosclerosis (hardening of the arteries), and cancer. This chapter examines collagen through the discussion of collagen structure, the classification of various types of collagen and the exploration of the biosynthesis of collagen. Subsequent chapters will focus on the regulation of collagen degradation in an effort to understand the common mechanisms involved in tissue remodeling, tissue destruction, and tissue invasion in a variety of normal and pathological conditions, especially cancer.

INTRODUCTION

Collagen is a protein that is found throughout the animal kingdom. It is the most abundant protein in humans and other mammals. The term collagen is derived from the Greek words "kolla" and "genos" which mean "glue" and "formation". The characteristic fibers of collagen are responsible for its functional integrity in tissues such as bone, skin and tendon. As an integral part of the extracellular matrix, collagen provides a structural framework in tissues. A great many human conditions, both normal and pathologic, involve the body's ability to repair and regenerate this collagenous framework.

The first studies in collagen research were performed by chemists, and involved hide tanning and gelatin production. Collagen was one of the first proteins to be investigated by x-ray diffraction by Wyckoff and Corey (1936). Modern research on collagen at the molecular level began in the 1950s. Highberger (1950) and Schmitt (1953) used the electron microscope to characterize the molecule. Ramachandran and Kartha (1954) and Rich and Crick (1961) developed models for the triple helical structure of the protein. Schmitt developed the model of quarter staggered arrays in the native fibril in 1956. The discovery of procollagen molecular forms, by Bellamy and Bornstein (1971) initiated the elucidation of the complex multistep biosynthesis of the collagen molecule. In 1971, Miller and Matukas, through the use of electron microscopy, identified three distinct but homologous types of collagen. The types of collagen identified were: type I collagen, the most abundant of collagens and originally thought to be the only type; type II collagen, shown to be the major protein associated with cartilage; and type III collagen, which is found in association with type I collagen in various ratios in most tissues throughout the body. These types I, II, and III collagens are considered to be the classical fibril forming collagens. In 1971, Kefalides (1973) discovered a specific type of collagen associated with basement membranes. This new type IV collagen was distinguished from the fibril forming classical collagens by its close association with basement membranes (Timpl 1981). Further identification of various types of collagen led to the need for better classification based on their structure and function.

This chapter begins its discussion of collagen with a detailed description of the collagen molecule. Structural information is important for the classification of the various collagen types. A classification scheme for the various collagen types, which includes functional roles and distribution, is presented. The complex biosynthesis of collagen is outlined to establish a general understanding of the genetics and biochemistry of collagens.

Once the structure, classification and biosynthesis of collagen have been described, the basic knowledge of collagen regulation and degradation of collagens in relation extracellular matrix turnover will be examined in subsequent chapters.

STRUCTURE OF THE COLLAGEN MOLECULE

The structure of collagen is critical to the function of collagen. The unique physical characteristics of collagen are conferred by this structure. Classification of the various collagen types is based on variations in this structure. This section provides a general overview of the structure of collagen, and discusses how structure is relevant to the function and classification of collagen.

Collagen triple helix

The ability to withstand proteolytic cleavage is one of the most important features of collagen. This feature is conferred by the collagen triple helix. Figure 1.1 represents the triple helical structure of the collagen molecule (Nimni 1988). All collagen molecules are characterized by this collagen triple helix, which is composed of three polypeptide α-chains. The three α-chains are coiled in a right hand direction to form a rope like molecule. The principle feature of the helical domains is the presence of a large number of glycine (gly) residues. Approximately every third amino acid in an α-chain is a glycine. This can be followed by any amino acid, but is often followed by proline (pro) and then by a hydroxyproline (hyp). The repeating sequence of gly-pro-hyp makes up about ten percent of the type I collagen molecule (Piez 1984). The presence of a glycine every third residue is absolutely critical to the formation of the triple helix. Glycine is the only amino acid which lacks a side chain. With the three-chain interaction, each chain alternates a glycine to form a shallow helix that runs down the middle of a super helical structure (Kucharz 1992). Side chains of other amino acids are pushed away from the center of the superhelical structure. The result of this three-chain interaction is the symmetrical

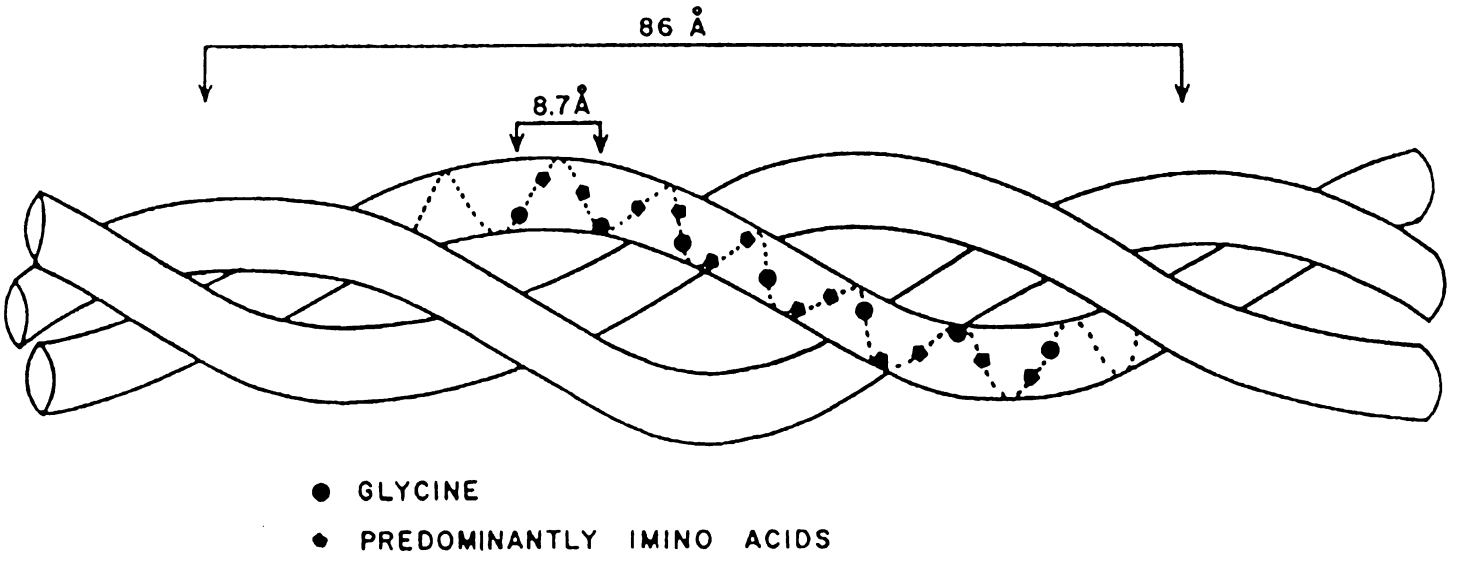

Figure 1.1. The triple helical structure of the collagen molecule. A glycine located every third residue in the primary structure, fold the molecule so that 8.6 Angstroms separate each glycine residue. The triple helical turn runs 86 Angstroms. (Nimni 1988).

pattern of a left-handed supercoil that possesses a pitch of approximately 8.6 nm (Figure 1.1). The approximate distance between amino acids is 0.291 nm, so each glycine residue is separated by an expansion of 0.873 nm. Individual residues are nearly fully extended in the collagen molecule (3.6 Angstroms) and this prevents interchain bonds from forming (Piez 1988). The triple helical structure and amino acid composition makes a collagen molecule resistant to proteolytic cleavage.

Nomenclature for α–chains

Collagen a-chains can have distinct primary sequences. Figure 1.2 shows the nomenclature scheme for collagen type 1. In this case the collagen type I triple helix is formed by two identical a-chains and one unique a-chain. The Arabic numeral 1 identifies the two identical a-chains, designated as a1(I) chain (Figure 1.2). The third chain is designated the a2(I) chain. The Roman numeral in parentheses indicate that these chains are encoded by genes associated with collagen type I.

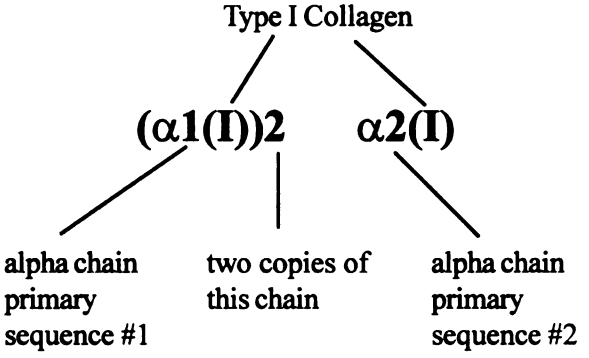

Figure 1.2. Nomenclature for α-Chains of Type I Collagen

Collagen non-helical domains

Although the collagen molecule is characterized by the α-chain triple helix, collagen can also contain non-helical domains. Variations in both α-chain primary structure and domain composition help to establish the various collagen types. Collagens can have long uninterrupted triple helical domains or the triple helix can be interrupted by any number of non-helical (globular) domains of various size. Figure 1.3 shows a schematic representation of different types of collagens and their various helical and globular domains (Nimni 1988). All of the collagen types have a collagen triple helix associated with their macromolecular structure. The structure of the collagen macromolecule is related to the function of collagen. In the classic fibril-forming collagens non-helical domains are positioned on either side of the collagen triple helix structure. In some types of collagens short non-helical portions interrupt the helical structure. Therefore, α-chain composition and domain structure used to classify various types of known collagen.

CLASSIFICATION OF COLLAGENS

Generally collagens are classified into two classes, fibril-forming and nonfibril-forming collagens. Table1.1 shows the fibril forming collagens and the non-fibril forming collagens. The fibril forming collagens have continuous or near continuous helical domains. This feature affects the way they aggregate into fibrils. The non-fibril forming collagens have helical domains that are separated by variously sized non-helical globular domains. These features allow these molecules to aggregate into non-fiber forming fibrous structures.

Currently there are 13 types of known collagen. Each newly purified collagen type is assigned a Roman numeral to designate its order of discovery. These collagens show distinct genetic as well as structural and functional differences. Table 1.2 shows some common types of collagen, identifies their α-chain composition and their distribution.

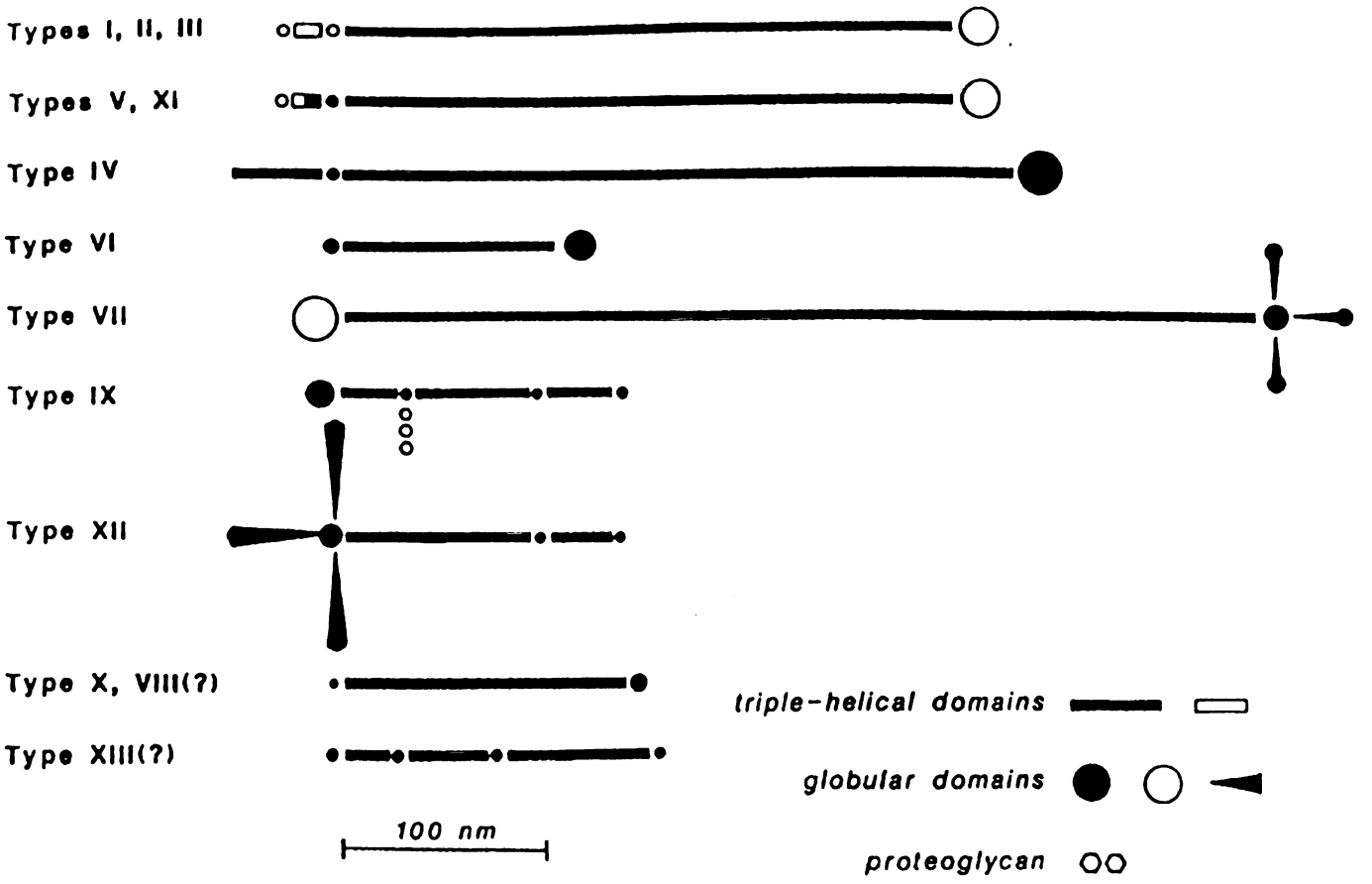

Figure 1.3. Collagens contain triple helical (solid and open rods) and globular domains (open and filled circles). Portions of the initially synthesized molecules are removed prior to their incorporation into insoluble matrices (open rods and circles) and the rest of the molecule remains intact in the matrix (closed rods and circles). The domains and their distributions are drawn approximately to scale (Nimni 1988).

Table 1.1. Fibril Forming Collagens and Non-fibril Forming Collagens

Fibril-Forming Collagens	Non-fibril-Forming Collagens
type I collagen	type IV collagen
type II collagen	type VI collagen
type III collagen	type VII collagen
type V collagen	type VIII collagen
type XI collagen	type X collagen
	type XIII collagen
	(Associated with fibril forming collagens)
	type IX collagens
	type XII collagens

Table 1.2. Chain composition and distribution for some human collagens

Collagen	Chain composition	Distribution
Type I	(α1(I))2, α2(I)	Skin, tendon, bone, cornea, fibrocartilage
Type II	(α1(II))3	Dentin, dermis, tendon, hyaline cartilage
Type III	(α1(III))3	Large vessels, uterine wall, dermis, intestine
Type IV	(α1(IV))2, α2 (IV)	Basement membranes

Each of these collagens shows distinct genetic structural and functional differences. As stated earlier, the three α-chains in a collagen molecule may have primary sequence variation among a type or genetic variation between types. These variations are indicated by Arabic numerals in the case of a primary sequence variation among a collagen type, and Roman numerals bounded by parenthesis in the case of genetic variation across collagen types. For example, type I collagen is made up of two α1(I) and one α2(I). The classification of this family of proteins segregates them into three classes based on general structural criteria. New collagens are being discovered and their classification is complex.

Fibril-forming collagens

This class of collagens includes some of the first discovered collagens. Types I, II, III, V, and XI collagens are in this class. These collagens are banded fibril forming collagens. All members of this class have lengthy, uninterrupted collagenous domains. As previously described, members of this group are evolutionarily related and show a common primordial gene unit. These collagens can be separated into two sub-categories, those that are found in cartilage tissue and those that are non-cartilaginous.

Collagen in cartilaginous tissues

Type II collagen is considered to be the major collagen associated with cartilage but its distribution is not limited to cartilage. Type II collagen molecules are made up of three identical alpha chains. These chains are similar to the α1 chains of type I collagen, showing similar electrophoretic characteristics. The cartilage collagen chains are designated α1(II), where the II is indicative of type II collagen. The most significant characteristic of cartilage collagen is its high hydroxylysine content and its glycosidically bound carbohydrates. Collagens from cartilage can contain up to five times more carbohydrates than the homologous collagen from the skin. This modification is thought

to limit fibril growth of the collagen aggregates (Huerre 1986). Type II collagen is synthesized during the chondrogenic stages of development from the mesoderm. In the process of amphibian limb regeneration, the tissues undergo three distinct stages of development during the transition from mesenchyme to cartilage to bone. A similar pattern is also seen in chondrogenic activity (Mayne 1990).

Type XI collagen is also found in cartilage and is characterized by three separate distinct α-chains that migrate slightly slower than α1(I) chains on SDS-PAGE. Suspected functions of type XI collagen include regulation of the diameter or growth of type II collagen fibrils. There is evidence to suggest that the banded cartilage collagen fibers are polymers of type XI and type II, where type XI is more concentrated at the fibril interior than at the surface. This layout indicates that type XI collagen is expressed early in the formation of cartilage (Mayne 1993).

Collagens in non-catrilaginous tissues

Type I and II collagens are the most common collagens found in non-cartilaginous tissues. Type I collagen was the first mammalian collagen discovered. It is composed of three chains, two identical α1(I) and one α2(I). Type I is most abundant in skin, tendon, ligament, bone, and cornea, where it comprises between 80 and 90% of the total collagen. Type I collagen is also the primary collagen associated with the bone matrix (Nimni 1988). The proportion of collagen in a particular tissue can vary at different sites during development, aging and pathology. Decreased levels of type I and type IV collagen has been seen in keratoconus cornea, a condition where the cornea undergoes abnormal growth and development that leads to impaired vision (Ihalainen 1986). In this disease, the outer lens of the eye, or cornea is malformed due to the thinning of the corneal periphery. The resulting cone shaped cornea can cause severe refractive errors. This disease is most likely the result of decreased collagen content or impaired collagen maturation.

Mutations in the α1(I) and α2(I) genes identify the importance of the normal type I collagen. The effect of insertions and deletions usually result in phenotypic changes in bone development characteristics, for example, in osteogenesis imperfecta (OI). In general, the gene defects involving OI lowers the amount of type I collagen associated with the bone matrix. This may be the result of intracellular degradation of defective collagen molecules or a failure of defective molecules to become normally incorporated into growing fibers (Kucharz 1992).

The molecule that is formed by three α1(I) chains is called the type I trimer collagen. It is stable and occurs in small quantities in various tissues. The type I trimer collagen is relatively resistant to fibroblast and neutrophil collagenases. The biological role of type I trimer has yet to be established. This collagen is synthesized under pathological conditions. Synthesis of the type I trimer is an early event in the de-differentiation of chondrocytes in culture. This may occur as a result of the tissue culture environment. The potential of observing type I trimer synthesis as a measure of abnormalities in collagen biosynthetic pathway maybe of significant interest (Mayne 1990).

Distribution of Type III collagen is normally associated with the skin where it accounts for 10-20% of the total collagen. It is also present in varying amounts in association with type I collagen in lung, heart muscle, heart valves, nerves, blood vessels, spleen, kidney, lymph nodes, bone and eye. Blood vessels are particularly rich in type III collagen, where 20-30% of the total collagen in the human aorta seems to be type III. The amount of collagen type III, in relation to type I, correlates with the extensibility of the tissue. This generalization has been seen in studies examining changes in the human aorta during atherosclerosis, where type III collagen shows a marked increase with atherosclerosis (Murata 1986). Type III collagen is composed of three identical chains. The pepsin resistant portion of α1(III) is similar in size to the α1(I) and α2(II) chains and show similar migration on SDS-PAGE (Burgeson 1992). Type III collagen shows a

relatively high degree of hydroxylation of proline and a high glycine content (more than 33% of the residues). The most significant characteristic of type III collagen is the presence of intermolecular disulfide bonds involving two cysteine residues close to the C-terminal region of the triple helix. These intermolecular disulfide bridge formations allow collagen type III to rapidly cross-link (Burgeson 1992). Such a rapid cross linking would be of great advantage during early development and wound healing, where collagen is deposited at a rapid rate.

Ehlers-Danlos type IV syndrome is associated with a gene mutation in the collagen type III gene. This result in decreased flexibility and increased fragility of the skin and micro-traumas result in wounds and atrophic scars. Patients with this disorder are subject to internal organ involvement including mitral valve regurgitation, vessel ruptures, gastrointestinal abnormalities, and ocular changes. (Kucharz 1992)

Types IX and XII collagens do not form fibril on their own but they are associated with fibril–forming collagens. These types do not appear to form supramolecular aggregates alone, however are often seen to participate in the formation of fibrils in conjunction with fibril forming collagens.

In cartilage, type IX molecules are localized in a periodic manner along cartilage collagen fibrils and are in fact cross-linked to collagen type II molecules within such fibrils. Type IX collagen is assembled by three α-chains, α1(IX) α2(IX) and α3(IX). Studies have indicated an inhibitory effect on lateral growth of type II collagen in cartilage, by type IX collagen (Shimokomaki 1990).

Non-fibril forming collagens

While half of the known collagens forms banded fibers or are associated with banded fibers, other collagens form structurally and functionally distinct supramolecular assemblies. The collagens that comprise class 3 have very different structure and function from the other two classes and from each other.

Type IV collagen is the major component of basement membranes. Basement Membranes are specialized structures that underlie epithelia and endothelia and perform many structural and functional roles, which include cell differentiation and orientation, cell polarization, selective permeability to macromolecules and barriers to cell movement. Other molecules that form the basement membrane include glycoproteins such as laminin, entactin/nidogen, proteoglycans, and other less well defined structures. Type IV collagen has two α1(IV) and one α2(IV) chains. Recent evidence suggests that type IV collagen forms homotrimers of either the α1(IV) or the α2(IV) chain (Oikawa 1989).

Type IV collagen does not form well-ordered microfibrils or "Segment Long Spacing (SLS) Crystals" as do the classic collagens. The characterization of their supermolecular structure remains elusive, partly due to the amorphous structure of basement membranes (Glanville 1987). The type IV collagen molecule is characterized by a long helical domain with several short interrupts. The amino-terminus has a short helical (7S) domain separated from a long triple helical domain (TH) by a small globular domain, and the carboxy-terminus possesses a globular non-collagenous (NC) domain known as NC1 (Figure 1.4) (Kucharz 1992). Two models of the supermolecular structure of type IV have been suggested and are diagrammed in Figure 1.5. These models are consistent with x-ray crystallography structural observations of type IV collagen (Glanville 1987). Two super molecular structures have been proposed for type IV collagen, the network model is dependent on the formation of tetramers at the 7S domain, before dimer formation at the NC1 domain. Subsequent NC1 binding results in a meshwork formation. The hexagonal model is dependent of the formation of the NC1 dimer before the 7S trimer, subsequent 7S binding results in the hexagonal meshwork formation (Kucharz 1992).

Genetic mutations affecting type IV collagen often result in glomerulonephropathy. For example in Alport's Syndrome, there is a defect in type IV collagen, the result of this defect is a systemic thickening of the basement membrane. Most of the research has

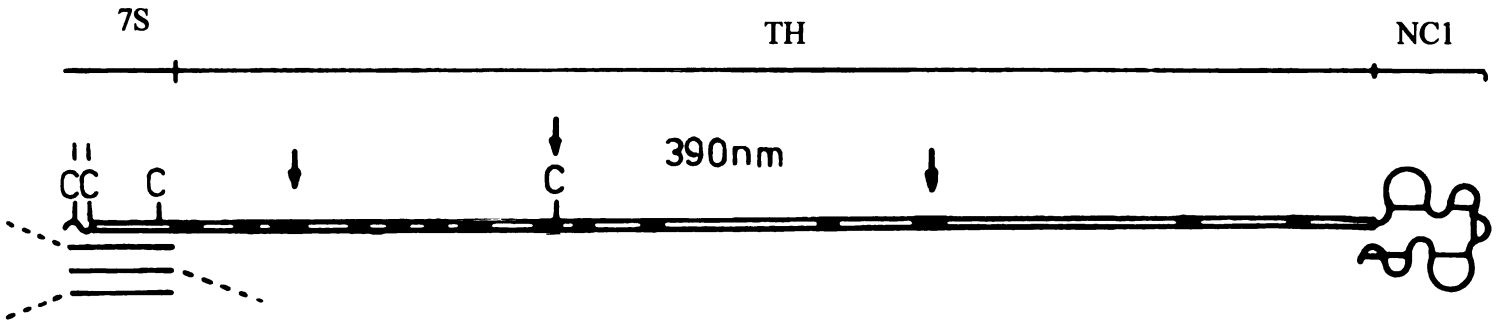

Figure 1.4. Type IV collagen schematic diagram showing the short helical domain (7S) in the C-terminus, the long triple helical domain (TH) and the N-terminus globular non-collagenous (NC1) domain (Kucharz 1992).

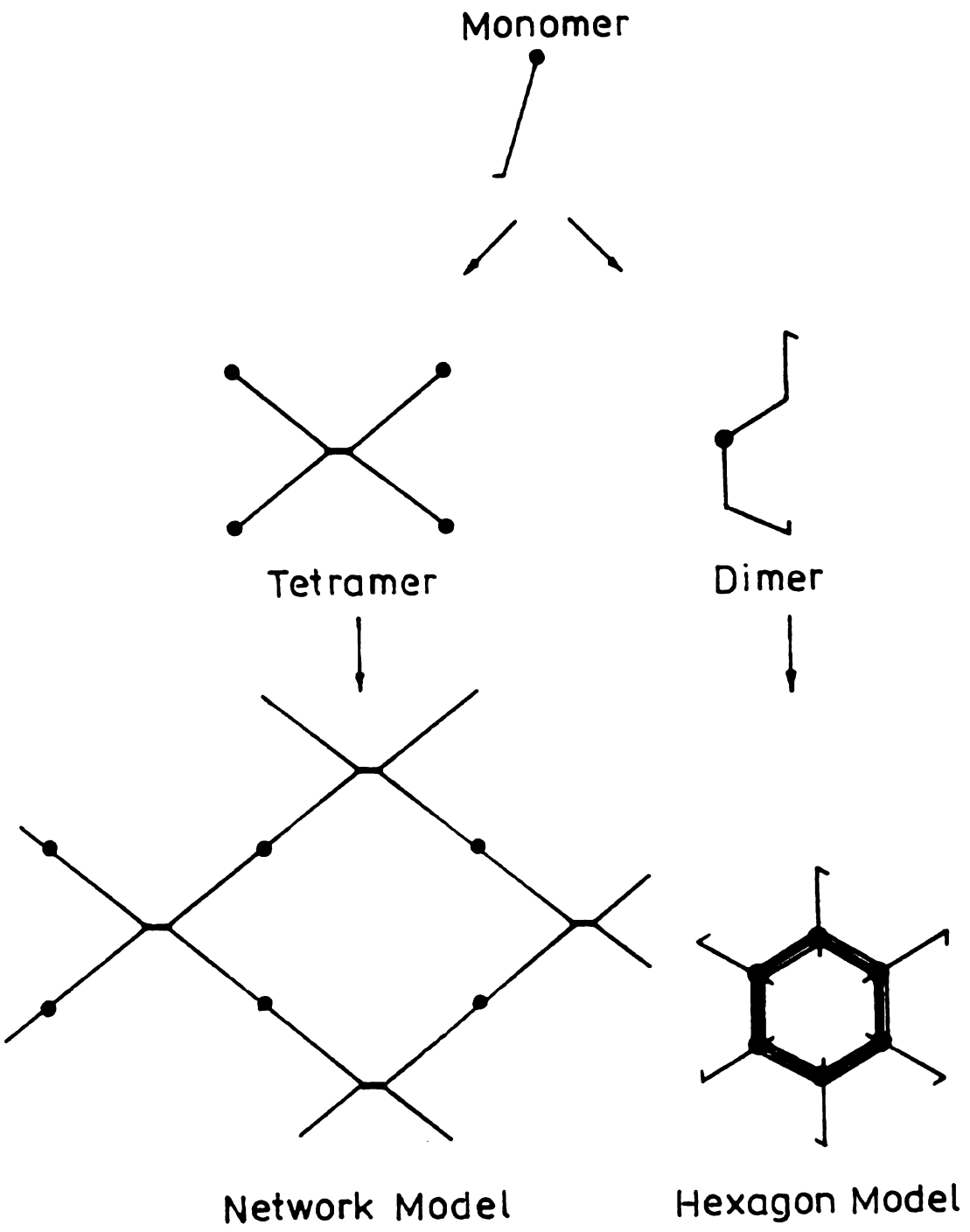

Figure 1.5. Proposed models for type IV collagen super structure (Glanville 1987).

focused on the basement membrane of the glomerulus. Patients with Alport's Syndrome often have thick basement membranes associated with the glomerulus of the nephron. This thickening is due to a genetic abnormality in type IV collagen, which result in a malfunction of the filtering activity of the glomerulus. In many cases this will lead to renal failure in patients with Alport's Syndrome.

In Nail-patella Syndrome there is an abnormal processing of type IV collagen that results in systemic pathology. Skeletal and ocular abnormalities as well as renal involvement characterize this syndrome. Banded collagen fibril formation is seen in the basement membrane of the glomerulus (Kucharz 1992). Considering its multiple functions, the malformation of basement membrane has broad reaching implications.

COLLAGEN BIOSYNTHESIS

Collagen Biosynthesis is a complex multistep process. This process was initially identified with the discovery of the procollagen molecule. The unique structures of collagens in the extracellular matrix are the result of a number of co-translational and post-translational modifications of the pre-procollagen molecule. Co-translation refers to the hydroxylation of an amino acid after a peptide bond is formed but before the protein is completely formed. Some of these events occur within collagen-producing cells, and are unique to collagen. Other modifications involved in collagen synthesis are common and are also seen in other examples of secretory protein biosynthesis. The following discussion considers the biosynthesis of fibril-forming collagens. Other genetically distinct collagen types employ slightly different mechanisms of biosynthesis.

The procollagen model

The formation of the intricate network of collagen fibers requires the synthesis of a precursor. This precursor is known as procollagen and the molecule that immediately precedes this molecule is known as pre-procollagen (Figure 1.6). Once the procollagen

Pre-procollagen

↓

Procollagen

↓

Collagen

Figure 1.6. The collagen synthesis pathway.

molecule is formed, this molecule can be enzymatically trimmed of its non-helical ends giving rise to a collagen molecule. The newly processed collagen molecule then spontaneously assembles into its native fiber in the extracellular space. Procollagen molecules have been identified for the three interstitial collagens (types I, II, and III) and many of the amino terminus (N-terminal) and the carboxyl terminus (C-terminal) peptides (propolypeptides) have been characterized and their primary sequences determined.

A schematic representation of the type I procollagen molecule is presented in Figure 1.7. Its synthesis is required for the proper formation of the intricate network of collagen fibers found in the extracellular matrix (ECM). Procollagen ensures delivery of the collagen molecule to the site of assembly, by preventing its premature assembly into fibers in improper locations. The non-helical terminal extensions of type I procollagen is shown in Figure 1.7. These extensions are later enzymatically cleaved, giving rise to a collagen molecule that can spontaneously assemble into fibers in the ECM. Type I collagen has (α1 (I))2 α2 (I) chain arrangement. Procollagen is formed by the precursors pro α1 (I) and pro α2 (I). Both of these propeptide chains have non-helical (globular) terminal ends. These extensions play a key role in the assembly of the triple helical procollagen molecule.

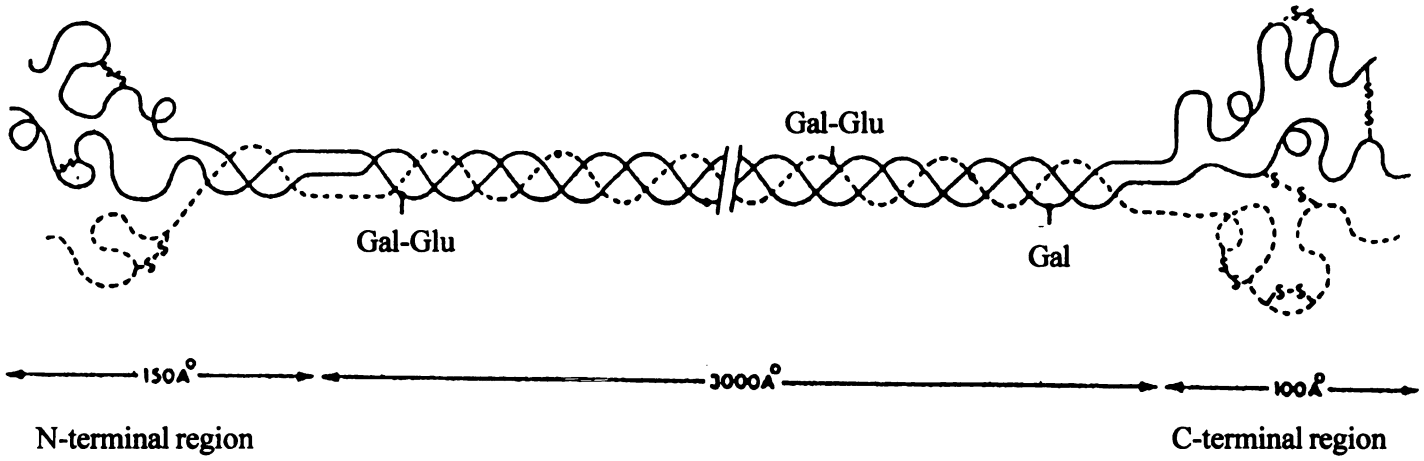

Figure 1.7. A schematic representation of type I procollagen, showing the "pro" segment, the helical domain, and the non-helical globular carboxy group (Burgeson 1992).

The C-terminal ends of procollagen propeptides of both the pro α-1 and pro α-2 chains have non-triple helical globular regions associated with them. These globular peptides have asparagine-linked oligosaccharide units composed of N-acetylglucosamine and mannose. This carbohydrate side chain is attached near an asparagine-Xaa-threonine (Asn-Xaa-Thr) sequence (where Xaa is any amino acid). This Asn-Xaa-Thr sequence is compatible with the structural requirement for glycosylation of asparaginyl residues by oligosaccharide transferases. The functional importance of this carbohydrate at the C-terminal is unknown, but it may be a recognition mechanism for alignment, secretion or assembly into microfibrils (Burgeson 1992). The central segment of this molecule has an α–helical arrangement and globular regions at both ends. The globular extensions in procollagen molecules play a key role in the assembly of the triple helical collagen molecule. Once the procollagen molecule assembly is completed and it is translocated to the cell surface, the extensions are enzymatically removed from some collagens by procollagen peptidases and then the fibrillogenesis begins. The procollagen peptidases are enzymes involved in the selective cleaving of these extensions. Subsequent spontaneous fibrillogenesis has been observed in many connective tissues and in culture media derived from collagen-secreting cells (Matuk 1980).

Events leading to the formation of the procollagen molecule and its subsequent processing can be divided into two categories, intracellular events, and extracellular events. Intracellular events are those processes that lead to the formation of the procollagen molecule before its secretion into the extra cellular space. Procollagen molecular processing continues after its release into the extracellular space and these modifications are referred to as extracellular events. This next section discusses the processing of the procollagen molecule and separates them into intercellular events and extracellular events.

Intracellular events

Collagen Genes

Significant progress has been made in the elucidation of the genetic heterogeneity involved in the formation of the procollagen molecule in recent years. The heterogeneity and the intron-exon arrangement of the gene family has been found to be more complex than expected for a molecule as uniform and regular as collagen. Collagen genes are identified by the letters COL with numbers following separated by the letter "A", which refers to the α chain. The first number codes for the type of collagen and the second number codes for the number of the chain. For example, COL3A1 represents the α1 chain of type III collagen.

The human collagen genes that code for the classic collagens have been characterized through the use of cloned DNA probes. The genes have been mapped to specific chromosome and these locations are summarized in table 1.3. These studies localized the gene COL1A1 to chromosome 17 (Huerre-Jeanpierre 1986). The other α-chain associated with the type I collagen, the COL1A2 gene, was found on chromosome 7 (Henderson 1983). COL2A1 codes for type II collagen the major protein associated with cartilage and is found on chromosome 12 (Huerre-Jeanpierre 1986). The gene for Type III collagen resides on chromosome 2 (Kucharz 1992). COL4A1 codes for the α1(IV) chain in type IV collagen, the basement membrane collagen and the gene has been located on chromosome 13 (Emanuel 1986). The localization of these collagen genes is useful when considering biosynthesis, expression and regulation of the various types of collagens.

The size of collagen genes varies from 18,000 base pairs in the COL1A1 gene of humans to 38,000 base pairs of the COL1A2 in the chicken (Kucharz 1992). The gene structure consists of several exons separated by the non-transcribed introns. The chicken COL1A2 gene contains at least 52 exons. These exons are 54 base pairs (bp) in length

and are separated by large introns that range 80- 2,000 bp. The conservation of base pairs suggests these exons developed from a primordial gene unit (Burgeson 1992). A possible model for the assembly of the ancestral collagen gene from a primordial gene unit in which the 54 bp exon codes for 18 amino acids, the minimum number required for the formation of a stable triple helical structure, is provided in Figure 1.8 (Kucharz 1992). A schematic representation of the human COL1A1 gene is displayed Figure 1.9 (Kuhn 1986). This gene is an example of the conservation of a primordial gene unit. Twenty-one exons are 54 bp long copies of the primordial gene unit. Nine exons are 108 bp and appear to be a pair of primordial gene units adjoined side by side. Five of the exons are modified primordial gene units, they show a deletion of 9 bp and have a length of 45 bp, and the last five exons are duplicates of the modified primordial gene unit and have 90 bp. The 9 deleted base pairs in the modified primordial gene unit correspond to one triplicate unit Gly-pro-hyp (Kucharz 1992). The related size of each of these exons in both the chicken and human suggests that these genes developed from a basic primordial gene unit (Kucharz 1992).

Regulation of the expression of the collagen gene is controlled at both the developmental and tissue-specific levels. Regulation of these genes continues to be elucidated. The promoter for the COL1A1 has been characterized (Brenner 1989) and evidence suggests that angiotensin-converting enzyme (ACE), angiotensin II and bradykinin are associated with the expression of the type I collagens (Weber 1995). Further studies in this area will help to provide a greater understanding of normal and pathological human conditions, such as, development, tissue fibrosis and wound healing.

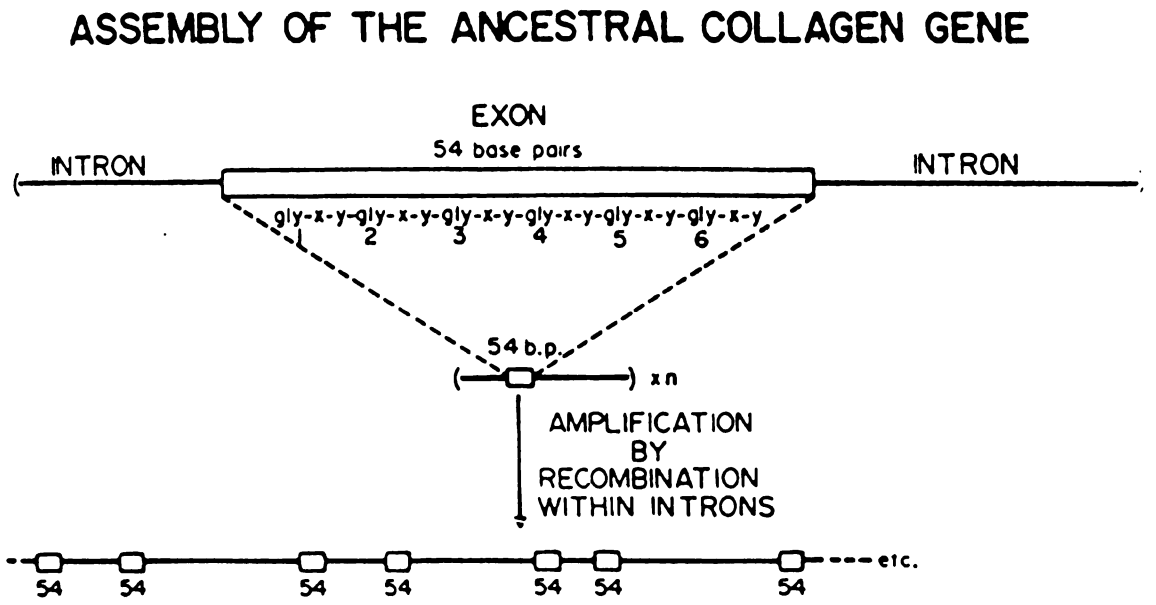

Figure 1.8. Diagram of ancestral Collagen Gene and a possible mechanism of collagen evolution (Kucharz 1992).

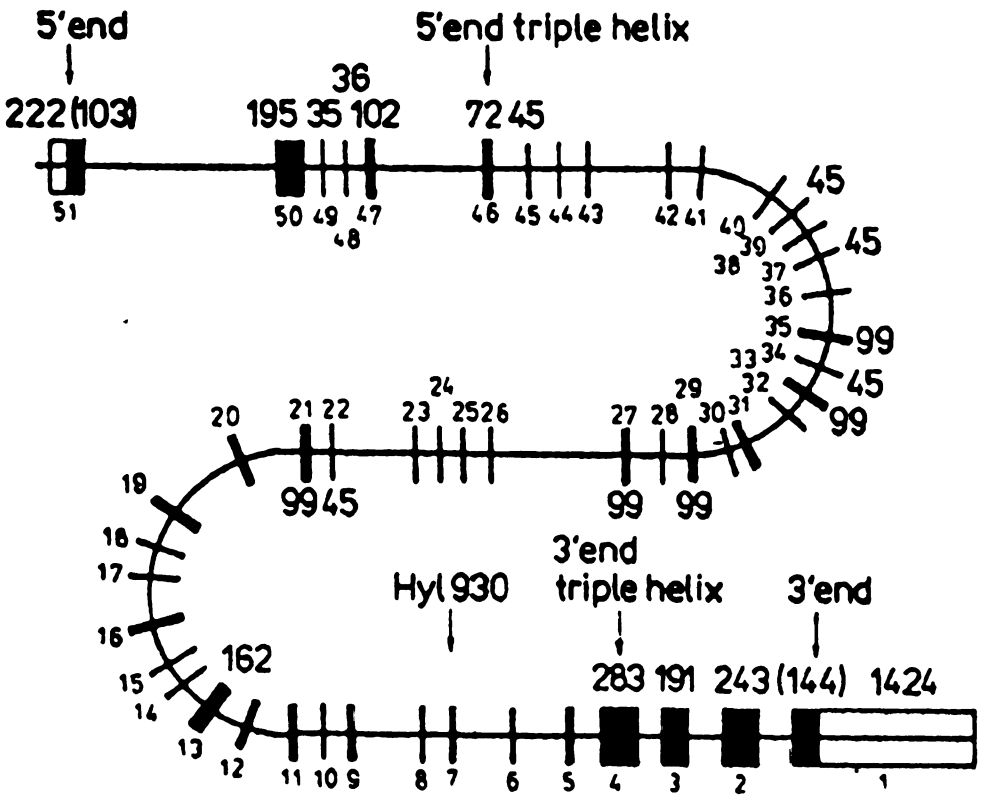

Figure 1.9. Schematic diagram of the human collagen type I alpha 1 chain (Kuhn 1986).

Translational, co-translational, and early post-translational events

The intracellular sequence of events leading to the formation of the procollagen molecule are shown in Figure 1.10 (Nimni 1988). Steps 1-8 show the formation of the procollagen molecule in the rough endoplasmic reticulum (RER). This begins once a gene that codes for a specific collagen type is transcribed to yield a functional mRNA. A specific mRNA is translocated to the RER and is translated by membrane bound polysomes (Figure 1.10, step 1-3).

Important co-translational modification accompanies translation of the mRNA (Figure 1.10, steps 3-4). The hydroxylation and enzymes involved in these steps are shown in table 1.3.

It is a well known observation that neither hydroxyproline nor hydroxylysine can be incorporated into a peptide before peptide bond formation. Therefore, hydroxylation of proline and lysine must occur subsequent to peptide bond formation. After the peptide bond is established, hydroxylation can occur and is mediated by two enzymes, lysyl hydroxylase and proyl hydroxylase in the presence of iron and ascorbate. The hydroxylation of proline at both the 3rd and 4th carbon positions is shown in Figure 1.11. These enzymes are highly specific and act only on chains in the non-helical conformation. This provides a reason for the time lapse between peptide synthesis and protein folding. This time varies considerably across various types of collagen. These differences in

Table 1.3. Hydroxylation Enzymes Involved in Collagen Biosynthesis (Nimni 1988)

Amino Acid	Hydroxylase	Symbol for Figure 10
proline	3-proline hydroxylase	(•)
proline	4-proline hydroxylase	(Δ)
lysine	lysyl hydroxylase	(o)

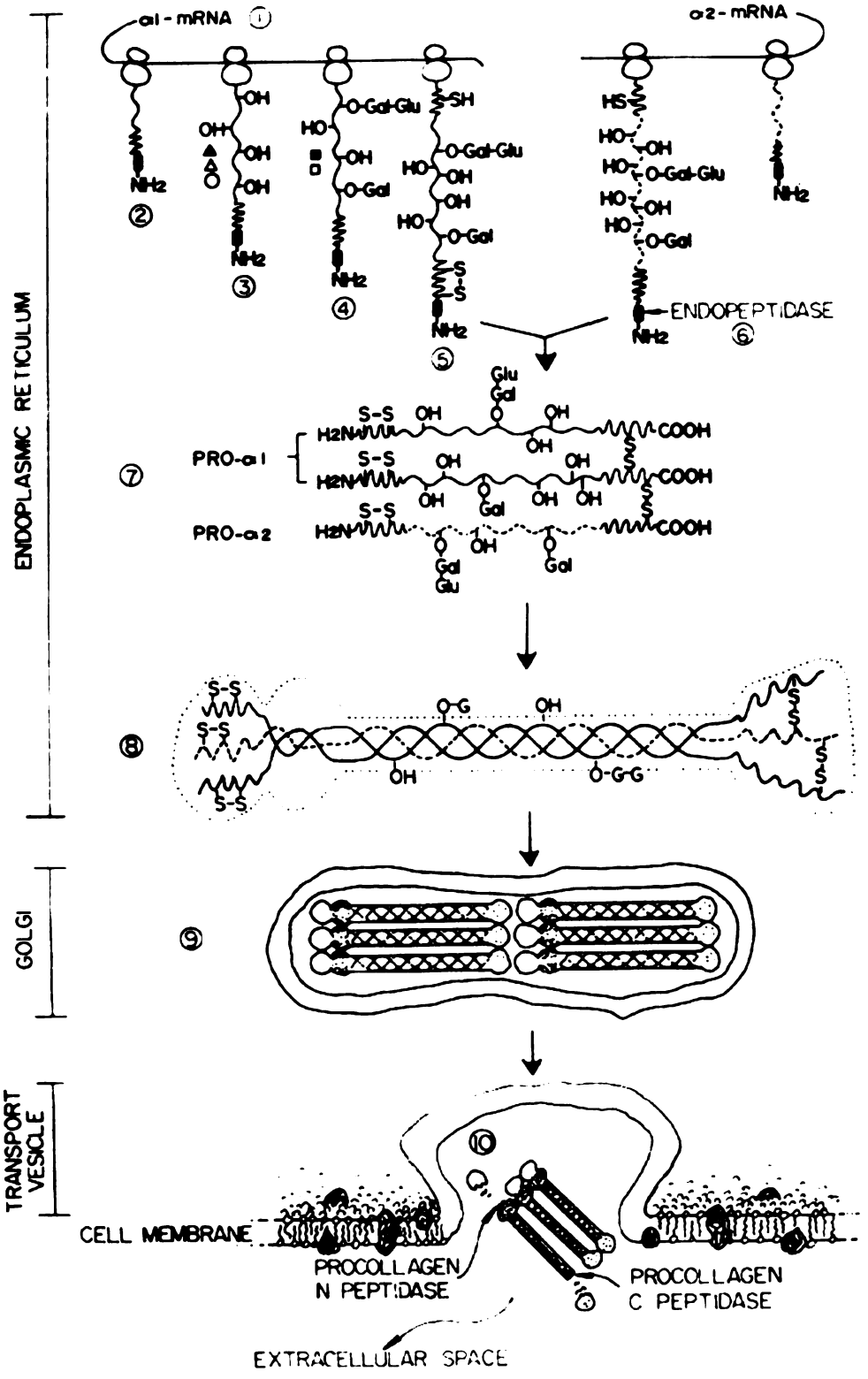

Figure 1.10. The intracellular sequence of events leading to the formation of the procollagen molecule (Nimni 1988).

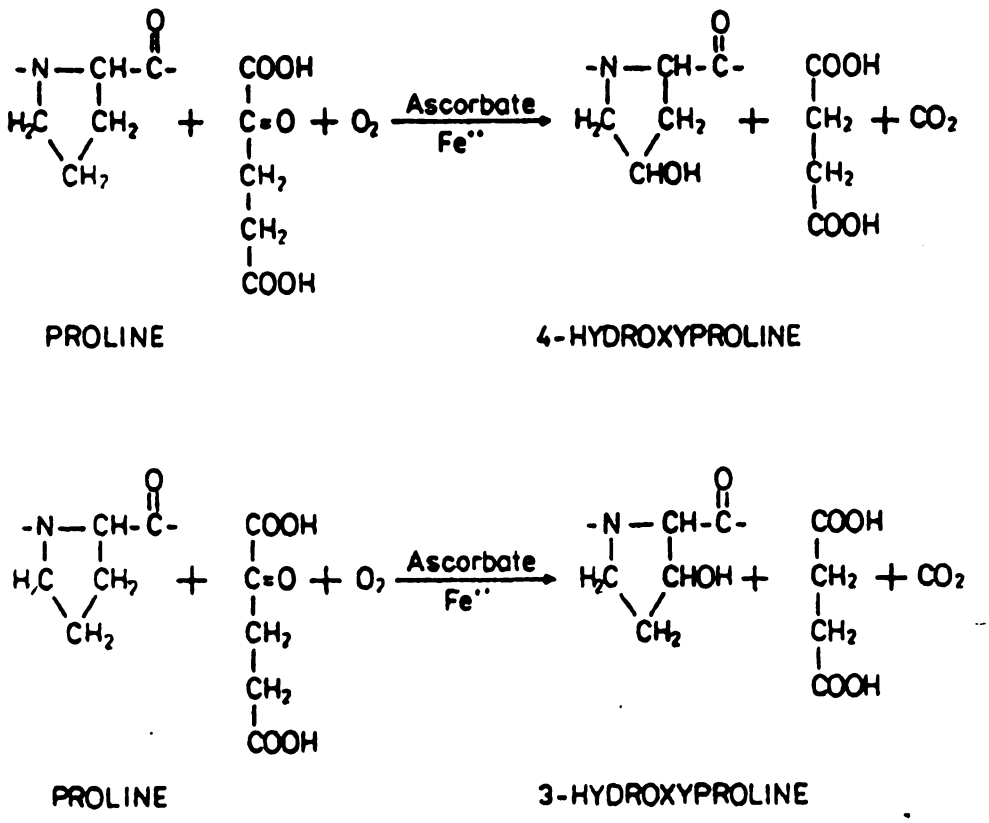

Figure 1.11. The hydroxylation of proline in collagen protein (Kucharz 1992).

time, coupled with the mechanism of hydroxylation and glycosylation, provide insight into the increased levels of hydroxylation and glycoslyation seen in the basement membrane collagen. They also may be valuable in the elucidation of collagen pathological states.

Glycoslyation is shown in Figure 1.10, step 4 and 5. Once the pro α-chain of the procollagen molecule is synthesized, sugar residues are post-translationally added to hydroxylysine. Galactotransferase and glucosyltransferase mediate this glycosylation. The function of the glycosylation of hydroxylysine is unclear. Studies suggest that this modification helps to regulate the fibril diameter. Observations on type IV collagen glycosylation of hydroxylysine indicate that this modification limits the diameter of the aggregate (Timpl 1981).

The next post-translational event is the removal of the amino terminal signal peptide (Figure 1.10, step 6) by endopeptidase. This type of cleavage is typical for proteins secreted as proenzymes. The function of the signal peptide and the enzyme responsible for its removal remains unclear. It has been suggested that the role of the signal peptide is to aid in the transfer of the pre-procollagen polypeptide chain across the RER membrane. It is generally accepted that a "signal peptidase" cleaves this extension in a nonspecific manner.

Once the amino-terminal signal peptide is removed, the completed α-chains are released from the ribosomes, and the α-chains begin to interact. Three α-chains associate through recognition of the carboxy-terminal non-helical domains and begin to form disulfide cross-links (Mechling 1996) (Figure 1.10, step 7). This initiates the formation of the triple helical supercoil.

With the linkage of the α-helices through disulfide bonds, the procollagen molecule can fold and form the triple helical supercoil (Figure 1.10, step 8). Then the procollagen is ready to be secreted into the extracellular space. Procollagen is translocated to the Golgi apparatus and packaged into segment long spacing (SLS) crystals (Figure 1.10,

step 9) and then placed into secretory vesicles. Fusion of secretory vesicles with the cell membrane and extrusion of the molecule are the last intracellular stages of collagen biosynthesis. This is accompanied by the removal of the carboxy-terminal non-helical extensions and part of the amino-terminal non-helical extensions by specific peptidases (Nimi 1988).

Extracellular events

Microfibrils form quarter staggered arrays

Microfibrils are formed in the process of extrusion. This is the result of a cross-linking catalyzed by the enzyme lysyl oxidase. The microfibril is made up of five strands arranged in a quarter staggered array (Figure 1.12)(Burgeson 1992)(Matuk 1980).

Aggregation

The extracellular aggregation of the collagen molecule and formation of the native fibril is summarized in Figure 1.12 (Burgeson 1992). Fibrillogenesis is a result of the quarter staggered arrays undergoing further aggregation. Microfibrils undergo lateral aggregation followed by end-to-end aggregation.

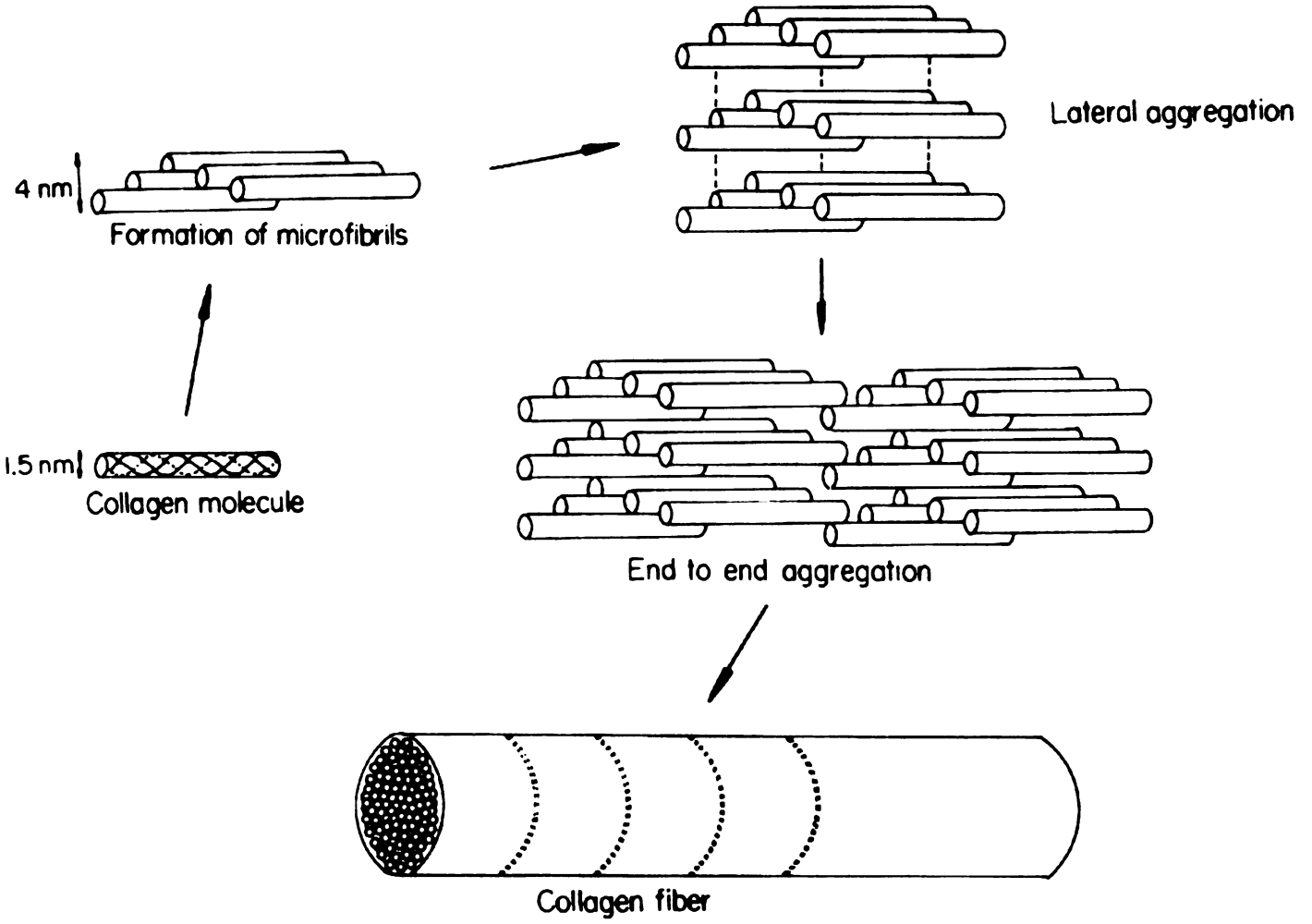

Figure 1.12. The extracellular aggregation of the collagen molecule and formation of the native fibril (Burgeson 1992).

CONCLUSIONS

It is important to examine collagens in order to understand the role of collagenases as proteolytic regulators of extracellular matrix turnover. Only with a basic understanding of the collagen substrate can the activities and the mechanisms of their specific proteolytic enzymes be truly understood. This chapter provided a basic understanding of collagen as a molecule that plays an integral role in the extracellular matrix. Tissue remodeling involves a complex interplay of collagens, matrix metalloproteinases and their inhibitors. Therefore, in subsequent chapters the matrix metalloproteinases (MMP) and their inhibitors (TIMP) will be discussed. The common mechanisms involving MMPs and TIMPs seen in many normal and pathological conditions will also be examined.

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CHAPTER 2

MATRIX METALLOPROTEINASES (MMPs): CLASSIFCATION AND BIOCHEMICAL PROPERTIES

ABSTRACT

Matrix metalloproteinases are a family of zinc and calcium-dependent proteolytic enzymes that have the ability to degrade components of the extracellular matrix (ECM) at physiological pH. The turnover of ECM contributes to the initial steps of tissue remodeling during morphogenesis, wound healing, angiogenesis, and tumor invasion. Degradation of the basement membrane type IV collagen by matrix metalloproteinases (MMPs) occurs in many normal processes involving tissue remodeling, such as, wound healing and tissue involution and tissue invasion during angiogenesis and inflammation. In addition, MMP's have been associated with several pathological conditions involving tissue destruction as in rheumatoid arthritis, cancer invasion and metastasis. At present there are 14 known members of the MMP family. These proteins are loosely classified on the basis of their substrate specificity. In this chapter a classification of the members of the matrix degrading metalloproteinases will be presented along with their substrate specificity, domain structure, and compositional analysis.

INTRODUCTION

Although several other proteases have the potential to degrade components of the extracellular matrix, matrix metalloproteinases (MMPs) appear to have the greatest physiological significance in matrix turnover. Matrix metalloproteinases are a family of secreted and membrane bound proteins that are capable of degrading components of the extracellular matrix under normal physiological conditions. Table 2.1 lists the human MMPs and shows some of their biochemical properties (Murphy 1995). MMPs require zinc and calcium for their activity. Their major proteolytic activity is directed toward important components of the extracellular matrix, specifically collagen.

Table 2.1. Human matrix metalloproteinases (Murphy 1995)

Enzyme	MMP No.	Mol.Wt.(Da)	Substrates
Collagenase	MMP-1 MMP-8 MMP-13	55,000 75,000 65,000	fibrillar collagens types I, II, & III, types VIII, & X collagens, proteoglycans, and gelatin
Gelatinase A	MMP-2	72,000	gelatins, Non-helical regions of fibrillar collagens
Gelatinase B	MMP-9	92,000	specific locus type IV collagen Types V, VII, XI collagens, elastin
Stromelysin 1	MMP-3	57,000	proteoglycan core protein. Non-helical regions type IV collagen
Stromelysin 2	MMP-10	57,000	procollagens I, II, & III, collagenase, gelatinase B
Matrilysin	MMP-7	28,000	strong stromelysin-like activity, elastin
Stromelysin 3	MMP-11	51,000	weak stromelysin-like activity
Metalloelastase	MMP-12	54,000	similar to matrilysin, elastin
Membrane-Type	MMP-14	63,000	progelatinase A

The first animal collagenases were described as enzymes responsible for the dissolution of the tadpole tail (Gross 1962). Although the existence of bacterial collagenase had long since been recognized, prior to 1962, there was little or no evidence for an animal collagenase. However, It was well accepted that the rapid turnover of collagen would be essential for normal development and tissue remodeling. Gross and Lapiere (Gross 1962) developed an assay for the detection of collagenolytic activity in living tadpole explants. It was suspected that during the metamorphosis from a tadpole to a frog, degradation of the ECM, involving collagenase, would occur particularly in dissolution of the tadpole tail. In these studies fresh tissue explants from tadpole tails were placed on culture dishes filled with a collagen gel. The gel was synthesized using a C^{14}-labeled proline. Figure 2.1 shows the dissolution of the collagen gel in the area of the tissue explant (Gross 1962). Quantification of free labeled proline confirmed collagenolytic activity. The protease in these experiments was subsequently identified as interstitial collagenase and is considered to be the prototypic member of the family of matrix metalloproteinases. The main criterion, by which a protease was identified as a "collagenase", was its ability to cleave a peptide bond in the triple helical region of the native collagen molecule. The ability of collagen to withstand degradation by proteolytic cleavage provided a clean definition for a collagenase. This basic definition of collagenases has been modified since the discovery of additional family members of MMPs.

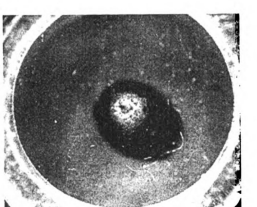

Figure 2.1 Tadpole tissue explant digesting collagen. The dark area around the tissue explant is the area of collagen lysis (Gross 1962).

Some collagen types have non-helical interruptions in collagen helical domain and these non-helical sections are susceptible to proteolytic degradation. Proteins that cleave these domains may be collagenolytic but would not be considered "collagenases" by the traditional definition as described above. With the discovery of more genetically distinct collagens and the importance of collagenolytic enzymes in normal and pathological conditions, a broader classification scheme must be applied when considering collagenolytic proteins. The modern classification scheme is based on substrate specificity and domain structure. In this scheme, the "collagenases" are defined as matrix metalloproteinases, which means that their catalytic activity is associated with the presence of calcium and zinc ions. Several other metalloproteinases have been associated with ECM degradation and matrix turnover. Many of these, although not considered to be "collagenases", have important collagenolytic function. Therefore, they merit consideration in the examination of ECM turnover.

With the discovery of animal matrix metalloproteinases, studies began to focus on these proteins and their association with various tissue remodeling events characterized by a dramatic extracellular matrix turnover. These include conditions such as endometrial cycling (Irwin 1996, Thompson 1994), mammary involution (Li 1997, Roswit 1988, 1992) wound healing (Okada 1997 Welgus 1980, 1983,), arthritis and cancer (Nakagawa 1994, Ries 1995).

Matrix metalloproteinases are widely distributed in mammals. They have been identified in tissues as diverse as skin, liver, uterus, and lung. They are typically present at basal levels throughout the body. Agents such as interleukin-1 (IL-1), tumor necrosis factor (TNF) and transforming growth factor-α (TGF-α) induce increased expression of MMPs. (Birkedal-Hansen 1993). Transforming growth factor β (TGF-β) and interleukin-4 (IL-4) have inhibitory effects on MMP expression (Stetler-Stevenson 1993).

Matrix metalloproteinases are secreted proteins and are usually present in a latent or inactive form (Willenbrock 1993). Initially, the nature of this latent form instigated controversy. Some investigators claimed that these enzymes were secreted as an enzyme-inhibitor complex,

while others suggested that the enzymes were secreted as a proenzyme. While there is evidence for the existence of MMP-inhibitor complexes (Goldberg 1992), several studies suggest that MMPs are secreted as proenzymes (Murphy 1995).

Matrix metalloproteinases are produced as proenzymes and require proteolytic cleavage for activation. Several enzymes such as trypsin, plasmin, plasminogen activator, cathepsin and kallikrein are capable of this cleavage. Tissue inhibitors of metalloproteinases (TIMPs) are associated with modulation of MMP activity. TIMPs inhibit MMPs by forming a 1:1 complex. TIMPs are further discussed in chapter 4. MMPs have the ability to autoactivate. Autoactivation of MMPs can result in accelerated collagenolysis (Jeffery 1985). In addition, several MMPs are capable of activating other MMPs via proteolytic cleavage (Matrisian 1992).

Although many proteases are capable of ECM turnover and basement membrane degradation, MMPs are believed to be the normal and physiologically relevant mediators of Matrix degradation. There are several reasons for this argument. First, MMPs are secreted into the ECM and are, therefore, in the proper location for degradation of ECM. Second, MMPs are active at neutral pH as would be required under normal physiologic conditions. Third, there are multiple levels at which MMPs are regulated suggesting a tight control over their proteolytic activity, as would be required *in vivo*. This chapter begins the examination of MMPs as modulators of ECM turnover by presenting a classification scheme for the family of MMPs and identifying some of their biochemical properties.

CLASSIFICATION OF MATRIX METALLOPROTEINASES

Traditionally, MMPs have been loosely classified into three classes according to their substrate specificity, collagenases, stromelysins, and gelatinases. This classification is limited because of the overlap in substrate specificity and variations in protein structure, however, this rough classification scheme is still useful when considering MMPs (Table 2.2). A combination of protein domain structure and substrate specificity now serves as a more

precise classification scheme for the MMP family. Figure 2.2 is a diagram of this new classification scheme and includes domain structure and major substrates for MMPs (Chambers 1997). This section uses both substrate specificity and domain structure in order to introduce the known human MMPs.

Collagenases

This class of MMPs has substrate specificity for fibrillar collagens type I, II, and III. There are three members of this class: interstitial collagenase, neutrophil collagenase, and collagenase 3 (Table 2.2). These MMPs cleave native fibrillar collagens at a single locus that results it two fragments. The first fragment, named tissue collagenase fragment A (TCA), is ¾ length of the original molecule, while the second fragment, tissue collagenase fragment B (TCB), is ¼ of the original molecule. The approximate site of cleavage is between a Gly-Ile peptide bond in the α1(I) chain and between a Gly-Leu peptide bond in the α1(II) chain. The location of these peptide bonds is around ¾ length from the amino terminus (Highberger 1979, Welgus 1982). These enzymes differ slightly in their substrate preference and origin. Interstitial collagenase is produced by fibroblasts and macrophages and has preference for type III collagen, while the neutrophil collagenase shows preference for type I collagen and has neutrophil derivation (Hasty 1987).

Gelatinases

The gelatinases have specificity for both denatured fibrillar collagen and type IV collagen. The members of this class include gelatinase A and gelatinase B (Table 2.2). Gelatinase A (MMP-2) is a 72-kDa protein, and is primarily secreted by human skin fibroblasts. This enzyme can be activated to catalyze the cleavage of a pepsin-resistant portion of type IV collagen. Its substrates, in order of preference are gelatin, type IV collagen, type V collagen, fibronectin, and type VII collagen. It does not cleave interstitial collagens (types I, II, and III) or laminin (Collier 1988, Seltzer 1976). A second gelatinase has a molecular

Table 2.2 Traditional Classification of Matrix Metalloproteinases

Matrix Metalloproteinases			
Collagenases	Stromelysins	Gelatinases	Other MMPs
Interstitial Collagenase (MMP-1)	Stromelysin-1 (MMP-3)	Gelatinase A (MMP-2)	Metalloelastase(s) (MMP-12) (MMP-18) (MMP-19)
Neutrophil Collagenase (MMP-8)	Stromelysin-2 (MMP-10)	Gelatinase B (MMP-9)	
Collagenase-3 (MMP-3)	Matrilysin (MMP-7)		MT-MMPs (MMP-14) (MMP-15) (MMP-16) (MMP-17)
			Stromelysin-3 (MMP-11)

weight of 92 kDa and is referred to as gelatinase B (MMP-9). Gelatinase B shares a virtually identical substrate profile with gelatinase A. The 92-kDa gelatinase B contains an additional 54 amino acid proline rich "collagen-like" domain. The 72-kDa gelatinase A (MMP-2) is expressed in a wide variety of tissues and is often elevated in malignant tumors (Nakagawa 1994, Liotta 1979). Gelatinase B is expressed by a variety of cells such as macrophages, neutrophils, and epithelial cells (Salo 1983).

Stromelysins

This class, based on substrate specificity, has at least three members: Stromelysin-1 and -2, and Matrilysin. Stromelysin-1 (MMP-3) and Stromelysin-2 (MMP-10) are highly homologous enzymes with molecular weights of 54.0 kDa and 54.1 kDa respectively. Their major substrates include proteoglycans and glycoproteins, such as, fibronectin and laminin (Welgus, 1990). Collagen type IV is cleaved by stromelysins at the globular domains only. A smaller molecule, Matrilysin (MMP-7), is also included in this group because it shares the same substrate specificity. It has been suggested that matrilysin can cleave native type IV collagen (Matrisian 1992). Although matrilysin shares a similar substrate profile to the stromelysins, it is much smaller and has a distinct domain structure, therefore, it may be useful to apply domain structure when classifying matrilysin (Figure 2.2). The domain structure of matrilysin is the minimum required for the degradation of ECM by an MMP. This domain structure includes a leader sequence (PRE), a pro-segment (PRO), and a catalytic domain (CAT) (Figure 2.2). It has been suggested that this domain structure is most closely related to the ancestral protein for the family of MMPs (Matrisian 1992).

Other MMPs

Recently, several other MMPs have been discovered that do not fall into the traditional categories and include, stromelysin-3, metalloelastase, and Membrane-Type MMPs (MT-MMPs). Since some of these MMPs have unique substrates or distinct additions to

their domain structure, it may be more useful to include domain structure in our classification scheme. This section identifies some of the new MMPs and briefly discusses their domain structure in relation to the other MMPs.

Metalloelastase (MMP-12) was first discovered in murine macrophages, and may represent a new class of elastin degrading MMPs that include MMP-18 and MMP-19. These MMPs share the same domain structure as the stromelysins and collagenases and have the PRE and PRO and CAT domains, a hinge segment (H) and hemopexin-like domain (HEM) (Figure 2.2). Hemopexin is a blood protein that is produced by the liver, its function is to scavenge free heme in the blood for retrieval back to the liver for processing. Metalloelastase appears to be more extensively processed at both the carboxy and amino terminal ends. Active metalloelastase, isolated from macrophages, has a molecular weight of 21 kDa, which is relatively small compared to the 55 kDa type I collagenase (MMP-1) (Matrisian 1992).

Stromelysin-3 (MMP-11) was first identified in stromal tissue surrounding breast adenocarcinomas (Basset 1993). Stromelysin-3 will degrade fibronectin and laminin weakly. The domain structure of stromelysin-3 is distinct from the other stromelysins and collagenases with the addition of a furin consensus site (F) (Figure 2.2). Furin is a ubiquitous mammalian Golgi enzyme that is involved in the proteolytic processing of a variety of proteins. The Furin consensus site has the sequence Arginine -Xaa-Xaa- Arginine (where Xaa is any amino acid.). This sequence is a recognition site for Furin cleavage. This activation by furin allows stromelysin-3 to be secreted in an active form.

Membrane-type metalloproteinases (MT-MMP) represent another group of MMPs that have recently been characterized (Sato 1994). The domain structures of MT-MMPs also have a furin consensus sites, PRE, PRO, CAT, and HEM domains. In addition, MT-MMPs have a transmembrane domain (TM) (Figure 2.2). The transmembrane domain results in a membrane bound MMP. Currently there are four types of MT-MMPs: MMP-14, MMP-15,

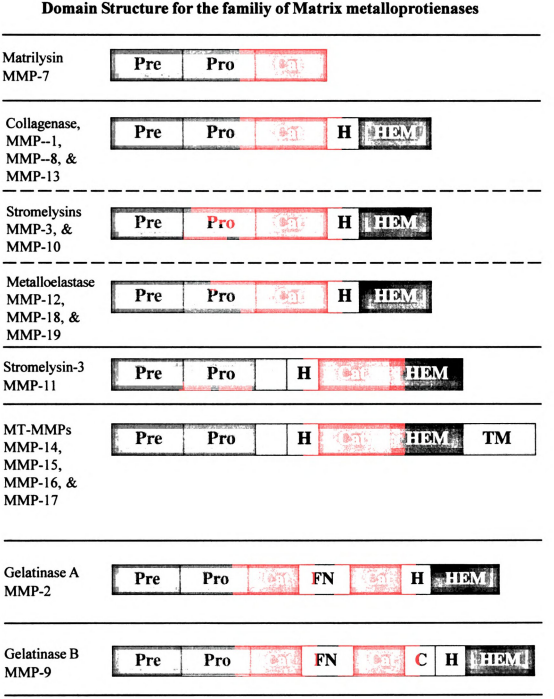

Figure 2.2. Domain structure for several MMPs. Pre, leader sequence; Pro, prodomain; Cat, catalytic domain; H, hinge sequence; HEM, hemopexin-like sequence; F, furin recognition site; TM, transmembrane domain; FN, fibronectin-like binding region; C, collagen-like binding region.

MMP-16, and MMP-17. The major substrates for MT-MMPs have yet to be identified, however, recent studies have strongly suggested their role in the activation of gelatinase A at the cell surface (Lim 1996).

BIOCHEMICAL PROPERTIES OF MMPs

All of the MMPs exhibit similar biochemical properties. They are secreted as proenzymes and require proteolytic cleavage for activation. They are evolutionarily related and show strong sequence similarity as well as protein domain conservation. All MMPs require metal ions for their function. This section discusses these biochemical properties of MMPs and how the differences between the types help to establish their substrate specificity.

Molecular weight characteristics

A comparison of molecular weight estimates in several MMPs is shown in Table 2.1. These weights are obtained through the use of sodium dodecyl sulfate polyacrylamide gel electrophorsis (SDS-PAGE). Stricklin (1977) found MMPs isolated from fibroblasts to have a molecular weight of 55,000 to 60,000 Daltons (Da) in the proenzyme form and, 45,000 Da to 50,000 Da in the trypsin-activated form. Similar values have been obtained for rat uterine collagenase and rabbit synovial collagenase (Roswit 1983). The molecular weight of human neutrophil collagenase is estimated to be 70,000 to 91,000 Da (Hastey 1986). In most cases we see a decrease in molecular weight with the activation of MMPs which suggests that the enzyme is secreted as a zymogen and requires a proteolytic event for activation.

Compositional analysis

Comparison of primary amino acid sequence of various members of the MMP family demonstrates that these proteins are divided into several groups which possess distinct and conserved domains among family members. The amino acid sequences have been identified

and show a remarkable degree of homology, despite the fact that these enzymes come from different cell types such as human fibroblasts, human neutrophils, and tadpole tails.

The amino acid sequence of all MMPs, as predicted by the complementary DNA (cDNA), demonstrates a high degree of conservation between each type of enzyme across several mammalian species (80% similarity amongst collagenases as well as amongst stromelysins). There is also a strong degree of conservation between MMP types (50% sequence similarity) (Murphy 1995). Some of these conserved domains have very specific functions and are critical for the degradation of ECM. A detailed profile of these domains is included in the following section.

MMP domain structure

The amino acid sequences of matrix metalloproteinases are well conserved across several mammalian species (Docherty 1992). The most conserved domains are the PRE PRO CAT and HEM domains. Figure 2.3 is a schematic representation of the conserved domains in collagenases, stromelysins and gelatinases (Docherty 1992). The following discussion describes these domains beginning from the amino terminus and moving toward the carboxy terminus.

Domain 1 is the propeptide domain and is approximately 80 amino acids in length. This domain is lost upon activation. As indicated in Figure 2.3, the conserved sequence is Proline – Arginine – Cysteine - Glycine - (Valine or Asparagine) - Proline – Aspartate (PRCGV/NPD) (Docherty 1992, Stetler-Stevenson 1989). This domain is responsible for maintaining enzyme latency. The conserved Cysteine in this region interacts with a zinc atom bound to the catalytic domain (CAT) or Domain 2.

Domain 2 is the catalytic domain and is just down stream from the pro-domain. This 170 amino acid catalytic domain contains the Histidine - Glutamate - (Phenylalanine or Lysine

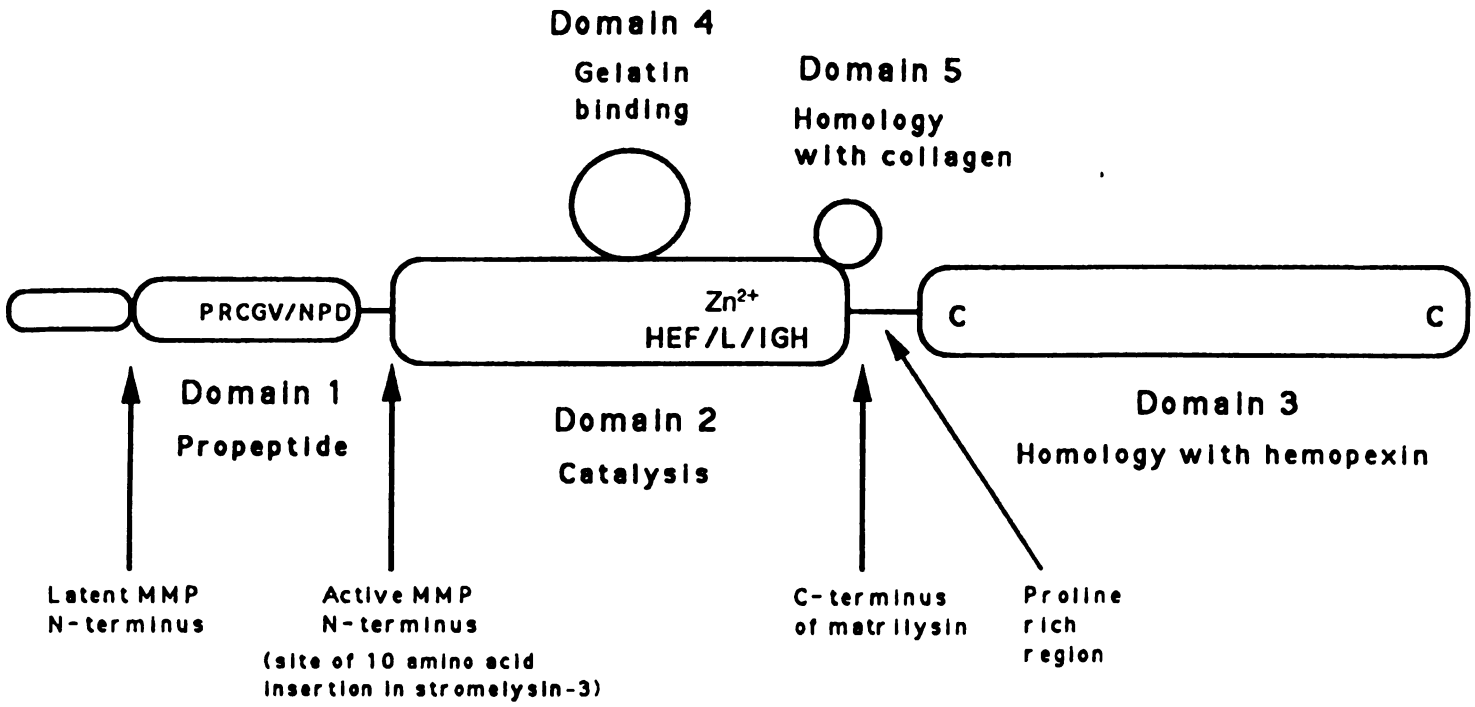

Figure 2.3. Schmatic diagram of conserved domain structure in MMPs (Docherty 1992).

or Isoleucine) - Glycine - Histidine motif (HEF/L/IGH). This motif has significant sequence similarity to thermolysin, a bacterial metalloproteinase. The histidine residues in this motif bind the zinc atom, required for MMP function. A pair of conserved Asparagine (Asp) residues located on either side of this domain act as binding sites for calcium, which serves to stabilize the enzyme.

Domain 3 is located at the carboxy terminus and has sequence similarity to hemopexin, a heme binding protein, and to the ECM component vitronectin (Matrisian 1992). This sequence similarity may direct MMPs to their substrates. This sequence is separated from the catalytic domain by a proline-rich hinge sequence (H) (Figure 2.2). Although this sequence is not required for catalytic activity, it appears to have a number of important functions. This sequence is required in interstitial collagenase for the binding and cleavage of fibrillar collagen, and in gelatinase A, this sequence aids in the activation of gelatinase A at the cell surface. The two cysteines in this region are thought to form a disulfide bond, resulting in the folding of this region.

Both gelatinase A and gelatinase B contain a gelatin-binding domain associated with Domain 2 (CAT), the catalytic domain (Figure 2.3 Domain 4) (Docherty 1992). This domain has sequence similarity to the gelatin-binding domain of fibronectin. This region is encoded by the addition of three exons not found in the genes for collagenases and stromelysins. This region mediates the sequestering of gelatinase within the extracellular matrix.

Domain 5 is found in gelatinase B (MMP-9). This domain is an additional insert before the hemopexin-like domain and shares homology to the collagen helix (Doherty 1993). The function for this insert is unclear, but it most likely mediates substrate specificity.

As previously stated, matrilysin (MMP-7) has the minimum conserved domains required for MMP activity. Its major substrates include proteoglycans and glycoproteins, which are important components of bacterial cell membranes. MMP-7 has a high sequence

homology to thermolysin, a bacterial metalloproteinase. It has, therefore, been suggested that matrilysin most closely resembles the ancestral protein amongst the known MMPs. The addition of exons to an ancestral gene, which encodes for a matrilysin-like ancestral MMP, could result in a phenotype with new substrate specificity. It is, therefore, conceivable that MMPs may have co-evolved with their substrates (Matrisan 1992,Woessner 1996).

Metal ion requirement

The mammalian collagenases are active at optimal pH between 7 to 8. Matrix metalloproteinases require zinc for their catalytic activity and calcium for stabilization. Chelating agents such as EDTA and 1,10 phenanthroline effectively abolish enzyme activity. A reversible loss of MMP activity is observed with the removal of calcium from human skin fibroblasts, rat skin fibroblasts and rat uterine cells grown in culture. The metalloproteinase nature of collagenase is evident in several pathological conditions. Ophthalmologists are able to control the rapid rate of corneal stromal degradation, following alkali burns, with the use of solutions containing EDTA. Patients with epidermolysis bullosa, a collagen disease that results in loose skin, secrete an abnormal collagenase that has a reduced affinity for zinc (Kucharz 1992).

Calcium functions both as an enzyme activator and it also stabilizes the tertiary structure of the enzyme at physiological temperatures (Seltzer, 1976). Asparagine (Asp) residues located on either side of the catalytic domain can bind calcium, which stabilizes the enzyme. Additionally, the inhibition of the MMPs by chelating agents, in the presence of excess calcium, suggests the participation of a second metal ion in the activation of the enzyme. Several transition metals have been shown to reverse the inhibition by chelating agents but zinc was shown to be the most effective (Seltzer, 1976).

The role of zinc in metalloenzymes can be divided into four categories: catalytic, structural, regulatory, and non-catalytic. In the case of matrix metalloproteinases, zinc has a catalytic function. Indeed, zinc is essential for and is directly involved in their enzymatic activity, and the removal of zinc by chelating agents yields an inactive apoenzyme (Vallee 1992). As previously described, MMPs have a zinc-binding site associated with their catalytic domains, the HEF/L/IGH motif. These binding sites have sequence similarity to the bacterial metalloproteinase, thermolysin. The zinc atom binds to the Hys residues in this motif. In an active matrix metalloproteinase this zinc interacts with a water molecule. The water molecule, upon entering the zinc coordination sphere, is activated for enzyme catalysis by either ionization or polarization, or is poised for displacement by the substrate, thus making it the decisive catalytic group (Figure 2.4) (Vallee 1992). In the pro-metalloproteinases the zinc atom binds with the Cysteine (Cys 73) residue of the pro-domain (Figure 2.5). Only with the disruption of this bond via the proteolytic cleavage of the PRO domain, or auto perturbation

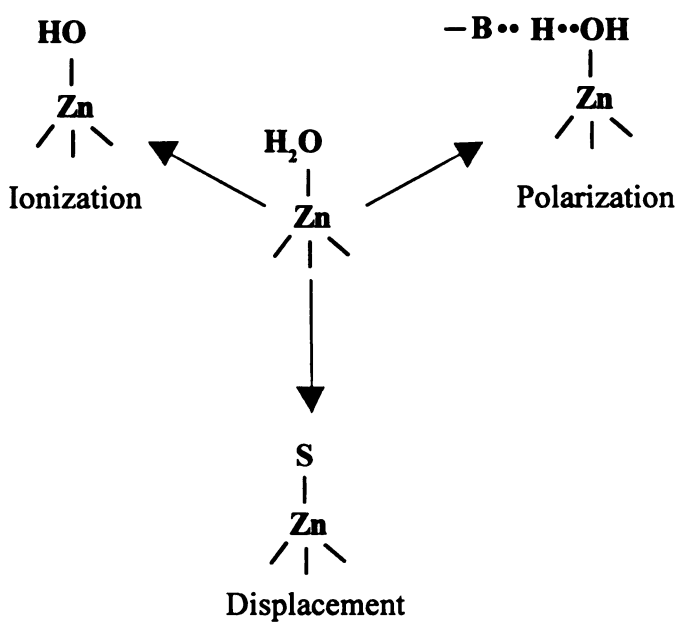

Figure 2.4 Schematic function of the H_20 ligand in active zinc site of MMP. S, substrate; B, base (Vallee 1992)

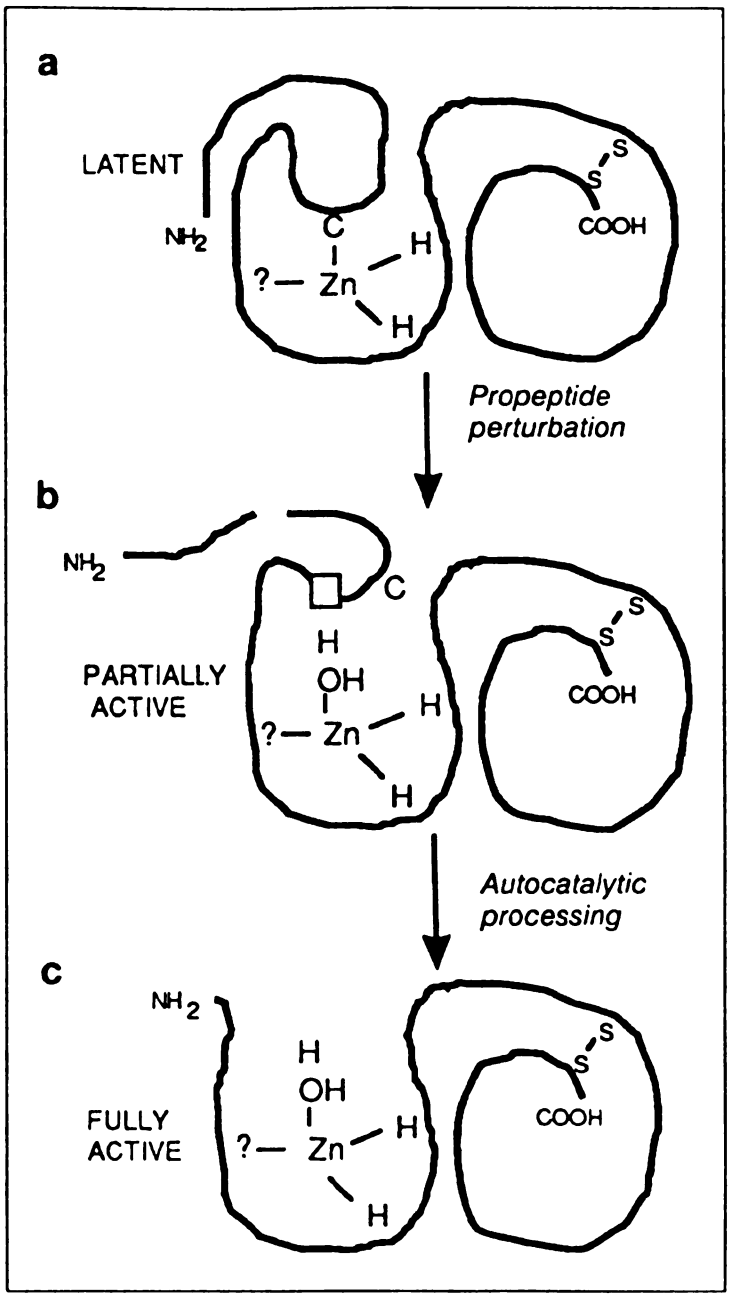

Figure 2.5. Schematic diagram of the "Cysteine Switch", the interaction of the zinc atom in the catalytic site, with a cysteine in the prodomain of MMP (Docherty 1992).

(autoactivation), can the zinc atom be freed to interact with a water molecule, thereby activating the enzyme. This mechanism has been described as a "Cysteine switch" and will be further considered in chapter 3 (Springman 1995).

MODULATION OF MMP ACTIVITY

The regulation of collagenolytic activity in the extracellular matrix is clearly important to organisms. Modulation of MMP activity occurs at several levels including: 1) biosynthesis and secretion, 2) pro-enzyme activation, and 3) inhibition of MMP by tissue inhibitors of metalloproteinases. This is the topic of chapter 3.

CONCLUSIONS

The study of collagen degradation, like that of collagen itself, has experienced an almost exponential growth in the past 20 to 30 years. Initiated by the pioneering work of Gross (Gross 1962), MMPs are being identified and characterized at a rapid rate. The activity of MMPs on the extracellular matrix justifies their study in physiological conditions involving ECM turnover. This chapter has introduced the family of MMPs by presenting a classification scheme based on protein domain structure as well as substrate specificity. It has also identified some of the basic biochemical properties associated with MMPs including molecular weight characteristics, composition analysis, domain structure, and metal ion requirements. Regulation of MMP activity is of critical importance, since ECM turnover is relevant to normal conditions such as, development, wound healing and regeneration, as well as pathological conditions such as arthritis, atherosclerosis, and cancer metastasis. The next chapter will explore in detail the regulation of MMP activity in these processes.

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CHAPTER 3

MODULATION OF MATRIX METALLOPROTEINASES

ABSTRACT

The turnover of extracellular matrix is regulated by the proteolytic activity of matrix metalloproteinases (MMP). The regulation of MMPs is modulated at three levels, expression, activation, and inhibition. The expression of MMP is under tight regulation and is controlled by a number of growth factors, tumor promoters, oncogenes and hormones. Proteolytic cleavage is required for the activation of MPPs and a number of proteolytic enzymes are capable of causing this cleavage, such as, plasmin, trypsin, and activated MMPs. Tissue inhibitors of matrix metalloproteinases (TIMPs) can block the degradation of extracellular matrix by MMPs. By examining the modulation of MMPs, this chapter provides insight into some of the common mechanisms involved in the turnover of ECM in normal and pathological conditions. Since MMPs are such a major regulatory force in the turnover of ECM, a study of their modulation is necessary.

INTRODUCTION

The extracellular matrix (ECM) plays a critical role in normal tissue development and remodeling. The turnover of ECM occurs during processes requiring tissue remodeling, such as, embryogenesis, organogenesis, wound healing, regeneration, and cancer metastasis. The enzymes capable of degrading components of the extracellular matrix, namely the family of metalloproteinases (MMPs), are thought to play a regulatory role in these processes. Matrix metalloproteinases are a family of zinc-dependent endopeptidases which are specific for different components of the ECM. The major members of this family include collagenases, stromelysins, and gelatinases. These proteins are secreted as zymogens and, once

activated via proteolytic cleavage, are capable of degrading specific components of the ECM (Howard 1991). Some of the major substrates for MMP are as follows: collagenases degrade types I, II, and III collagens, gelatinases degrade types IV, V and VI collagens, and stromelysins degrade laminin, fibronectin, and proteoglycans (Matrisian 1992). The rate of ECM turnover is determined by the net activity of the secreted MMP. This activity is controlled at three levels: 1) the rate of production of the proteolytic enzymes, 2) the activation of zymogens by proteolytic processing, and 3) the production of specific protease inhibitors (Nomura 1989). This multi-level control meets the requirement of strict regulation for normal function, and implies that MMP are the physiologically relevant mediator of ECM turnover. The apparent consequences of abnormal regulation are expressed in several pathological conditions, such as, rheumatoid arthritis, chronic wounds, osteoporosis, corneal ulceration, certain genetic diseases and migration of tumor cells through the basement membrane (Bullen 1995, Schorpp 1995).

Regulation of MMPs is complex and occurs at three different levels; expression, activation, and inhibition. Several agents including growth factors, cytokines, oncogenes, UV irradiation and tumor promoters control this regulation (Angel 1987, Wasylyk 1991).

EXPRESSION OF MMPs

Expression is the first modulation point of MMPs. Increased expression of MMPs is often seen during tissue remodeling or cellular invasion, as is the case during development, wound healing, cancer invasion and metastasis. The genes for MMPs can be induced by a variety of agents including, cytokines, growth factors, oncogenes, and phorbol esters (Stearns 1993). Individual members of the family of matrix metalloproteinases do not all respond to the same stimulatory factors. This point is understandable because not all MMPs need to be expressed at the same

time. The expression of any MMP is transient, and is normally under tight regulation. There is evidence for the conservation of regulatory features in MMP. However, the transcriptional regulatory mechanisms have not yet been determined for all MMPs. This section identifies some of the regulatory transcription elements common to several MMPs, and discusses some of the differences observed in MMP transcription.

The promoter regions for genes that encode for human stromelysins and collagenases have important common transcriptional regulatory features (Hagmeyer 1993). Each of these genes has a TATA element associated with its promoter which is located approximately 30 nucleotides up stream from the transcriptional start site (Schorpp 1995). Figure 3.1 is a schmatic diagram of the promoter region of an MMP gene. These genes are characterized as having activator protein transcription factor binding sites (AP-1) and polyomavirus enhancer A binding Protein 3 sites (PEA-3). These response sites, AP-1 and PEA-3, are the most conserved response elements in the promoters of MMPs.

The AP-1 site is transactivated by AP-1. AP-1 consists of a dimerization of the proto-oncogenes *c-fos* and *c-jun*. The AP-1 element can be activated by treatment with the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) (Angel 1987). This induction is due to the activation of protein kinase C (PKC) by TPA. This activation of PKC results in the activation of *c-fos*, which results in the formation of AP-1 and the induction of MMP via the AP-1 element. Of course PKC induction does not explain the whole story of AP-1 activation. Tumor necrosis factor α (TNF-α) has been identified as an activator of collagenase through its prolonged activation of *c-jun* (Brenner 1989). An analysis of the effects of TNF-α on collagenase gene expression is presented in Figure 3.2. In these experiments human fibroblasts, in confluent primary cultures, were treated with TNF-α for 0, 6, and 24 hours (panel a, lanes 1, 2, & 3 respectively). An increase in the expression

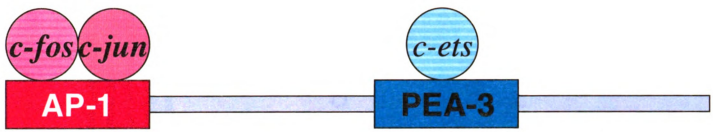

Figure 3.1. Schematic diagram of the promoter of MMP genes, showing the presence of the AP-1 and PEA-3 response elements. The AP-1 response element is transactivated by the dimerization of *c-fos* and *c-jun*. The PEA-3 response element is transactivated by the *c-ets* family of nuclear transcription factors.

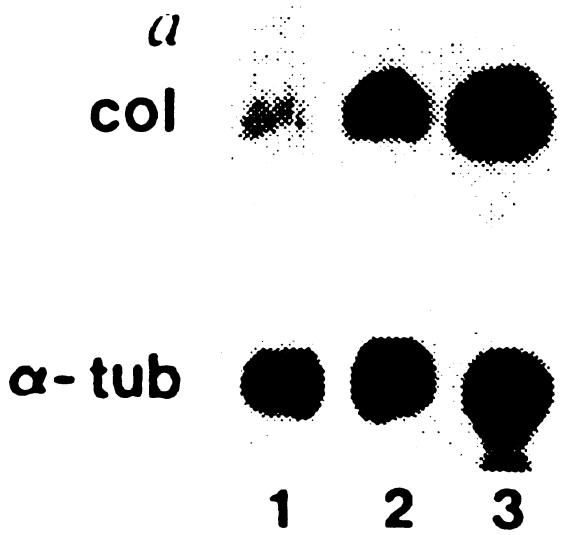

Figure 3.2. Human fibroblasts were treated with TNF-α for 0, 6, and 24 hours (lanes 1, 2, & 3 respectively) in confluent primary cultures. An increase in the expression of collagenase is seen with prolonged exposure to TNF-α. TNF-α activates the AP-1 element resulting in an increased expression of collagenase. Col = collagenase, α–tub = α–tubulin, used as a control (Brenner 1989).

of collagenase is seen with prolonged exposure to TNF-α. TNF-α activates the AP-1 element resulting in an increased expression of collagenase (Brenner 1989). Growth factors, such as, epidermal growth factor (EGF) and transforming growth factor-α (TGF-α) have been shown to elicit proliferative and dedifferentiation effects by stimulation of proto-oncogenes. Transcription factors c-*fos*, and c-*jun* are activated in the early response to the EGF, and TGF-α cascade of events (Bauknecht 1993). Activation of the AP-1 is also mediated by UV irradiation via c-*jun*. Both UV irradiation and TPA cause post-translational modification of AP-1 within minutes (Radler-Pohl 1993). The transactivation of the AP-1 element alone can lead to increased expression of MMPs, however, other response elements in the promoters of MMPs may serve to fine-tune the modulation of MMP expression.

The PEA-3 response element contributes to the fine tuning mechanisms that modulate the inducibility of the MMP genes by agents such as growth factors, oncogenes, and cytokines (Matrisian 1992). Another proto-oncogene, *c-ets* recognizes the PEA-3 element (Wasylyk 1991). Several growth modulators affect *c-ets* activity, including growth factors, non-nuclear oncoproteins, activators of protein kinase, intracellular calcium and cyclosporin (Wasylyk 1990). Activation of PEA-3 and AP-1, mediated through *c-ets* and *c-fos/c-jun*, results in a synergistic increase in the expression of collagenase (Wasylyk 1990). In contrast, activation of the stromelysin promoter by both *c-ets* and *c-jun/c-fos* is efficient but the combined effect is not synergistic. These findings suggest that PEA-3 may serve as a fine tuning mechanism for differential response to transcriptional regulatory factors. This is further complicated by the fact that *c-ets* may serve to activate *c-jun* and *c-fos* (Wasylyk 1991).

It is important to note that gelatinase A lacks the AP-1 site which is conserved in most MMPs. In fact treatment with TPA will actually decrease expression of gelatinase A. Unlike most other MMPs, gelatinase A is constitutively expressed in

a number of cell types. As a result of this basal expression of gelatinase A, transcriptional elements in the promoter region of gelatinase A have been difficult to identify. The promoter region of gelatinase A has a noncanonical TATA box site and two SP1 response elements (Stetler-Stevenson 1996). The noncanonical TATA sequence in the gelatinase A promoter region has the sequence TACATCT, this sequence has been suggested to be the basal promoter of gelatinase A (Templeton 1991). In 93% of TATA box consensus sites, position 3 is a T for the canonical configuration, however there is a 7% possibility for position 3 to be a C, A, or G. For position 6, A is the base in 83% of TATA-box sequences and there is a 17% possibility for this position to be held by a C, A, or G. The TACATCT sequence associated with the gelatinase A promoter differs from the canonical TATA box sequence at position 3 and 6 only and is considered to be and non-cannonical. Since the possibility exists for the 6 position to be a C, the TACATCT can be considered an acceptable, but non-canonical TATA box. In addition, this non-canonical TATA box site is surrounded by G-C rich sequences, as are most TATA box sequences. This non-canonical TATA box site allows greater flexibility for polymerase or necessary transcription factors to recognize the transcription start site (Templeton 1991). Overexpression of gelatinase A may be due to the loss of negative control circuits. Cell type-specific regulation of gelatinase A has been proposed. The presence of negative circuits such as DNA methylation or the existence of a silencer in the DNA has been proposed (Templeton 1991). This cell type-specific regulation is important since high levels of gelatinase A are associated with pathological conditions, such as, glomerular nephritis and cancer invasion and metastasis.

In the kidney, gelatinase A is important in inflammation reactions. The over expression of gelatinase A (MMP-2) in glomerular mesangial cells can lead to an inflammatory phenotype (Mertens 1997). Recent studies have demonstrated that glomerular mesangial cells (GMC) exhibit unique stimulation patterns including,

stimulation by interlukin-1, tumor necrosis factor, transforming growth factor-β and phorbol esters (Harendza 1995). The transcriptional element associated with this stimulation is a unique 40 base pair (bp) sequence located at –1322 to-1282 bp relative to the translational start site of gelatinase A (Harendza 1995). This sequence (CTGCTGGGCAAG) was found to interact with the YB-1 transcription factor (Mertens 1997). These findings indicate that YB-1 is the cell type-specific regulator in glomerular mesangial cells (Mertens 1997).

In prostate cancer, gelatinase A is overexpressed in the human prostate carcinoma cell line, PC-3 cells, after treatment with invasion-stimulatory factor (ISF). This 78 kDa protein was purified from the conditioned medium of a bone-metastasizing human prostatic cell line PC-3ML (Stearns 1994). In human lung carcinoma cells, mRNA for gelatinase A is observed when cells are treated with the tumor cell derived "collagenase stimulatory factor" (CSF) (Polette 1997). The mechanism for the transcriptional response to these tumor derived stimulatory factors has yet to be determined, however, their activity supports the notion of cell specific regulation of gelatinase A expression.

ACTIVATION OF MMPs

Once the MMP has been secreted into the ECM, the next regulatory stage is activation of the MMP by proteolytic cleavage. Matrix metalloproteinases are secreted as latent proenzymes. These latent pro-MMPs require proteolytic cleavage of the prodomain for activation. The prodomain is a highly conserved region in the amino terminus, and has the sequence of PRCGVPD. There are several enzymes that can cause this proteolytic activation, such as trypsin, plasmin, urokinase-type plasminogen activator, furin (Golgi enzyme), other MMPs, and Cathepsin G. Several MMPs have the ability to autoactivate and include, collagenases, stromelysins, and

gelatinases. Autoactivation of the 72 kDa progelatinase (gelatinase A) can be induced by treatment with organomercurial compounds (Howard 1991). A "cystine switch" is involved in the activation of MMPs.

The cysteine switch

The proteolytic cleavage of the prodomain of the MMP leads to the activation of the catalytic site of MMP by the "cysteine switch". The conserved catalytic domain in MMP binds a zinc metal ion. This zinc ion is the catalytic element of MMPs. When the MMP is in the proform and the prodomain is still associated with it, the cysteine in this PRCGVPD sequence interacts with the zinc ion bound to the catalytic domain. The cysteine and zinc interaction prevents zinc from interacting with a water molecule, which is required for MMP catalytic function. Proteolytic cleavage of the prodomain of proMMP leads to the disruption of this cysteine and zinc interaction, freeing zinc to interact with a water molecule. In the case of autoactivation there is chemical or mechanical perturbation that causes a disruption in the conformational structure of the proMMP. This results in the disruption of the cysteine and zinc interaction. At this point the catalytic domain of MMP is active and can cleave its own pro-domain as well as that of the surrounding MMPs. This mechanism, involving the conserved cysteine of the prodomain and the zinc atom in the catalytic domain described in the above scenarios, has been referred to as the cysteine switch.

The urokinase-type plasminogen activator (uPA) system is a major contributor to MMP activation *in vivo*. Plasminogen is a regular component of the plasma and interstitial fluid and can be converted by uPA to the active enzyme plasmin. Plasmin is a serine protease capable of cleaving a part of the prodomain of MMPs. The partial cleavage of the prodomain by plasmin results in the active MMP. Once the MMP has been activated by plasmin it can cleave the rest of the prodomain by

autocatalysis. The MMP is then fully activated. Like MMPs, uPA has the AP-1 and PEA-3 elements associated with the promoters of the urokinase-type plasminogen activator gene (Matrisian 1992). The coordinate transcriptional activation of collagenase, stromelysin and urokinase may, therefore, result in a cascade of events that lead to ECM degradation. Basic residues of the "pro"domain of both collagenase and stromelysin are recognized and cleaved by plasmin. The subsequent removal of a part of the "pro" domain destabilizes the association of the cysteine residues in the conserved PRCGVPD region with zinc resulting in a conformational change. This cascade of events might serve as a control mechanism to facilitate the total degradation of several components of the ECM.

INHIBITION OF THE ACTIVATED MMP

After MMPs have been expressed and activated, there remains a third level of modulation. Matrix metalloproteinases can be inhibited by the general serum proteinase inhibitor α2-macroglobulin (Murphy 1995), however, a family of specific tissue inhibitors also block the ability of MMPs to degrade the ECM, and appear to be the most physiologically significant factor. Tissue inhibitors of metalloproteinases (TIMPs) are a family of ubiquitous proteins and are considered to be the major players in the regulation of MMP. At present there are at least four known members of the TIMP family. The classification, structure, and function of these are outlined in more detail in chapter 4. The primary functional role of TIMPs is to inhibit ECM degradation by MMPs. This inhibition of MMP by TIMP is mediated by a blockage of the catalytic domain of MMP. This activity is notably due to the conservation of 12 cysteine residues in TIMP. These cysteine residues form high affinity and essentially irreversible complexes with the active forms of MMP (Declerck 1991).

This interaction of TIMP and active MMP results in the formation of a 1:1 complex (Declerck 1993). Because of the suspected role in collagen metabolism regulation, it is important to understand the activity and regulation of TIMPs.

It is interesting to note that TIMP-2 appears to form stable complexes with procollagenase and progelatinase A. The formation of the procollagenase-TIMP-2 and progelatinase-TIMP-2 complexes prevents the autocatalysis of both pro-forms. Activation of the procollagenase-TIMP-2 complex can be achieved by proteolysis of the prodomain by stromelysin. In the case of the progelatinase A-TIMP-2 complex, stromelysin is not an effective activator, however, TIMP-2 appears to mediate the activation of progelatinase A at the surface of cells by interaction with membrane type-MMPs (Howard 1991a).

The balance of TIMP to MMP is very important in the turnover of the extracellular matrix. Any disruption in this balance can lead to pathological condition, such and arthritis, sclerosis, and cancer metastasis. Like collagen, MMPs and TIMPs are expressed in a variety of tissues. Transcriptional expression of TIMP is regulated by a number of factors that, interestingly enough, mirror factors involved in MMP activation. An AP-1 element, responsive to serum and the phorbol ester TPA, has been identified in the promoter of the TIMP gene. Figure 3.3 highlights the restriction map sequence of the 5′-end of the murine TIMP gene (Campbell 1991). The TPA-responsive element (TRE) is the element responsible for the activation by TPA as seen in both the stromelysin and collagenase genes. This element most likely binds the AP-1 protein as observed in MMP activation. In addition to the AP-I element, a TATA box is also present in the promoter of TIMP. The TRE element also responds to TGF-β (Campbell 1991). Despite the similarity

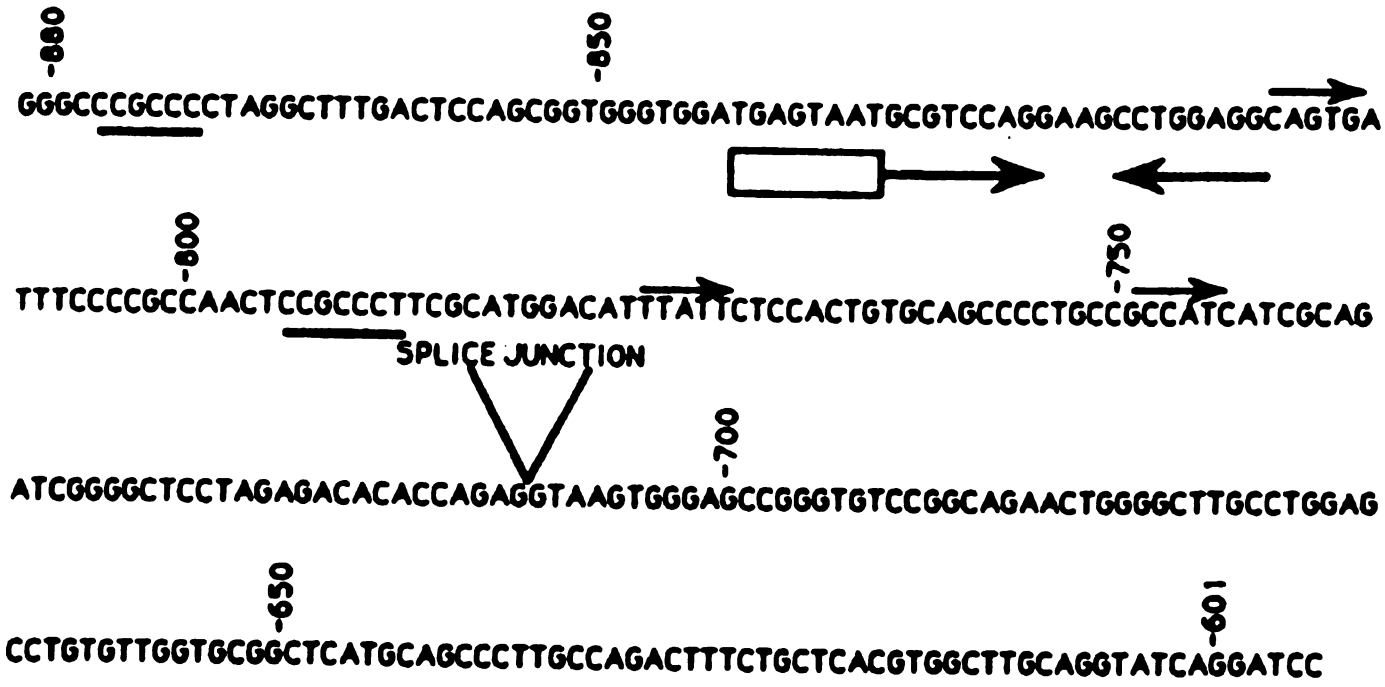

Figure 3.3. Sequence of the 5′ end of the murine TIMP gene. AP-1 is represented by the open box. All positions are numbered in relation to the transcriptional start site (Campbell 1991).

in MMP and TIMP modulation of transcription, MMPs and TIMPs are often differentially expressed, both spatially and temporally. The fact that TIMPs and MMP are regulated by similar factors emphasizes a tight level of control over MMP function.

ROLES FOR MATRIX METALLOPROTEINASES

The complex regulation of MMPs and the importance of ECM turnover suggest that MMPs play a critical role in many physiological processes. The balance of MMP to TIMP is important in the spatial and temporal control of ECM turnover. Several normal and pathological conditions are dependent on this balance.

Extracellular matrix (ECM) remodeling accompanies cell migration, cell-cell interactions, growth, blastocyst implantation, and tissue invasion during mammalian embryogenesis. During development, MMPs and TIMPs are differentially expressed to facilitate cell migration. Mouse embryos secrete functional ECM-degrading metalloproteinases, including collagenase and stromelysin. These are inhibited as well as regulated by the tissue inhibitor of metalloproteinases (TIMP) during peri-implantation development and endoderm differentiation (Brenner 1989). The expression of collagenase, stromelysin, and TIMP was detected as maternal transcripts in the unfertilized egg, which were present at the zygote and cleavage stages, and increased at the blastocyst stage and with endoderm differentiation (Brenner 1989). These data suggest that metalloproteinases function in cell-ECM interactions during growth, development, and implantation of mammalian embryos. MMPs are also associated with several other reproductive processes. Ovulation and the release of the mature ovum require the activity of MMPs and TIMPs. Mammary gland involution, following lactation, requires the expression of MMPs and a decrease in the expression of TIMPs. These findings suggest that MMP and

TIMPs are important modulators of ECM turnover required for these processes. The coordination of MMP and TIMP expression in these conditions is remarkably similar to wound healing.

In wound healing MMPs and TIMPs are temporally and spatially differentially expressed. It is likely that the balance between proteolytic enzymes and their inhibitors, namely neutrophil collagenase and gelatinases A and B, plays a major role in the maintenance of this critical control. Inflammatory cells secrete neutrophil collagenase and gelatinase B. Epithelial cells express Gelatinase A. In normal wound healing, proteolysis occurs in the first few days followed by a shift to matrix deposition. Studies of chronic wounds show significantly higher levels of gelatinase A and B enzymes and lower levels of TIMP than those observed in normal healing wounds (Bullen 1995). Since wound healing requires a balance between synthesis and degradation of ECM components, it follows that a disruption in the balance between MMP and TIMP will lead to chronic wounds. Tumors have been described as wounds that do not heal, and it is expected that these same mechanisms apply to invasion and metastasis by cancer cells.

During metastasis, there are a series of collagen-containing structural barriers that a tumor cell must pass. Extracellular matrix and basement membranes must be breached for cells to intravasate and extravasate. The basement membrane underlying endothelial cells presents a continuous collagen-containing barrier to the metastatic process. Within a tissue, at either a primary or a secondary tumor site, degradation of extracellular matrix must occur in order to permit tumor cell invasion and spread. Therefore, MMPs are the likely candidates that facilitate the ECM turnover required by cancer metastasis. Increased expression of MMPs has been associated with several malignant phenotypes of cancers such as, breast, lung, skin and the prostate. Several generalizations can be made concerning the expression of MMPs in these malignant phenotypes: 1) the number of different MMP family

members that can be detected tends to increase with cancer progression; 2) the relative level of any individual MMP family member tends to increase with the stage of cancer and 3) MMPs can be made by either tumor cells themselves or, quite commonly by stromal cells, as a host tissue response to the tumor. The expression patterns for MMPs, therefore, support a role for these MMPs at later stages of tumor progression.

CONCLUSIONS

The turnover of extracellular matrix is a critical event in many normal and pathologic conditions. Matrix metalloproteinases are a family of zinc dependent enzymes capable of degrading the ECM. This chapter reviewed some of the MMP-mediated mechanisms involved in modulation of ECM turnover. The activity of MMPs is modulated at three levels, expression, activation and inhibition. Disruption of MMP modulation at any of these levels can lead to uncontrolled destruction of ECM observed in many pathologic states including, arthritis, chronic wounds, and cancer metastasis. Chapter 4 will examine the third level of MMP modulation involving TIMPs.

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CHAPTER 4

TISSUE INHIBITORS OF MMPs (TIMPs): STRUCTURE, EXPRESSION, GENETIC REGULATION, AND FUNCTION

ABSTRACT

Tissue inhibitors of metalloproteinases (TIMPs) are secreted proteins that have inhibitory effects on the extracellular matrix-degrading matrix metalloproteinases (MMPs). Both TIMPs and MMPs are key determinants of the establishment, maintenance and turnover of the ECM, and are directly involved in normal and abnormal human conditions, such as, development, regeneration, and cancer. Since the ECM is important to cell communication, TIMPs may play an indirect role in ECM-associated cell signaling. Recent studies have proposed a secondary effect of TIMPs on cell proliferation, independent of their MMP inhibitory activities. This chapter examines the most recent studies on TIMPs, including structure, function, expression, and their regulation.

INTRODUCTION

Tissue inhibitors of metalloproteinases are currently the subject of considerable interest in many fields of biological research. They play a key role in the modulation of ECM turnover by virtue of their inhibitory action on matrix metalloproteinase (MMP) (Matrisian 1992, Murphy 1995). The primary function of TIMP is to inhibit the activity of MMP. This is preformed by an interaction of 12 conserved cysteine residues of TIMP with the active sites of MMPs. These cysteine residues form noncovalent bonds forming a MMP:TIMP complex. One MMP and one TIMP molecule forms this MMP:TIMP complex. The spatial and temporal balance of MMP to TIMP is essential for normal function. Any prolonged imbalance of the MMP:TIMP ratio can lead to pathological tissue destruction as seen in cancer

and arthritis, or conversely, to an aberrant ECM accumulation as see in fibrotic states (DeClerck 1992, Edwards 1996, Gohji 1996). A study examining the mean serum MMP:TIMP ratio in patients with urothelial cancer is presented in Figure 4.1. This study showed that patients with cancer recurrence had significantly higher mean serum MMP:TIMP ratios than those patients who had no recurrence (Gohji 1996). This study illustrates the importance of a normal balance between MMP and TIMP. However, the role of TIMP in the modulation of MMP activity is not completely understood. In some cases TIMPs play a role in the stabilization of MMPs, and in others, to the activation of MMPs. Recent studies have also indicated that TIMPs may have additional functions separate from their MMP inhibition, including the ability to stimulate cell growth. This chapter reviews TIMPs by introducing some of the members of this family of proteins, followed by an examination of the primary function of TIMPs. Next, some of the recent studies on TIMP are discussed including their controversial mitogenic activity and their clinical application in cancer treatment.

THE TIMP FAMILY

The growing family of mammalian TIMPs is currently composed of three members. TIMP-1, TIMP-2, and TIMP-3. These TIMPs differ in their structure, biochemical properties and *in vivo* expression, suggesting that each form may have a specific physiological role. The first member of the TIMP family of proteins is TIMP-1, a glycosylated 28-kDa protein. The second member, TIMP-2, is a smaller 21kDa protein, which has less glycosylation. There is a 40% amino acid sequence homology between TIMP-1 and TIMP-2 (Hammani 1996, Williamson 1993). Although TIMP-3 shows sequence homology to the other TIMPs, initial

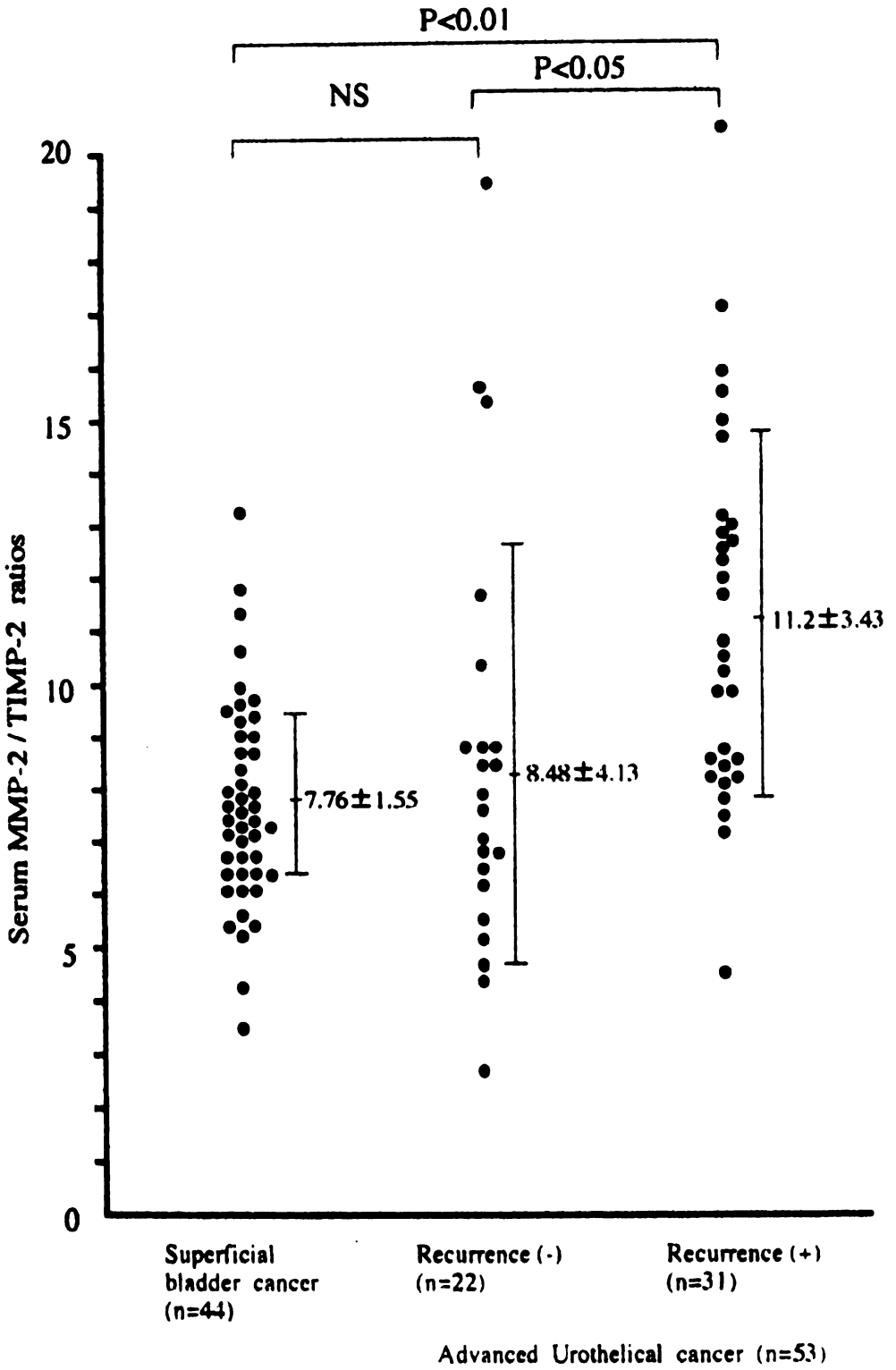


Figure 4.1. The mean serum MMP:TIMP ratio in patients with urothelial cancer. Patients with recurrence have a significantly higher mean serum MMP:TIMP ratios than those patients who had no recurrence (Gohji 1996).

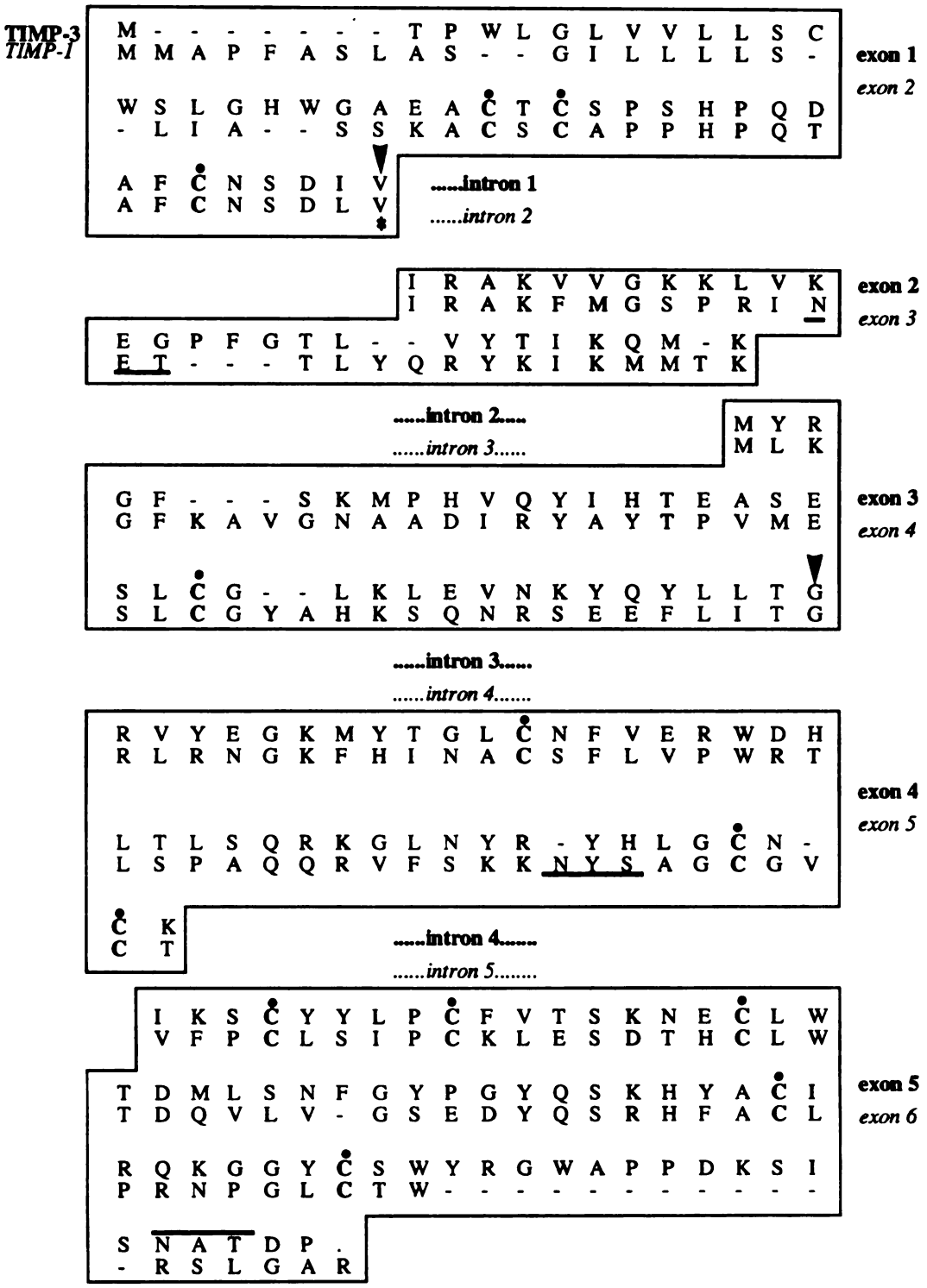
characterization of this protein reported a closer relationship to the chicken protein chTIMP-3 (chicken TIMP). Like chIMP-3, TIMP-3 is localized in the ECM, in contrast to the freely diffusible TIMP-1, and TIMP-2 (Uria 1994). The amino acid composition and sequence for TIMP-1, TIMP-2, TIMP-3, and chIMP-3 is displayed in Figure 4.2 (Uria 1994). The conservation of amino acid sequence in these proteins is important to their common primary function to inhibit MMPs. All of these TIMPs are able to inhibit the ECM proteolysis by MMP (Murphy 1995, Sato 1992).

All of these TIMPs show the precise spatial conservation of 12 cysteine residues. These cysteine residues aid in the formation of interchain disulfide bonds. These bonds are involved in the protein tertiary structure and folding (Figure 4.3). (Williamson 1993). The disulfide bonds of highly conserved residues are located in the following sequence: Cys-1-Cys-70, Cys-3-Cys-99, Cys-13-Cys-124, Cys 127-Cys-174, Cys-132, Cys-137 and Cys-145-Cys166. The tertiary protein structure has two domains, made up of six loops (Murphy 1995, Wilison 1996, Williamson 1993). The N-terminal domain (loops 1-3) of TIMP-1 and TIMP-2 is sufficient for MMP inhibition (Willenbrock 1993). The first 22 amino acids at the N-terminus are present in all three TIMPs (Uria 1994). However, it is likely that multiple sites of TIMPs have the ability to interact with MMPs.

DISTINCT SECONDARY SITES OF MMP/TIMP INTERACTION

Although all TIMPs can inhibit all MMPs, TIMP-2 forms specific complexes with the latent forms of MMP (proMMP) (Goldberg 1992, Imai 1996, Murphy 1995). In addition to the catalytic domain of MMPs, TIMPs may bind to other distinct sites on the MMP. The function of these multiple sites of interaction is not yet fully understood but these interactions may reflect additional functions for TIMPs in controlling the stability and activation of specific MMPs. Studies have suggested

Figure 4.2.Comparison of the structures of the Timp-3 (top row) and Timp-1 genes (bottom row). All features of the Timp-3 gene are indicated in bold type, whereas those of the Timp-1 gene are italicized. The exons are represented by the amino acid sequences they encode and are enclosed in boxes. Conserved cysteine residues are in bold type and indicated by solid circles. Arrowheads indicate that the splice junction splits the codon and that the split is after the first nucleotide in this codon. The asterisk indicates the only published splice site for the TIMP2 gene, this splits the relevant codon of TIMP-2 in an identical fashion. Other splice junctions are between codons. Potential N-linked glycosylation sites in TIMP-1 are underlined, while a single site in TIMP-3 is overlined. The sequences of murine TIMP-3 and TIMP-1 were aligned using the DNASTAR software with gaps introduced for optimal alignment (Apte 1995).


TIMP-3
TIMP-1
M - - - - - - - - T P W L G L V V L L S C
M M A P F A S L A S - - G I L L L L S -
exon 1
exon 2
W S L G H W G A E A C T C S P S H P Q D
- L I A - - S S K A C S C A P P H P Q T
A F C N S D I V
A F C N S D L V
.....intron 1
.....intron 2
I R A K V V G K K L V K
I R A K F M G S P R I N
exon 2
exon 3
E G P F G T L - - V Y T I K Q M - K
E T - - - T L Y Q R Y K I K M M T K
.....intron 2.....
.....intron 3.....
M Y R
M L K
G F - - - S K M P H V Q Y I H T E A S E
G F K A V G N A A D I R Y A Y T P V M E
exon 3
exon 4
S L C G - - L K L E V N K Y Q Y L L T G
S L C G Y A H K S Q N R S E E F L I T G
.....intron 3.....
.....intron 4.....
R V Y E G K M Y T G L C N F V E R W D H
R L R N G K F H I N A C S F L V P W R T
exon 4
exon 5
L T L S Q R K G L N Y R - Y H L G C N -
L S P A Q Q R V F S K K N Y S A G C G V
C K
C T
.....intron 4.....
.....intron 5.....
I K S C Y Y L P C F V T S K N E C L W
V F P C L S I P C K L E S D T H C L W
exon 5
exon 6
T D M L S N F G Y P G Y Q S K H Y A C I
T D Q V L V - G S E D Y Q S R H F A C L
R Q K G G Y C S W Y R G W A P P D K S I
P R N P G L C T W - - - - - - - - - - -
S N A T D P .
- R S L G A R

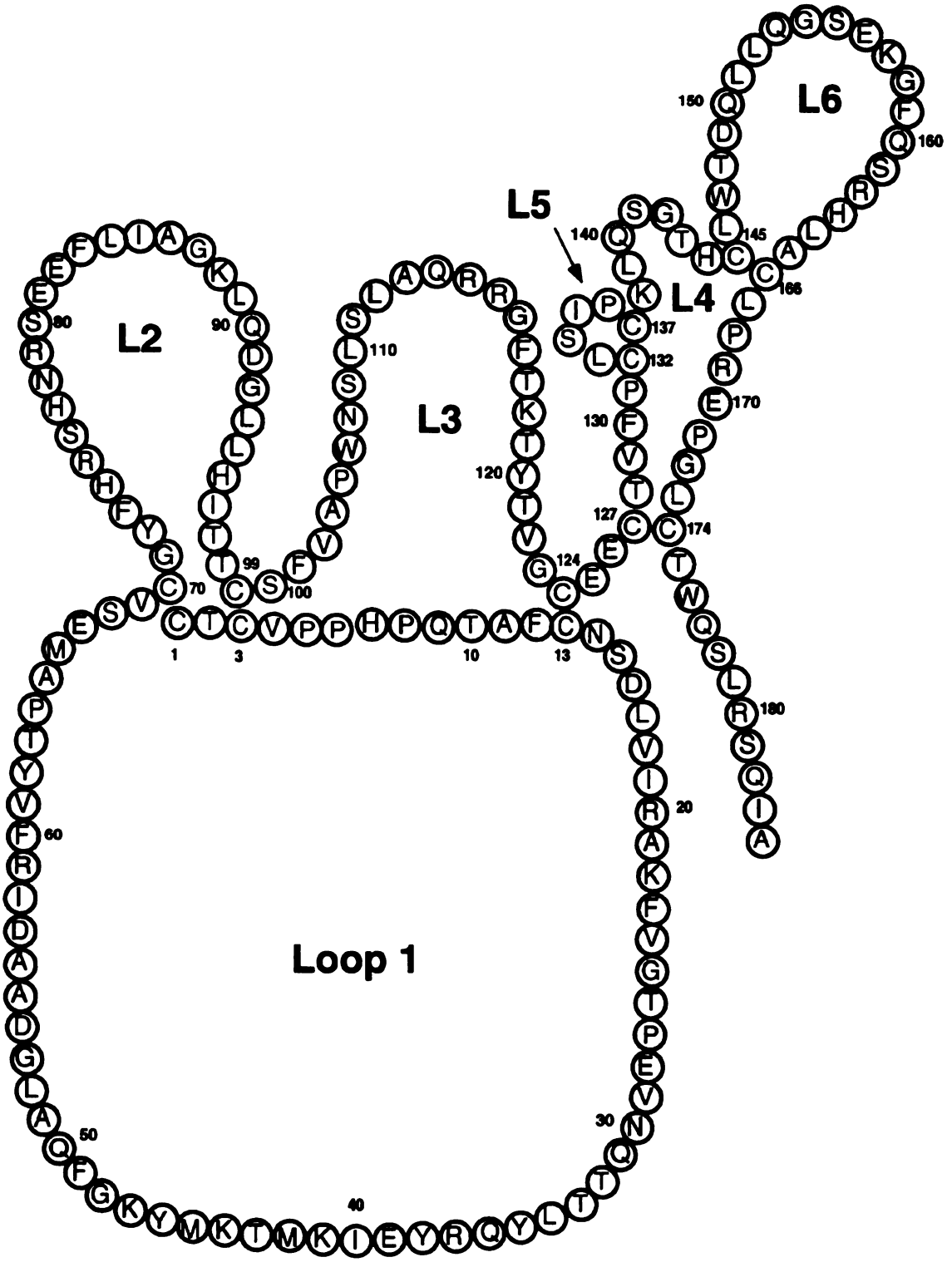


Figure 4.3. Protein folding in TIMP-1, showing the conserved cysteines and formation of loops in TIMP (Caterina 1997).

that TIMP-2 has the ability to form complexes with the pro form of type I collagenase and gelatinase A. In the case of type I collagenase, TIMP appears to stabilize the enzyme, however, this stabilization is not observed in gelatinase A

The interaction of TIMP-2 and the proform of type I collagenase (proMMP-1) results in the stabilization of the enzyme. TIMP-2 binds to the hemopexin domain of proMMP-1. This interaction blocks autoactivation of MMP-2, however, the proMMP-1:TIMP-2 complex can still be activated by cleavage by stromelysin. Once the MMP-1:TIMP-2 complex is activated, a second TIMP (TIMP-1, -2, or –3) is required for inactivation.

The carboxy-terminus of TIMP-2 will interact with the carboxy-terminus of gelatinase-A (MMP-2) (Willenbrock 1993). The proMMP-2:TIMP complex is a product of the C-terminus domain of TIMPs, as show via studies using truncated proteins that contain only the N-terminal domain (Murphy 1991). This interaction was shown to yield an increased rate of MMP-2 inactivation by a factor of 100. A schematic representation of the interaction of TIMP-2 and gelatinase A by their C-termini is presented in Figure 4.4. This interaction is involved in the activation of gelatinase A at cell surfaces, it is mediated by MT-MMPs, and is discussed in greater detail in chapter 6.

TIMP-3 IS BOUND TO THE ECM

A striking feature of TIMP-3 and chIMP-3 is that they are ECM-bound, whereas both TIMP-1 and TIMP-2 are freely diffusible, extracellular proteins (Hammani 1996, Uria 1994, Williamson 1993). The restricted localization of these proteins suggests that they may have the greatest potential to modulate pericellular proteolysis (Uria 1994). The molecular basis for the ECM association of TIMP-3 continues to be elusive but one interesting possibility has surfaced. Both TIMP-3 and chIMP-3 have motifs composed of seven amino acids flanked by two basic

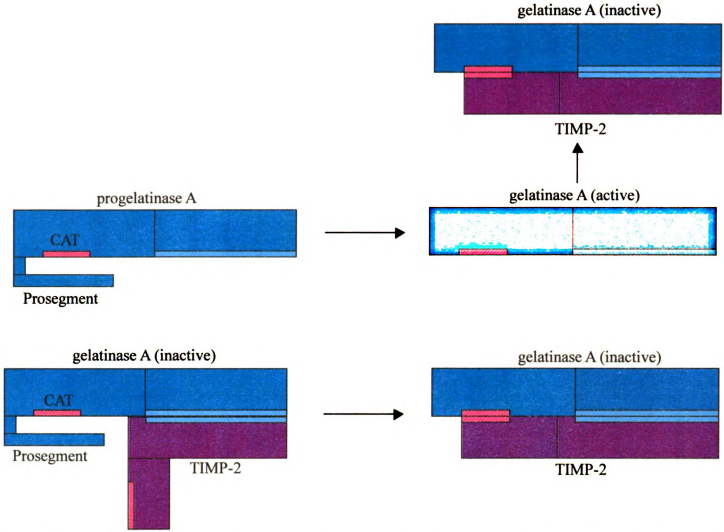


Figure 4.4. A schematic representation of the interaction of TIMP-2 and gelatinase A by a site distinct from the catalytic site of MMP-2.

amino acids, or B(Xaa7)B motifs, B is a basic amino acid, here lysine (K) or arginine (R), and Xaa is any other amino acid) These motifs have been implicated as having a close association with hyaluronic acid (Yang 1994). TIMP-3 has three of these B(Xaa7)B motifs, one is in the N-terminus domain and two are juxtaposed in the C-terminus domain. Since these B(Xaa7)B motifs are not found in TIMP-1 and TIMP-2, a strong possibility exist that TIMP-3 association with the ECM is meditated by the interactions of this B(Xaa7)B motif and hyaluronic acid or other related glycosaminoglycans (Yang 1994).

PREFERENTIAL INHIBITION OF MMP BY TIMP

Despite the fact that all MMP family members, by definition, are inhibited by TIMPs, whether different TIMP species show preferential inhibition of particular active MMPs, is still very much open. TIMP-2 has been shown to be more effective at inhibiting both gelatinase-A and gelatinase-B, than TIMP-1 (Howard 1991). TIMP-1, on the other hand, is more effective in the inhibition of interstitial collagenase than TIMP-2. TIMP-2 will bind to gelatinases approximately 10 times faster that TIMP-1 (Howard 1991). The preferred inhibition of gelatinase B by TIMP-3 has also been observed in mouse embryos in the process of implantation. Studies have identified gelatinase-B as an enzyme that is essential for the cellular invasion involved in embryo implantation. Gelatinase B is expressed principally by trophoblast giant cells in implanting (7.5 days post coitum) mouse embryos (Harvey 1995). At this time, there is a high level of expression of TIMP-3 that is restricted to maternal decidual cells immediately adjacent to the implanting embryo (Harvey 1995). This suggests that TIMP-3 forms a protective shield that is deployed by the uterus to contain the highly invasive embryo. Thus, TIMP-3 may be the inhibitor of choice in tissue remodeling situations that involve transient, high level production of gelatinase-B, the added advantage being that it provides a mechanism for spatial

restriction of MMP activity by virtue of its ECM-association. Preferential inhibition of the various TIMPs for different MMPs provides a fine tuning mechanism by which to control specific ECM turnover.

DIFFERENTIAL EXPRESSION OF TIMP

Additional information regarding the differential expression of TIMPs during development has provided further evidence for the specific role of these inhibitors. In adult mice TIMP-1 is preferentially expressed in epithelial tissue, cartilage and muscle. In contrast, during murine embryonic development, TIMP-3 is preferentially expressed in these same tissues (epithelial, cartilage, and muscle), while TIMP-1 is expressed specifically in the bone (Hammani 1996). The phorbol ester, 12-O-tetradecanoylphorbol-13-acetate (TPA), can induce the expression of TIMP-1 and TIMP-3. A similar induction of TIMP-1 and TIMP-3 is observed by treatment with transforming growth factor β (TGF-β). However, TPA and TGF-β down regulate TIMP-2 production (Khokha 1992, Lim 1996, Wilson 1996). In macrophages, lipopolysacharides have been identified as up-regulators of TIMP-2 and the down-regulators of TIMP-1 (Lim 1996). Together, these observations suggest that each member of the TIMP family has a specific physiological role.

REGULATION OF THE TIMP GENES

The *in vivo* patterns of expression of the three TIMP genes are distinct (Nomura 1989). In the mouse, the expression of TIMP-1 has been localized to areas of active ECM remodeling, such as, sites of osteogenesis in the developing embryo, and the uterus and ovaries of adult females. TIMP-2 shows a distinct accumulation in the placenta just prior to birth. TIMP-3 mRNA transcripts are found at high levels in certain adult tissues that lack expression of other TIMPs, including the kidney and the brain (Leco 1994). In the developing mouse embryo,

TIMP-3 transcripts are abundant in the surface epithelia of organs, such as, the developing bronchial tree, kidney, colon and esophagus that have extensive tubular structures (Apte 1994). These studies further support the notion that the individual TIMPs perform specific and non-interchangeable tasks. The patterns of stimulus-responsiveness and tissue-specific expression of the three TIMPs differ dramatically. The transcription sites AP-1, and PEA-3 are found within the promoters of the many TIMP gene promoters. Interestingly, AP-1 and PEA-3 sites are also found in the promoter regions of several MMP genes. The production of TIMP-1 and TIMP-3 is induced by the activation of AP-1 and PEA-3 sites in response to treatment with TPA or TGF-β (Lim 1996, Fabunmi 1996, Grant 1996). This suggests that AP-1 is a critical factor that may serve to coordinate the responses of MMP and TIMP genes. In contrast, TIMP-2 is not induced by TPA and TGF-β and has a very diffrent regulation mechanism (Campbell 1991), indicating the possibility that different elements are at work in the promoter of TIMP-2. The anti-inflammatory glucocorticoid dexamethasone strongly induces TIMP-3, whereas it inhibits TIMP-1 and TIMP-2 expression (Leco 1994). A comparison between the 5′-flanking regions of the three TIMP genes is presented in Figure 4.5. The following section describes some of regulatory elements associated with the promoters of TIMP genes.

The expression of TIMP-1 is largely confined to the adult bone and the ovary and to tissues undergoing remodeling or inflammation. In culture, serum and growth factors modulate the expression of TIMP. The promoter of the TIMP gene contains approximately six exons and does not possess a TATA box sequence. Elements associated with the TIMP-1 gene confer a transcriptional activation by viruses, serum, phorbol esters, and TGF-β. TIMP-1 has two AP-1 sites and PEA-3 response element. The AP-1 sites are inducible by interaction with both heterodimers of c-*fos*/c-*jun* or homodimers of c-*jun*. The PEA-3 response element is activated by c-*ets*, however, this element is dependent on AP-1 activation. Therefore, this PEA-3

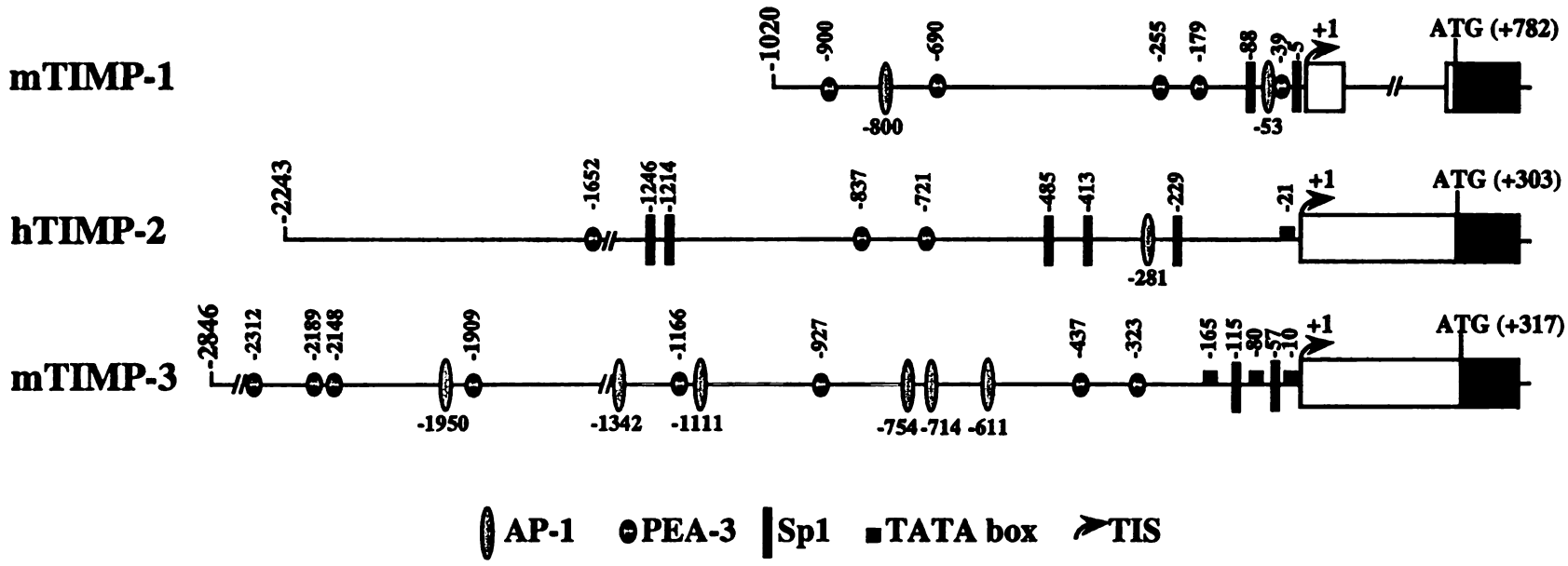


Figure 4.5. Comparison between the 5-flanking regions of the three TIMP genes. Putative transcriptional regulatory elements are shown in the promoter of the hTIMP-2 gene and compared with those found in the murine TIMP-1 gene and the murine TIMP-3 gene. The positions of these elements are determined from the position of the major transcriptional start site in the three genes. The first two exons in the murine TIMP-1 gene and the first exon of the hTIMP-2 and murine TIMP-3 genes are represented by boxes in which the coding part is shown as a black box with the position of the initiator ATG codon indicated. A 0.8-kb sequence corresponding to the 5′-end of the human TIMP-1 gene contains features similar to those found in the murine TIMP-1 gene (Hammani 1996).

response element may have the same fine tuning function as PEA-3 in the collagenase gene. The interaction of AP-1 and PEA-3 elements in the transcription of TIMP-1 suggests that multiple signal transduction pathways coordinate and regulate TIMP-1 production.

The promoter region of TIMP-2 is associated with one AP-1, two AP-2 and three PEA-3 binding sites. The AP-1 biding site appears non-responsive to treatment with TPA. The position of this AP-1 site is relatively close to the transcriptional start site, and may be non-responsive due to its position. The production of TIMP-2 has been shown to be down regulated by lipiopolysaccharides in macrophages and upregulated by cAMP in human fibroscarcoma (HT1080) cells. The AP-2 binding sites have been shown to mediate the cAMP response in many genes and two of these sequences are found in the promoter of TIMP-2. Lipopolysaccharides have been shown to affect gene regulation via interleukin 6 (IL-6). IL-6 activates a nuclear factor (NF) known as NF-IL6 and there are two NF-IL-6 recognition sequences associated with the promoter of TIMP-2 (Hammani 1996). These differences of TIMP-2 gene expression are likely to result in the specific role TIMP-2 plays in the inhibition of MMP.

The promoter region of TIMP-3 gene is very extensive. Computer analysis of cloned TIMP-3 promoter has revealed six AP-1 binding sites, two nuclear factor NF-κB sites, one *c-myc* site, and two copies of a p53-binding motif separated by eight base pairs with two mismatches at the second motif, along with many other *cis* elements (Sun 1995). TIMP-3 gene expression is inducible by TPA, and TNF-β just like TIMP-1. These stimulatory agents transcactivate via AP-1 and NF- κB sites. It is, however, possible that the NF-κB site provides the mechanism for the distinct temporal pattern of expression seen between TIMP-1 and TIMP-3.

TIMP EFFECTS ON CELL GROWTH

In addition to its structural role, the ECM is an information-processing medium that controls cell position, identity, proliferation and fate. This functional role can be the result of a direct interaction with cells since cells are often dependent on their contact with the ECM. In addition, the ECM also provides a contextual framework for the interaction of growth factors and cytokines with their signaling receptors (Murphy 1995). Given the importance of TIMPs as regulators of the rate of ECM turnover, it is reasonable to propose that they exert some influence on ECM-dependent cell signaling.

In the mammary gland, cells depend on instructive signals from the underlying basement membrane. Are the activities of mammary cells directed by the expression of TIMP or MMP? In the case of mammary gland, TIMPs are expressed at high levels during lactation where they promote cell growth and milk production (Talhouk 1992). Increased expression of MMP-2 is seen during mammary involution with a decrease in TIMP production. By protecting the basement membrane, TIMPs maintain the ECM integrity required during lactation. Lactation ceases when the protective role of TIMP is overcome by excess MMPs leading to basement membrane destruction, and mammary gland involution. Several proteins expressed during mammary gland lactation and involution such as casein, gelatinase A, gelatinase B, stromelysin-1, and TIMP. Casein is a milk protein and its expression is indicative of lactation. The expression of casein (lactation) appears to be protected by the expression of TIMP against the expression MMPs, initially. However, as the levels of gelatinase A, gelatinase B, and stromelysin-1 increase the level of casein decreases. The levels of TIMPs appear to go up initially in response to MMP expression but in a short time the TIMPs inhibition is overcome, lactation ceases and mammary involution begins.

In all of the situations discussed above, any effects of TIMPs on cell growth would be exerted indirectly via the ability of TIMP to modulate MMP. Perhaps the strangest aspect of the family of TIMP is that they may be directly involved in the control of cell proliferation. This controversial function was first identified in TIMP-1. TIMP-1 is an Erythroid Potentiating Activator (EPA) and can stimulate both *in vivo* and in *in vitro* growth of erythroid precursors (Hayakawa 1992). Several subsequent studies have suggested TIMP-1 is mitogenic for a number of cell types including fibroblasts, keratinocytes and a number of hematopoetic cell types. The EPA function has also been observed for TIMP-2. A broad-spectrum mitogenic activity, optimally stimulating the growth of human gingival fibroblasts and Raji Burkitt's lymphoma cells at small concentrations, has been proposed as additional functions of TIMP-2 (Hayakawa 1994). Alklinization of TIMP-1 and –2 blocks their inhibitory effects on MMPs, but does not affect their growth promoting activities. This suggests that this activity is distinct from their MMP inhibitory activity. Progelatinase-A/TIMP-2 complexes, which retain anti-MMP activity against endogenous active MMPs, are ineffective growth promoters. Since TIMP-2 interacts with progelatinase A by the carboxy terminus, this finding suggests that the growth promoting feature of TIMP-2 is associated with the carboxy terminus (Hayakawa 1994). These studies argue that at least some of the effects of TIMPs on cell growth involve functions that are distinct from their MMP inhibiting activity.

There is also evidence to suggest that under certain conditions TIMP-1 may inhibit cell growth. Originally, Khokha (1992) suggested the importance of the maintenance of TIMP balance by showing that an artificially-mediated reduction of TIMP-1 in Swiss 3T3 cells conferred an oncogenic phenotype (Khokha 1992). Later

studies revealed that TIMP-1 overexpression in B16FI0 mouse melanoma cells reduced their metastatic abilities, but that this effect might not be solely attributed to diminished cellular invasiveness (Khokha 1994). Rather, there is evidence for a suppressive effect of TIMP-1 on the growth of melanoma cells after extravasation from the circulation (Koop 1994). A study using intravital videomicroscopy, to measure the time course of extravasation, was used to examine B16F10 cells transfected with the TIMP-1 gene and the wild type B16F10 cells. It was expected that the wild type B16F10 cells would extravasate faster because they expressed MMPs. In contrast, this study showed that both cells extravasated at similar rates. However, they did see an increased time for the initial division for the B16F10 cells that were tranfected with the TIMP-1 gene (Koop 1994). This observation indicates TIMP-1 has some inhibitory effects on cell growth.

Nothing is currently known about the effects of mammalian TIMP-3 on cell growth but chicken TIMP-3 promotes the growth of non-transformed chicken fibroblasts in low serum conditions. Currently, the mechanisms for both the induction of cell growth as well as the modulation of this activity are unknown. Elevation of cAMP levels following activation of adenylate cyclase by a G-protein-coupled mechanism is suggested to be a potential signaling pathway involved in the mitogenic effects of TIMP-2 on human HS68 fibroblasts and HT1080 fibrosarcoma cells (Tanaka 1995). Despite these intriguing findings there is still widespread skepticism concerning the growth promoting effects of TIMPs. This issue is unlikely to be resolved until TIMP receptors and signal transducers are identified and characterized at the molecular level.

APPLICATIONS

Matrix metalloproteinases play an important role in a number of normal and pathologic human conditions. TIMPs appear to block the destruction of ECM by MMPs. TIMPs have been show to block invasion and metastasis (Declerck 1992, Duncan 1996, Ito 1996, Motgomery 1994, Nuovo 1995). They also have anti-angiogenic and tumor suppressive properties (Dunsmore 1996). Clinicians and researches endeavor to apply the knowlege of TIMP to human health care. MMPs have become targets for anticancer, antiarthritic, and opthalmological therapies. Several agents, namely tetracyclines and synthetic inhibitors of MMP, such as batimastat (BB-94), are currently under clinical investigation for the treatment of periodontal disease, arthritis and cancer.

Batimastat, ({4-N-hydroxyamino}-2R-isobutyl-3S-{thienylthiomethyl Succinyl}-L-phenylalanine-N-methylamide) (BB-94) is a member of the rapidly expanding class of synthetic inhibitors of MMPs. These drugs are designed to block MMP activity, and are currently under clinical investigation as anticancer agents. Through its ability to block stromelysin, gelatinase-A, gelatinase-B, and matrilysin, BB-94 has been shown effective in the inhibition of human cancers. The effect of BB-94 on breast cancer regrowth, using MDA-MB-4435 breast carcinomas in nude mice, is presented Figure 4.6 (Sledge 1995). The data show that BB-94 has a suppressive effect on tumor growth. Other studies indicate that treatment with BB-94 causes inhibition of ovarian cancer cell growth, prevention of B16-BL6 murine melanoma and human colon cancer cell metastasis, and inhibition of angiogenesis (Sledge 1995).

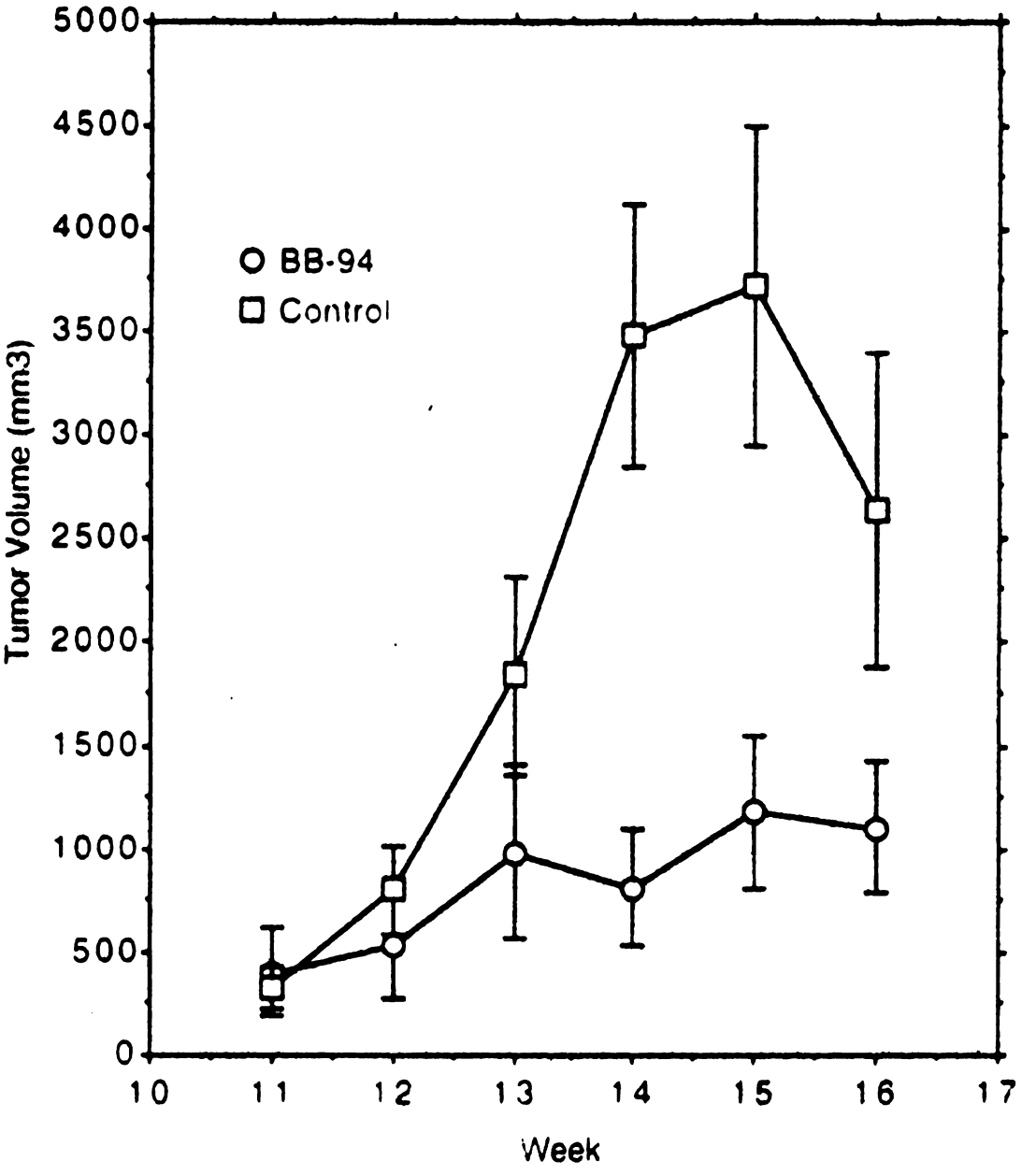


Figure 4.6. Effect of batimastat (BB-94) treatment on MDA-MB-435 primary tumor regrowth in athymic nude mice. Tumor growth is measured by tumor volume, treatment with batismastat decreased tumor growth (Sledge 1995).

CONCLUSIONS

TIMPs are multifunctional proteins. It is well established that TIMPs modulate the activity of MMPs by the formation of a one to one complex that interferes with the catalytic activity of MMPs. The role of TIMP in the regulation ECM turnover is well accepted and researchers and clinicians are rushing to apply the current knowledge to modern medicine. Conversely, In some cases, TIMP-2 actually aids in the activation of progelatinase A. In addition, it has been suggested that TIMPs play an important role in cell proliferation. It is clear that the maintenance of the ECM could help to maintain some cell growth or at least inhibit cell death as is seen in mammary gland involution. However, one controversial observation suggests that TIMP may have mitogenic function independent of their MMP regulatory function. Further study of this multifunctional protein will provide important information that can be applied towards the treatment of connective tissue diseases as well as cancer. The next chapter examines the roles of MMP and TIMP in cancer invasion and metastasis.

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CHAPTER 5

THE ROLE OF MATRIX METALLOPROTEINASES IN INVASION BY CANCER CELLS

ABSTRACT

Remodeling of the extracellular matrix (ECM) is a prerequisite to cellular invasion. Matrix metalloproteinases (MMPs) have been associated with the process of ECM turnover, and are thought to play a critical role in tumor cell invasion and metastasis. The overexpression has been correlated with tumor progression in many cancers. Specifically, the uncontrolled expression of gelatinase A, gelatinase B, and stromelysin-1 is believed to be critical because of their ability to degrade type IV collagen, a major component of basement membranes. This review will focus on gelatinase A and gelatinase B, and the stromelysins (stromelysin-1, -2, -3 and matrilysin), because of their ability to degrade type IV collagen. This review also examines MT-MMPs, an integral membrane MMP, that has been implicated in the activation of gelatinase A. By examining the distribution, expression, and activation of these MMPs, this review sheds light on the their role in invasion and metastasis.

INTRODUCTION

A benign tumor grows within the confines of a basement membrane, can progress to a malignant tumor, which traverses the basement membrane, and invades the surrounding stromal tissue. Further progression of the carcinoma from invasion to metastasis requires the intravasation into, and the extravasation out of the blood circulatory and lymphatic systems. These systems serve as highways for the dissemination of malignant tumor cells to potential secondary, metastatic tumor sites. The cancer cells must now invade a new site, again crossing basement membranes to seed themselves into the secondary tissue. In this metastatic site the

tumor must grow and establish itself away from its original cellular environment. The ability of cancer cells to penetrate the barriers of multiple basement membranes and the stromal extracellular matrix strongly indicates the presence of proteolytic enzymes capable of extensive degradation of matrix proteins.

The matrix-degrading metalloproteinases (MMPs) are a family of secreted, zinc and calcium-dependent, proteolytic enzymes that have the ability to degrade components of the extracellular matrix at physiological pH. MMP activity is thought to be important in processes that involve tissue remodeling and tissue invasion (Edwards 1996). In addition, MMPs have been associated with several pathological conditions involving tissue destruction, including rheumatoid arthritis and cancer invasion and metastasis (Mignatti 1996). Based loosely on their substrate specificity the family of MMPs has been traditionally divided into at least three major classes, which include the collagenases, the gelatinases and the stromelysins (Pajouh 1991). All of the members of the MMP family possess conserved functional and structural domains, which translate into similar mechanisms of latency, activation and proteolysis (Murphy 1995). The amino terminal or activation locus and the catalytic site that binds the zinc atom are the sites of greatest homology within this family (Matrisian 1990). Except for matrilysin, the carboxy terminal region of all MMPs has a hemopexin/vitronectin-like domain that has been shown to be important in the binding of substrates and inhibitors. A review of the MMP family is presented in Table 5.1 (Corcoran 1996). The gelatinases have a fibronectin type gelatin-binding domain which contributes to their substrate specificity. Both gelatinase A and gelatinase B degrade type IV collagen (Conway 1996). Stromelysins are made up of four MMPs, three of which are normally secreted by stromal cells (Stromelysins -1, -2, -3). All of the stromelysins, including matrilysin, have a broad substrate specificity that includes the non-helical region of type IV collagen as well as additional components of the extracellular matrix, such as, fibronectin and vitronectin

Table 5.1. MMP Family, including subgrouping, nomenclature, mRNA & protein size and major substrates

Subgroup	MMP	Nomenclature	mRNA kb	Protein kD	Substrate(s)
Interstitial collagenase	MMP-1	Fibroblast	2.0	52,57	Fibrillar collagen (III > > I)
	MMP-8	PMN	3.3	75	Fibrillar collagen (I > > >III)
	MMP-13	Collagenase-3	2, 2.5, 3	54	Fibrillar collagen
Stromelysin	MMP-3	Stromelysin-.1	1.9	52, 58	Lam, FN core protein, nonhelical collagen
	MMP-10	Stromelysin-2	1.7	58	Same as above
	MMP-11	Stromelysin-3	2.2	29	α1 Antitrypsin
	MMP-7	Matrilysin	1.1	28	Similar to MMP-3
Gelatinases	MMP-2	Gelatinase A	3.1	72	Gelatin, Col I and IV, FN, Vn, elastin, β-emploid
	MMP-9	Gelatinase B	2.3	92	Gelatin, Col IV, Col V
MTMMP	MMP-14	MTMMP-1	4.5	63	Pro-gel A
	MMP-15	MTMMP-2	3.6	72	?
	MMP-16	MTMMP-3	2.1	64	Pro-gel A
Elastase	MMP-12	Metalloelastase	1.8	53	Elastin

(Corcoran 1996).

(Conway 1996). Matrilysin, a special member of the stromelysin group, has strong proteolytic activity and is produced by glandular epithelia (Wilson 1996). Stromelysins are also important because they have the ability to activate other MMPs including gelatinase B. Membrane-type matrix metalloproteinases (MT-MMPs) contain a transmembrane domain which facilitates cell-surface activation of gelatinase A (Imai 1996). This review will focus on the gelatinases, and stromelysins because of their ability to degrade type IV collagen and other components of the basement membrane. Although MT-MMPs have not been identified as MMPs that degrade type IV collagen, they will be considered in this review because of their role in the activation of gelatinase A (MMP-2).

DISTRIBUTION OF MMPS INVOLVED IN BASEMENT MEMBRANE INVASION

The normal distribution patterns of MMP expression divides the family of MMPs into three distinct categories (Table 5.1). First, there are MMPs that are primarily expressed by the stromal cells, these include, stromelysins-1, -2, -3, the interstitial collagenases, and gelatinase A (Crawford 1995, Sato 1992). Second, inflammatory cells express MMPs including neutrophil collagenase, gelatinase B, and macrophage metalloelasetase. Expression of gelatinases A and B has also been observed in epithelial cell types. Third, matrilysin is expressed in normal epithelial tissue. Matrilysin is the only known MMP constitutively expressed by normal epithelial cells (Wilson 1996). This section examines the distribution patterns and substrate preferences of gelatinases, stromelysins and MT-MMPs.

Gelatinase A

A 72 kDa type IV collagenase, called gelatinase A, or MMP-2 (Table 5.1) is constitutively secreted by human skin fibroblasts and a number of human normal and tumorgenic cell types. This enzyme catalyzes the cleavage of the α-helical

domain of type IV collagen, a major component of the basement membrane. The substrates for gelatinase A in the order of preference are gelatin, type IV collagen, type V collagen, fibronectin, type VII collagen, and elastin (Murphy 1993). Gelatinase A has been isolated from normal skin explants and several tumor cell types (Docherty 1992).

Gelatinase B

Gelatinase B (MMP-9) is a 92 kDa gelatinolytic enzyme (Table 5.1). Neutrophils and macrophages typically express this enzyme. Its normal expression is often associated with the inflammatory phase of wound healing. Overexpression of gelatinase B has been observed in epithelial cells and several invasive tumor cell types (Webber 1995). The substrates, in order of preference for gelatinase B are gelatin, type IV collagen, type V collagen, and elastin.

Stromelysins

Stromelysin-1 and –2 expression has been normally observed during tissue remodeling. In human wound healing both stromelysins and interstitial collagenases are expressed at sites of wound healing (Crawford 1996). Stromelysin-1 has been proposed to be involved in the remodeling of dermis as in the removal of granulation tissue, resolution of scar tissue as well as in the restructuring of the basement membrane around basal keratinocytes (Crawford 1996). It is expressed in the stroma and has the ability to cleave components of the ECM, such as fibronectin and vitronectin. Stromelysin-1 can activate several other MMPs including gelatinase B. Stromleysin-3 is expressed in the connective tissue of developing digits, involuting mammary glands, and the inflammatory stage of wound healing (Dochtery 1995).

Matrilysin

Human matrilysin expression is found in epithelial cells at the sheath of hair follicles, mammary and parotid glands, pancreas, liver and prostate (Wilson 1996). In contrast to other stromelysins, matrilysin expression is constitutive, and usually independent of tissue remodeling. This indicates a function distinct from other stromal MMPs, such as, proteolytic processing or degradation of peptides in the luminal space of glandular epithelium. Matrilysin has been found in a large number of cancers including breast, colon, stomach and prostate. It has also been associated with invading portions of squamous and basal cell carcinoma of the skin where its expression has been observed in early and late stages of tumor progression and shown to affect tumor invasion and tumorigenicity (Sato 1992)

MT-MMPs

A new subfamily of matrix metalloproteinases, the membrane-type matrix metalloproteinases (MT-MMPs) has been recently described. Membrane type MMPs (MT-MMPs) have the addition of a transmembrane domain in their protein structure. The presence of this domain results in their integration into the cell membrane so that the amino-terminus is positioned outside the cell while the carboxy-terminus is positioned inside the cell. MT- MMPs are activated by the Golgi serine protease furin. This is facilitated by the presence of a furin consensus sequence in the domain structure of MT-MMPs, resulting in their activation before they are expressed at the cell surface. It has been proposed that the function for MT-MMPs is to activate gelatinase A at the cell surface in order to have gelatinolytic activity at the leading edges (invadapodia) of invasive cells. The expression of MT-MMPs is temporally and spatially regulated, for example, in vascular and urogenital development, and in the development of osteocartilaginous and musculotendinous structures (Apte 1997). MT-MMPs are also expressed in human placental cells (Takino 1995). These MMPs are often expressed at the invadapodia of metastatic tumors (Corcoran 1996).

EXPRESSION OF MMP GENES

The expression of MMP genes is modulated by a number of factors including cytokines (IL-2, IL-6, and IL-10), growth factors (TGF-β, TNF-β, and bFGF) and proto-oncogenes (*c-fos, c-jun*, and *c-ets*). The observed distribution of MMPs in normal cells is, in part, reflected in the promoter elements associated with the genes that encode them. The signal transmitted by the activated protein kinase (PKC) is thought to be one of the common mechanisms of regulation in the expression of several MMP and TIMP genes (Howard 1991). This has been realized through studies involving the expression of these genes by treatment with the tumor promoter, TPA (Stearns 1993). The promoters of most human MMPs, except MMP-2 and Stromelysin-3, contain a TPA responsive element (TRE). This element binds to the *fos/jun*-containing complex of the AP-1 transcription factor (Sato 1992). This TRE facilitates the response of MMPs to TPA, IL-1, TNF-α, interferon-β (INF-β), and growth factors, such as, EGF and PDGF. However, the PKC pathway alone cannot explain the expression pattern of the genes in tumor cells.

All of the MMP promoters, aside from gelatinase A, contain multiple PEA-3 sites. The PEA-3 site was originally identified as an oncogene responsive *cis*-element in the polyoma virus enhancer (Pajouh 1991). The PEA-3 site binds members of the *c-ets* family of nuclear proto-oncogenes, which respond to *ras* and *src* activation (Gutman 1991). This PEA-3 site has been shown to be important for TPA-induced transcription. Studies have shown cooperativity between AP-1 and PEA3 sites (Gutman 1991). Mutation of either the AP-1 site or PEA-3 in the MMP promoter can reduce TPA and oncogene inducibility by greater than 50%, indicating a synergistic rather than additive relationship (Matrisian 1992). It is possible that PEA-3 serves as a fine tuning mechanism for AP-1 induction of MMP. Similar results have been observed in non-MMP enhancers and promoters (Crawford 1996).

The expression of Gelatinase A is not inducible by TPA. This is most likely due to the lack of a TRE in its promoter. Therefore, gelatinase A expression is not the result of AP-1 activity (Murphy 1995). In fact TPA treatment actually inhibits MMP-2 expression (Corcoran 1996). In addition, the promoter for the gelatinase A gene also lacks the PEA-3 transcription element (Corcoran 1996). In general, TGF-β decreases the synthesis of MMP. It has been suggested that this TGF-β suppression is mediated via a TGF-β-inhibitory element (TIE) on most of the promoters of MMP genes. The promoter for the gelatinase A gene lacks this TIE. In fact treatment by TGF-β appears to increase the expression of progelatinase A in a number of cell types (Corcoran 1996).

Unlike other MMPs, gelatinase A appears to be constitutively expressed in many cell types (Stearns 1993). Gelatinase A has a non-canonical TATA box and two SP1 sites (Templeton 1991). Other MMPs have canonical TATA boxes. The advantage of having a non-canonical TATA box is that it is easier to be recognized by transcription factors. This sort of promoter is typical of a "house keeping" gene, and exhibits a rather promiscuous expression pattern, suggesting a role in cell survival. This sequence has been presented as the basal promoter of gelatinase A (Templeton 1991).

While general growth-related elements are relevant to MMP expression, it is important to note that this more restrictive style of promoter lends itself to tight regulation by a variety of potential promoter and enhancer elements that have yet to be identified in MMP genes. Therefore, it is expected that elements that control tissue-specific expression will be identified, in addition to elements that are specific targets of other signal transduction pathways.

MECHANISMS OF MMP ACTIVATION

The biochemical mechanism for initiating the catalytic activity of mammalian MMPs is believed to occur via a cysteine switch mechanism (Murphy 1995). According to this theory, an unpaired cysteine residue in the pro-domain of the enzyme keeps the zymogen in the latent pro-form by direct coordination with the zinc atom bound to the catalytic site of MMPs. Four mechanisms for the *in vivo* activation of these MMPs have been proposed. The first is by the extracellular activation of MMPs by proteolytic enzymes. The second is by an autoactivation mechanism. The third proposed method of activation involves the Golgi network serine protease furin. Recently, a fourth mechanism of activation has been described, as the activation of MMP-2 via MT-MMP. Each of these suggested methods of MMP activation will be explored in relation to degradation of the basement membrane in cancer invasion.

Activation of MMPs by proteolytic enzymes

Proteases such as, trypsin, plasmin, urokinase, and stromelysin-1 can cleave the prodomain, of most MMPs. The plasmin cascade was one of the first *in vivo* mechanisms identified for the activation of MMPs. This mechanism appears to be involved in the activation of progelatinase B, and prostromelysin-1 and -2. Plasminogen, when exposed to urokinase-type plasminogen activator (uPA), yields the active serine protease plasmin (Waghray & Webber 1995). Plasmin can cleave 84 amino acids (the prodomain) from prostromelysin resulting in a fully active stromelysin (Figure 5.1) (Corcoran 1996). This cascade may occur locally at the cell surface via the uPA receptor or in the ECM. Active stromelysin will also cleave the prodomain of MMPs. Stromelysin-1 has been observed to activate type I collagenase and gelatinase B. Recently, matrilysin was reported to activate stromelysin-1 and gelatinase B. The activation of stromelysin-1 by matrilysin was

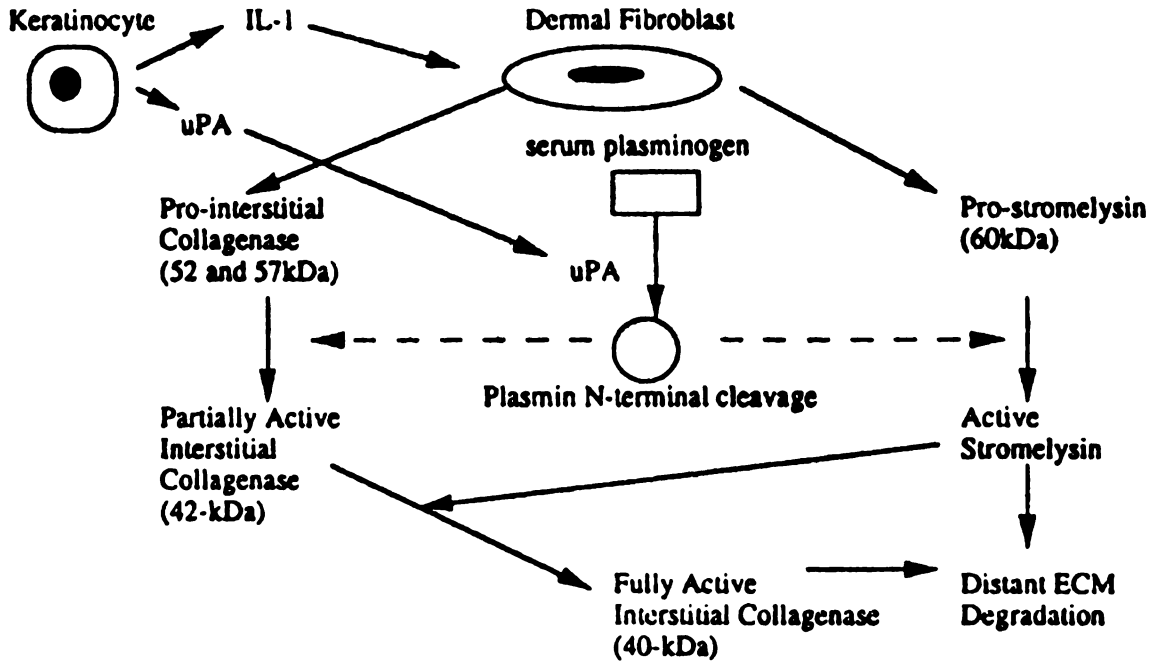

Figure 5.1. Actvation of MMP by plasmin (Corcoran 1996).

100% efficient and is similar to plasmin activation of stromelysin. Conversely, proteolytic activation of pro-gelatinase B by matrilysin is only 50% efficient, this may be due to the fact that matrilysin cleaves progelatinase B at a site distinct from the plasmin cleavage site. Gelatinase A and plasmin also activate pro-gelatinase B (Corcoran 1996).

Autoactivation of MMPs

Autoactivation of MMPs is also possible. The "cysteine switch" mechanism can be triggered by perturbation of the pro-MMP by certain chemicals, such as, 4-aminophenylmercuric acetate (APMA) (Bu 1995). This disruption initiates a cascade of events that alters the proMMP protein conformation and allows for the autoproteolytic cleavage of a portion of the pro-domain, yielding a partially activated enzyme. Autoactivation of the protease can also be caused by the deletion of the arginine or cysteine residues in the pro-domain of MMPs. Loss of the amino-terminal pro-domain is evidence of, but not equivalent to, an active enzyme species. The autoproteolytic activation of proMMP suggests that the ability to cleave peptide bonds is acquired prior to removal of the pro segment. Synthetic peptides that mimic the pro-domain (PRCGXPDV sequence) have been previously shown to inhibit not only enzymatic activity (Nomura 1993) but also invasion by cancer cells (Melchiori 1992). Although *in vitro* studies have aided in understanding some details for activation of these MMPs, the mechanisms of *in vivo* activation of these enzymes is still under investigation.

Activation of MMP by furin

Activation of MMPs by furin occurs prior to secretion of the MMP. Furin is a Golgi network serine protease that recognizes proteins with a RXKR sequence. This furin recognition sequence is present in MT-MMPs and stromelysin-3 and is

located between the pro-domain and catalytic domain. Cleavage of this domain by furin results in a fully active form of stromelysin-3 and MT-MMP. Since furin activates these MMPs in the secretory pathway, their proforms are not normally present in the extracellular space.

Mechanism for the activation of progelatinase A

While some investigators report that the plasmin cascade is also responsible for activation of progelatinase A , others have found that plasmin actually degrade this enzyme. Since cellular activation of this enzyme occurs in the presence of inhibitors of serine proteases and plasmin, the plasmin cascade may not be the mechanism utilized *in vivo*. Unlike other MMPs, the activation of progelatinase A has been shown to occur at the cell surface. A membrane-associated specific activator of progelatinase A has recently been described (Imai 1996). The activation of progelatinase A by membrane preparations from cells treated with these agents can be blocked by treatment with metalloproteinase inhibitors (including metal chelators such as EDTA or O-phenanthroline), as well as TIMPs, suggesting that the receptor involved in this activation may be an MMP.

Membrane Type Metalloproteinase (MT-MMP) has been suggested as the possible *in vivo* activator of progelatinase A. MT-MMP activates progelatinase A (Imai 1996). Molecular manipulation of MT-MMP has revealed that its transmembrane domain is needed for gelatinase activation. When this domain is deleted or replaced, MT-MMP is soluble and fails to activate progelatinase A (Chen 1996). The working hypothesis for MT-MMP activation of gelatinase A, as suggested by Corcoran and collaborators (Corcoran 1996), is presented in Figure 5.2. This Figure describes the membrane-associated activation of progelatinase A. TIMP-2 mediates the binding of pro-gelatinase A to the cell surface by virtue of an interaction between either MT-MMPs directly or by an unknown separate integral

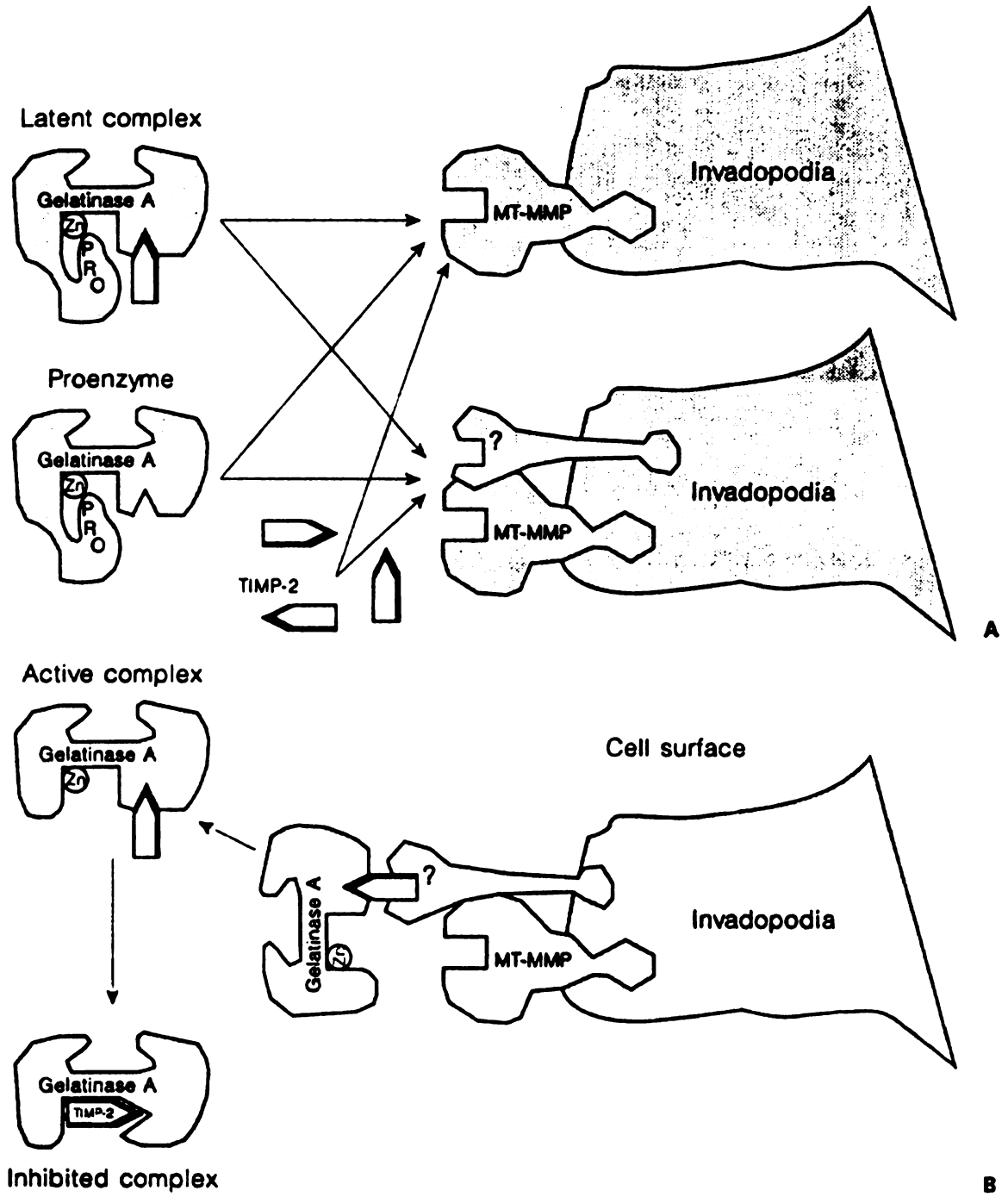

Figure 5.2. The activation of gelatinase A by MT-MMP and TIMP-2. The biniding of TIMP-2 may be mediated by an unknown integral membrane protein (?) (Corcoran 1995).

membrane protein. Once localized to the cell surface, MT-MMP acts on the progelatinase A / TIMP-2 complex, and the result is a cell bound activated gelatinase A / TIMP-2 / MT-MMP complex. When this complex dissociates from the cell surface TIMP-2 rapidly inactivates gelatinase A. This cell-mediated mechanism for activation of gelatinase A would favor the degradation of the basement membrane at the leading edge of invasive cells.

EVIDENCE FOR THE ROLE OF MMPS IN INVASION THOROUGH THE BASEMENT MEMBRANE

The mechanisms controlling the metastatic progression of a localized tumor are very complex, and involve many biochemical and cellular events. One likely event is the expression of MMPs capable of degrading the basement membrane. Degradation of the basement membrane, which is mainly composed of type IV collagen and laminin is mediated by a set of secreted MMPs that include gelatinase A (MMP-2), gelatinase B (MMP-9), Stromelysin-1 (MMP-3) and Matrilysin (MMP-7). Aberrant expression and distribution of these MMPs is a critical part of the multifactorial invasive nature of tumor cells. The increased expression of MMPs that degrade the basement membrane has been associated with increasing degree of invasiveness of tumor cells. In mesenchymal cells, MMP-2 MMP-9 and MMP-3 mRNA transcripts are expressed directly in proportion to their invasive potential (Sato 1992).

Late stage malignant cell types have been shown to express stromelysins and interstitial collagenase (Sato 1992). Stromelysin-1 and interstitial collagenase are not normally expressed in differentiated keratinocytes, however, late stage squamous cell invasive carcinoma cells express these stromal MMPs. It is important to note these cancer cell types have acquired a spindle shape and they no longer express normal epithelial markers like E- and P- cadherins, keratins and desmoplakins and have begun to express the mesenchymal marker vimentin (Sato 1994).

Among the MMPs, stromelysin-1 (MMP-3) possesses the broadest substrate specificity. Transgenic mice that express an autoactivating mutant of rat MMP-3, targeted to the epithelial compartment of the mammary gland, have been generated (Lochter 1997, Sympson 1994). Phenotypically, MMP-3 transgenic mice display increased branching morphogenesis and lactogenic differentiation at pre-puberty stages and premature involution during late pregnancy. Branching morphogenesis requires the invasion of epithelial cells into the adipose tissue, a process reminiscent of invasion of stromal compartments by tumor cells. Strikingly, a large number of MMP-3 transgenic mice also develop mammary tumors of various histotypes, including invasive adenocarcinomas. Studies performed with synthetic inhibitors of MMP activity and tissue inhibitors of metalloproteinases (TIMPs) have shown that suppression of MMP activity also suppresses tumor growth and metastasis (DeClerck 1992). In many cases, the level of MMP-3 expression in tumors of the mammary gland and other tissues is positively correlated with the degree of malignancy (McDonnell 1990). An investigation was conducted to determine whether MMP-3 plays a direct role in invasion of the ECM. Two mammary carcinoma cell lines, TCL1 and SCg6 that formed rapidly growing, invasive tumors *in vivo* (Figure 5.3) and migrated through Matrigel and collagen gels in culture, were used. Antisense oligodeoxynucleotides (ODNs) against MMP-3 inhibited Matrigel invasion by TCL1 and SCg6 cells by more than 80% and collagen invasion by about 50% (Figure 5.4) (Lochter 1997). These data suggest that MMP-3 expression is associated with invasive properties of these breast cancer cells.

Basement membrane degradation is a key event in tumor invasion and metastasis. Type IV collagen and laminin are the most abundant of the basement membrane structural proteins in normal prostate tissue. Selective degradation of these components of the basement membranes has been observed in the prostate (Waghary & Webber 1995). A study of the metastatic prostatic adenocarcinoma

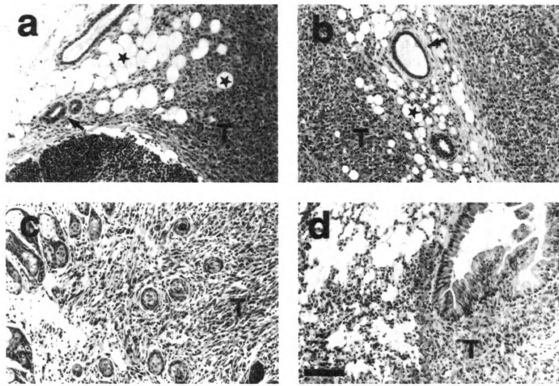

Figure 5.3. Histology of tumors formed by TCL1 and SCg6 cells. Hematoxylin/eosin-stained paraffin sections of primary tumors (panels a and b), invaded skin (panel c) and lung metastasis (panel d) generated by TCL1 (panels a and c) and SCg6 (panels b and d) cells injected into the mammary gland of nude mice. The spindle cell tumors (T), entrapped adipose tissue (asterisks) and epithelial ducts (arrowheads) in the mammary gland are indicated. Comparison of the regulation of MMP-3 expression by ECM in TCL1 and SCg6 cells with the nonmalignant, functional cell line SCp2 revealed striking differences that could play a role in the acquisition of an invasive tumor phenotype (Lochter 1997).

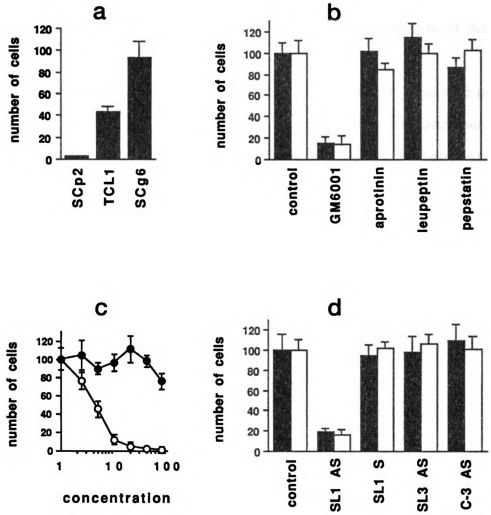

Figure 5.4. Invasion of Matrigel by SCp2, TCL1, and SCg6 cells and inhibition by proteinase inhibitors and antisense ODNs. SCp2, TCL1, and SCg6 cells were plated on Matrigel in modified Boyden chambers. After fixation with 5% glutaraldehyde and removal of cells that had not migrated, the number of cells that had migrated through Matrigel after 18 h of culture was quantified by counting the number of toluidine blue-stained cells per visual field. Panel a, comparison of invasion by SCp2, TCL1 and SCg6 cells. Panel b, TCL1 cells (black bars) and SCg6 cells (white bars) were maintained in the absence of proteinase inhibitors (control) or with GM6001 (10 μM), aprotinin (0.3 μM), leupeptin (1 μM), or pepstatin (1 μM). Panel c, SCg6 cells were maintained in the presence of GM6001 (white circles) or GM1210 (black circles) at increasing concentrations given in μM. Panel d, TCL1 cells (black bars) and SCg6 cells (white bars) were maintained in the absence of ODNs (control) or in the presence of SL1 antisense (3.2 μM, SL1 AS) or sense (3.2 μM, SL1 S) ODNs, SL3 antisense ODNs (8 μM, SL3 AS), or collagenase-3 antisense ODNs (32 μM, C-3 AS) ODNs. Results were normalized with control values set to 100 in b-d. Error bars indicate standard deviations from three experiments. (Lochter 1997)

cell line PC-3 showed increased gelatinase A activity. This study indicated that invasion through the basement membrane was a critical step toward metastasis (Webber 1995, Webber 1996).

It is important to note that cell invasion is dependent on a cascade of events that involves the participation of several factors. There are several enzymes capable of degradation of the basement membrane and other components. The overexpression and increased activity of MMPs are clearly not the only factors involved in this process. The turnover of ECM and the expression of MMPs are also involved in a number of normal conditions. Metastatic invasion is likely to be due to an imbalance of positive and negative control factors. One such negative factor may be TIMP. The loss of TIMP expression might also lead to a disregulation of ECM turnover, giving the same result as MMP overxpression. Results show that several malignancies have little or no TIMP expression (Murphy 1993). Therefore, the possibility of treatment of cancer metastasis by TIMP is promising.

CONCLUSIONS

It is now clear that proteases play an integral role in physiological conditions that require turnover of the extracellular matrix. It has been established that some MMPs, specifically gelatinases, stromelysins, and matrilysin, will degrade type IV collagen, a major component of the basement membrane. These MMPs have been implicated in the invasive behavior of many tumor types. Further study of the distribution patterns, expression, and activation of these select MMPs will provide valuable insight into cancer progression. The next chapter will examine the role of MMPs in prostate cancer.

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CHAPTER 6

MATRIX METALLOPROTEINASES AND PROSTATE CANCER

ABSTRACT

Prostate cancer is the most common cancer currently diagnosed in American men (Landis 1998). In 1998, 39,200 deaths are expected from this disease, making prostate cancer second only to lung cancer as the leading cause of death from cancer among men. Prostate tumors display a high degree of biological variability, ranging from benign tumors to aggressive adenocarcinomas, and currently there is no method for determining whether a tumor will remain latent or invade and metastasize. The biological variability of prostate cancer is measured by the ability of tumor cells to invade the surrounding basement membrane. This invasion is considered to be the first step toward metastasis. Matrix metalloproteinases (MMP) are a family a zinc dependent endopeptidases that degrade components of the extracellular matrix. MMPs have been strongly implicated in cancer cell invasion and metastasis. This chapter defines the role of MMPs in prostate cancer by examining the expression patterns of MMPs and their inhibitors in normal and malignant prostate, especially in invasive cancer. Further, clinical applications of this work are also explored.

INTRODUCTION

Prostate Carcinoma is one of the leading causes of cancer-related death in several industrialized countries. In American men, it ranks as the second leading cause of death from cancer (Powell 1993) after lung cancer. These statistics are significant because a large segment of the American male population has recently entered the age group affected by prostate cancer. Prostate cancer is occurs predominantly in men over the age of fifty. It is estimated that the percentage of

men over age 55 will increase from 18% to 25% of the total male population by the year 2010 (Powell 1993). This increase in the number of affected men will cause prostate cancer to become a large public health problem.

Prostate cancer displays a large degree of biological variability, ranging from slow growing to rapidly growing and aggressive metastatic cancer. Distant metastases to the lymph nodes and bone are the principal causes of death (Festuccia 1995). At the time when prostate adenocarcinoma is first detected it is not currently possible to determine whether the tumor will remain latent for the patient's lifetime or invade and metastasize within 5 years. Despite the existence of several modern techniques in clinical management of these tumors, little or no progress has been made in identifying reliable prognostic parameters for predicting the invasive behavior of an individual tumor. The majority of patients with prostate cancer are often diagnosed with already disseminated disease (Stephenson 1992). The lack of good diagnosis and prognostic tools, combined with the relative lack of effective therapies, makes this disease one of the major causes of cancer related death in men today. Identification of cellular features that predict the biological behavior of the individual tumors is one of the major challenges of modern medicine.

The term, invasiveness, describes the ability of cells to cross anatomic barriers such as basement membranes, interstitial stroma, and intercellular junctions. Normal invasive processes occur in the embryo and in the adult organism, including trophoblast implantation, formation of blood vessels or angiogenesis, extravasation of leukocytes in inflammation, and wound repair. Other invasive processes occur under pathological conditions, such as bacterial infections and tumor spread. Invasion is a recurrent theme in the development of malignant tumors. During tumor growth, while a malignant cell invades normal adjacent tissues, nearby normal vascular endothelial cells invade the neoplasm itself, by capillaries or venules. These cells form a tumor's vascular network (Montironi 1996). A common feature of the

invasive process is the degradation of the basement membrane, extracellular matrix and interstitial stroma (Conway 1996). The ECM-degrading proteinases produced by the most invasive cells can be divided into three classes, serine proteases, metalloproteinases, and cystine proteases.

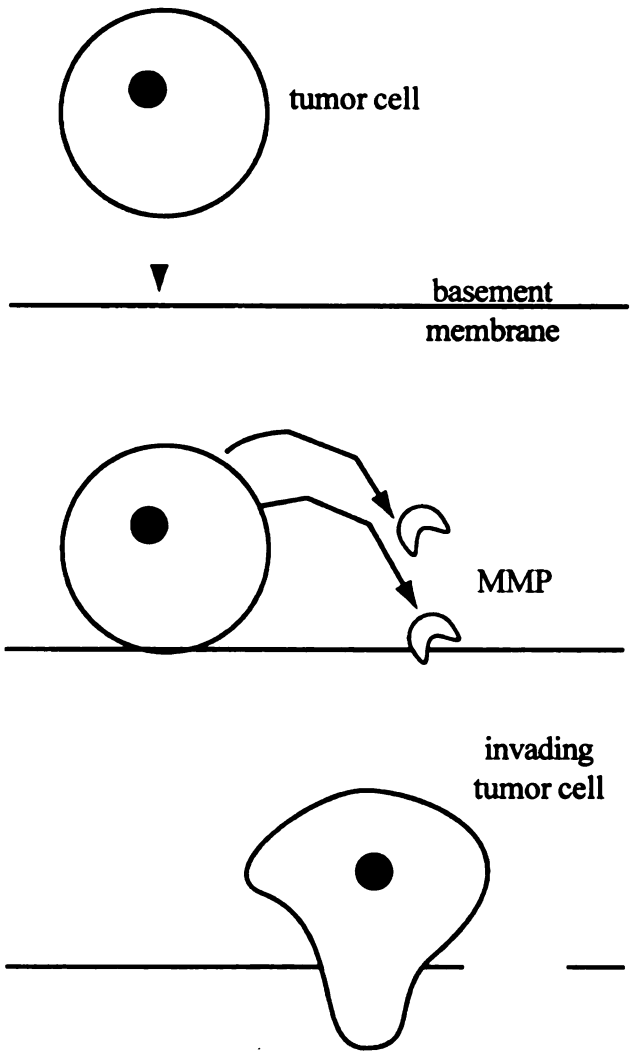
It is apparent that the biological variability of prostate adenocarcinoma manifests itself in the extent of tumor cell invasion through the epithelial basement membrane. Destruction of the basement membrane has been observed in many different cancers including prostate cancer (Conway 1996). Mechanisms controlling the metastatic spread of a localized tumor are very complex and involve many events. One such event is the secretion of proteolytic enzymes, capable of degrading the extracellular matrix by invading tumor cells. Penetration of the epithelial basement membrane by cancer cells, that can secrete and locally initiate a proteolytic cascade, is the first step in tumor invasion. The invading cells must also subsequently penetrate and degrade the basement membrane underlying the vascular endothelium to enter the circulation (intravasation). The cancer cell must then exit the vascular network by invading the blood vessel wall of the host target organ (extravasation). Matrix metalloproteinases and their inhibitors have been proposed as modulators of these steps in the process of invasion and metastasis. Increased proteolytic activity of MMPs, specifically gelatinases and stromelysins, has been observed in several invasive prostate tumor types (Stearns 1993, Wang 1996, Webber 1995). Gelatinases and stromelysins can use components of the basement membranes as their substrates and are, therefore, capable of degrading the basement membrane. Studies have shown a progressive loss of the basement membrane with increasing grade of human prostate carcinoma (Stearns 1993). These results support the hypothesis that MMP expression may be indicative of a change from a benign to a malignant state in prostate cancer.

TUMOR CELL INVASION OCCURS IN THREE STEPS

A three-step process has been suggested for describing the sequence of events during tumor cell invasion and metastasis (Figure 6.1). The first step is the attachment of the tumor cell via receptors to the ECM glycoproteins, such as, fibronection and laminin. The next step, following attachment, is the secretion of proteolytic enzymes by cancer cells and their induction in neighboring stromal cells and inflammatory cells. These proteases can degrade the components of the basement membrane. The third step in the process of invasion is the migration of the tumor cell through the partially degraded ECM. Autocrine motility factors as well as chemotatic factors can modulate this process. The process of tumor cell-mediated degradation of the ECM appears to involve a complex series of proteases that may be secreted and activated in a cascade of activation of latent proteases (Conway 1996).

EXPRESSION OF MMP IN PROSTATE CANCER

The MMP family is divided into three subclasses based on their homology and substrate specificity. The three subclasses are the collagenases, the gelatinases, and the stromelysins. Proteins known as tissue inhibitors of metalloproteinases (TIMPs) can inhibit the activated form of these MMPs. The process of regulating tumor cell invasion and metastases may depend, in part, on the balance between MMPs and their inhibitors. The MMP expression can be considered a positive influence on invasion while inhibition by TIMP can be considered a negative influence on invasion. It is thought that the uncontrolled activity of MMPs result in the degradation of the extracellular matrix and the loss of integrity of the basement membrane, which facilitates tumor cell dissemination. Disregulation of MMPs has



1. Attachment of Tumor Cell to the basement membrane

2. Secretion of Proteolytic Enzymes that degrade the basement membrane

3. Migration of tumor cell through the partially degraded basement membrane

Figure 6.1 Tumor cell invasion occurs in three steps.

been observed in many tumor cell types and has been associated with progression toward metastasis (Stearns 1992). This section will examine the role of MMPs in metastatic prostate cancer.

Matrilysin (MMP-7) is expressed normally in epithelial cells of the prostate gland. Its normal function is proteolytic processing of glandular secretions. Matrilysin can degrades gelatins and fibronectin, and has also been shown to activate gelatinases and stromelysins. Increased expression of the matrilysin gene has been associated with progression of adenocarcinoma of the prostate (Pajouh 1991). Using both Northern blot analysis and *in situ* hybridization, expression of the matrilysin gene was observed in 14/18 (72%) samples studied (Pajouh 1991). Gelatinase A was expressed in more than 60% of the sample of prostate adenocarcinomas examined (Pajouh 1991). *In situ* hybridization showed that matrilysin was only expressed in luminal epithelial cells, and not by the surrounding stromal cells (Pajouh 1991). These data suggest that an increase in the expression of matrilysin can lead to an increase in gelatinase A activation, resulting in a more invasive prostate adenocarcinoma.

Normal prostate epithelial cells express gelatinase A (Webber 1996). This expression of gelatinase A has been shown to be upregulated in benign prostatic hyperplasia and prostate cancer (Stearns 1993). The Gleason score is a measure of the invasive potential of cancer cells. The tumor is assigned a number 1-10, where 10 represents a highly invasive cancer. Northern blot analysis indicated quantitative differences in the levels of cytoplasmic gelatinase A (MMP-2) expression between benign prostatic hyperplasia and prostatic tumors (Figure 6.2). These data indicated BPH samples showed low expression of gelatinase A, conversely, increasing expression of gelatinase A was associated with increasing Gleason score in prostatic carcinomas (Stearns 1993). *In situ* hybridization showed weak staining for gelatinase A in BPH, and progressively stronger staining with increasing Gleason score prostate

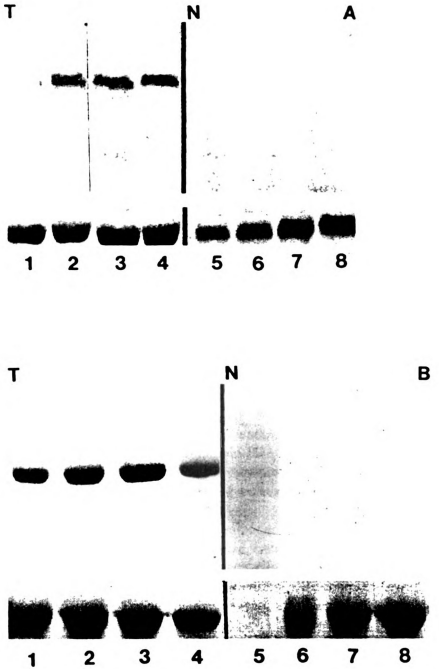


Figure 6.2. Northern blot analysis of gelatinase A mRNA from prostate tumor tissue (T, lanes 1-4) and adjacent normal stromal tissue (N, lanes 5-8) respectively. In panel A, the tissue was from benign prostatic hyperplasia (BPH) tissue (lanes 1 and 5): and from prostate tumor tissue with a Gleason score (GS) of 1 (lanes 2 and 6); 2 (lanes 3 and 7); or 3 (lanes 4 and 8). In panel B the Gleason score was 4 (lanes 1 and 5); 5 (lanes 2 and 6); 6 (lanes 3 and 7); or 8 (lanes 4 and 8) (Stearns 1993).

cancer tumors (Stearns 1993). Stromal tissues surrounding these tumors showed no expression of gelatinase A. These data associate the increased expression of gelatinase A with the increasing invasive potential of a cancer cells.

A comparative study of normal prostatic tissue from adults (28-35 years) and juvenile (4-12 years) patients and diseased prostatic tissue from patients with benign prostatic hyperplasia (BPH) and prostatic carcinoma (PC) was performed (Lokeshwar 1993). These studies showed that the secretion of active forms of MMP was limited to the malignant and juvenile samples (Figure 6.3) (Lokeshwar 1993). The increased expression of MMP seen in these samples indicate a favorable proteolytic condition for rapid changes in their basement membranes. In the juvenile prostate, this probably represents normal enlargement of the prostate during the development of secondary sexual characteristics, while in the adult this change indicates the onset of BPH or cancer.

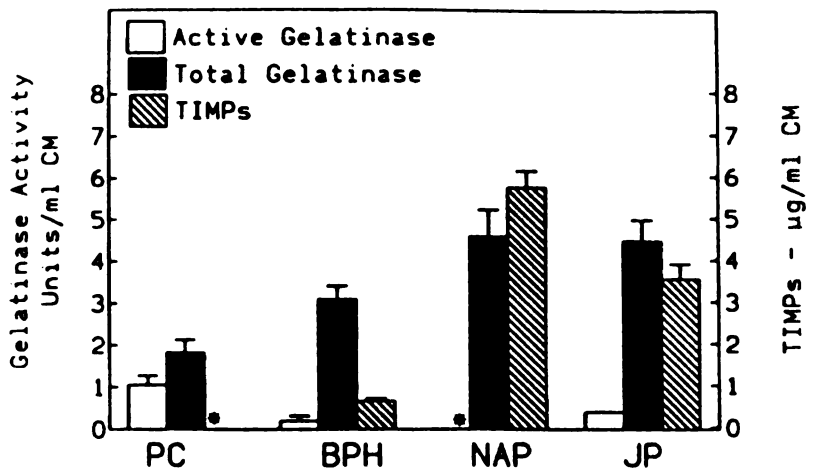


Figure 6.3. Examiniation of gelatinase expression, gelatinase activity and TIMP expression in PC primary carcinoma of the prostate; NAP, normal adult prostate; and JP, juvenile prostate. *indicates no significant reading (Lokeshwar 1993).

The induction of MMP genes can result in the transformation to an invasive phenotype. DU-145 cells do not express matrilysin (Powell 1993). Powell transfected DU-145 with a matrilysin cDNA (Powell 1993). These transfected DU-145 cell overexpressed matrilysin. They were injected into SCID mice to test their *in vivo* invasive potential when placed on the mouse diaphragm. The controls for this experiment were DU-145 cell transfected with vector only. While the control cells appeared to be inhibited from crossing the diaphragm basement membrane, DU-145 cells transfected with matrilysin cDNA were able to invade the basement membrane *in vivo* (Figure 6.4) (Powell 1993).

Recent studies show an increase in gelatinase A (MMP-2) activity in human prostate cancer (Wang 1997). This expression of MMP-2 was found to have an increasing trend corresponding to the Gleason score of the carcinoma. The expression of MMP-2 in cancers with Gleason scores from 5-8 was low to moderate, and a noticeable increase was seen in cancers with Gleason score of 9-10. Lymph node metastases also showed a significant increase in the expression of MMP-2. This increase was twice as much as the increase observed in Gleason score 9-10 tumors. ELISA measurements, comparing the ratios of activated MMP to proMMP, showed that this ratio increased as Gleason scores increased (Wang 1997). These data indicate that both the expression of MMP-2 as well as the activation of MMP-2 are good prognostic measures of the progression of prostatic carcinoma toward a aggressive phenotype.

MODULATION OF MMP ACTIVITY IN PROSTATE CANCER

The complex process of tumor cell metastasis is thought to be due to several genetic changes in at least one primary tumor cell subpopulation. These mutations in tumor cells can yield increased sensitivity to autocrine or paracrine growth factors or decreased sensitivity to growth inhibitors. Either of these changes results in a

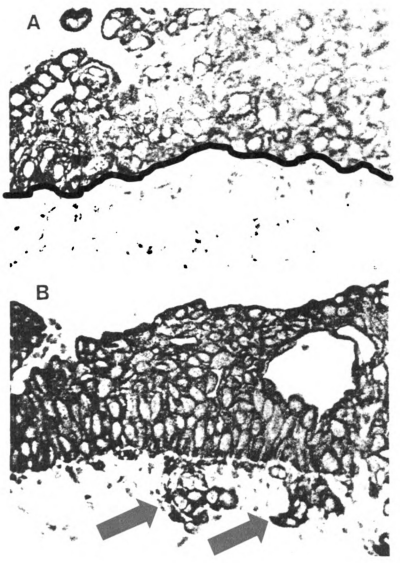


Figure 6.4. Immunohistochemical staining of SCID mouse diaphragmatic tumors. DU-145 cells are shown in panel A, and DU-145 cells transfected with matrilysin cDNA are shown in panel B. Panel B shows cells crossing the basement membrane (arrows) (Powell 1993).

selective advantage for the mutated cell subpopulation within both the primary or secondary (metastatic) tumors. It is possible that a cell with a mutation that results in an increase in MMP activity would result in an invasive or metastatic phenotype. This section examines the modulation of MMPs by growth factors or growth inhibitors in the progression of metastatic prostate cancer.

LOSS OF INHIBITION BY TGF-β

Tumor progression to the stage of metastasis may result, in part, from the selection of certain primary tumor cell clones, which are phenotypically competent for survival, invasion, and growth at secondary sites. Selection for traits such as loss of growth inhibitory responses, enhanced motility and increased collagenase activity are likely to contribute to cancer progression and may be regulated through the action of growth factors. TGF-β is known to inhibit growth of prostatic epithelial cells and inhibit most MMPs (Sehgal 1996). Therefore, the elimination or subversion of TGF-β-responsive pathways should be considered as a mechanistic framework for metastatic events. One study examined the growth and extracellular matrix responses to TGF-β in six metastatic and six primary tumor-derived cell lines in a mouse model of prostate cancer (Sehgal 1996). Tumor cell lines derived from focal pulmonary metastasis secreted relatively greater quantities of total TGF-β, lost most or all TGF-β growth inhibition, but responded to TGF-β through induction of gelatinase B. Cell lines derived from tumors that proliferated at the primary site retained the growth inhibition and did not produce gelatinase B (Sehgal 1996). These results suggest that acquisition of differential responses to the TGF-β family could result in phenotypic traits, which facilitate tumor metastasis from certain primary site clones. It is conceivable that a mutation occurred in one of the cell subpopulations of a primary tumor that lead to a metastatic phenotype. This mutated cell subpopulation was able to invade the basement membrane and enter and exit

the circulation to establish a secondary tumor. Any cells collected from these metastatic tumors would be derived from the mutated cell subpopulation and would most likely display this similar aggressive phenotype.

MMP SECRETION AND INVASION

Kinesin is a microtubule associated ATPase that powers macromolecule and organelle transport to the cell surface. Kinesin is ubiquitous and may be universally important for vesicle transport and secretion in eukaryotic systems. It has been postulated that enhanced kinesin levels might be a prerequisite for accelerated protease secretion, as required for cell invasion. Studies examining both invasive and noninvasive PC-3 human prostate carcinoma cell sublines have demonstrated interesting results (Stearns 1991). Each of the non-invasive PC-3 sublines failed to express detectable levels of gelatinase A, while each of the invasive PC-3 sublines overexpressed gelatinase A. The invasive PC-3 sublines had a significant increase in the basal expression of kinesin over the non-invasive PC-3 sublines (Stearns 1991). It is suspected that this increase in kinesin expression results in elevated secretion of gelatinase A. The elevated kinesin expression observed in invasive PC-3 sublines can be further increased when treated with conditioned medium collected from any of the invasive clones. In addition to enhanced kinesin expression, cells treated with conditioned medium showed increased gelatinase A secretion and invasion (Stearns 1991). This coordinately enhanced kinesin expression, gelatinase A secretion and invasion was not induced by treatment with bovine serum albumin, fetal calf serum, PDGF, EGF, or IGF. The enhancement of these three factors, kinesin, gelatinase A and invasion, by conditioned medium, was not observed in non-invasive PC-3 sublines. These findings suggested the possibility of an autocrine stimulatory factor as well as the presence of response mechanisms unique to the invasive PC-3 subpopulations. Trypsinization or treatment with pertussis toxin

could inhibit the enhanced response to condition medium (Stearns 1991). Inhibition by trypsin indicates that the autocrine factor(s) might bind to the surface receptors of cells to regulate kinesin expression. Inhibition by pertussis toxin suggests that the receptor may be G-protein-dependent. It is suspected that invasive PC-3 sublines have acquired the ability to secrete an autocrine growth factor and produce a receptor to respond to it (Stearns 1991).

Cytokines, such as, interleukin–2 (IL-2), interleukin-6 (IL-6), and interleukin-10 (IL-10) have been identified as regulators of ECM degradation. Several studies have demonstrated cytokines as downregulators of MMP expression (Sokoloff 1996, Stearns 1995, Wang 1996). Currently, the mechanisms and the nature of the second messenger pathways involved in growth factor and cytokine regulation of MMPs and TIMPs remain unclear. It is thought that differential regulation of genes that encode MMP and TIMP may be due to various cytokines and growth factors that allow responsive elements to either cooperate with, or antagonize each other. Both IL-2 and Interferon gamma (IFN-γ) were demonstrated to down regulate type IV collagenase in DU-145 and PC-3 human prostate carcinoma cell lines (Sokoloff 1996).

Five different cytokines (IL-2, 4, 6, 10, and IFN-γ) and their effects on MMP-2, MMP-9, TIMP-1 and TIMP-2 expression in HPV-18 immortalized human prostate carcinoma epithelial cell lines have been recently examined (Wang 1996). Seven sample immortalized cell lines were derived from three different low-grade prostatic tumors (Gleason score 5) (HPCA-5a-HPV18, HPCA-b-HPV18, and HPCA-c-HPV18) and four high grade prostatic tumors (Gleason score 10) (HPCA-10a-HPV18, HPCA-b-HPV18, HPCA-c-HPV18, and HPCA-d-HPV18). All of the low Gleason score samples (HPCA-5a-HPV18, HPCA-5b-HPV18, and HPCA-5c-HPV18) showed high levels of expression of TIMP-1 and low levels of MMP-2 while the high Gleason score samples (HPCA-10a-HPV18,

HPCA-10b-HPV18, HPCA-10c-HPV18, and HPCA-10d-HPV18) showed low TIMP-1 expression and high MMP-2. ELISA and Northern blot analysis showed that IL-10 and to a lesser extent IL-4 and IL-6 stimulated significant increases in the expression of TIMP-1 in all immortalized cell lines, but particularly in the HPCA-10-HPV18 group. A decrease in the expression of MMP-2 was also observed in the HPCA-10-HPV18 a, b, c cell lines (Figures 6.5 and 6.6) (Wang 1996). The induction of TIMP-1 in response to IL-6 and IL-10 was found to be both dose and time dependent (Stearns 1995). These studies purpose that IL-10, IL-6 and IL-4, in order of effectiveness, can stimulate the expression of TIMP-1 and simultaneously decrease the levels of MMP-2 expression in epithelial cells derived from the human prostate.

APPLICATIONS

The expression of MMPs may be used as a biomarker to monitor cancer progression (Meyers 1996). Prostatic lesions are often identified as atypical (benign) or dysplastic (possible cancer), based on light microscopic morphological criteria. The term prostatic intraepithelial neoplasia (PIN) is generally used to describe these lesions within the ductal and glandular epithelium of the prostate. Frequently advanced PIN lesions are indistinguishable from malignant prostatic carcinoma only on the basis of cell morphology. This similarity may suggest a premalignant role of PIN. A comparison of both phenotypic and genotypic characteristics of PIN versus prostatic cancer cells would be useful in the elucidation of this likely transformation. As a result of the focal nature of PIN, studies of biomarkers such as growth factor receptors, oncogene products, and glycoslyated tumor antigens are difficult, because it is almost impossible to isolate pure preparations of PIN. One technique that has been used is micro-dissection. This procedure is tedious and may still yield tissue samples contaminated by surrounding stroma or normal epithelial cells. This

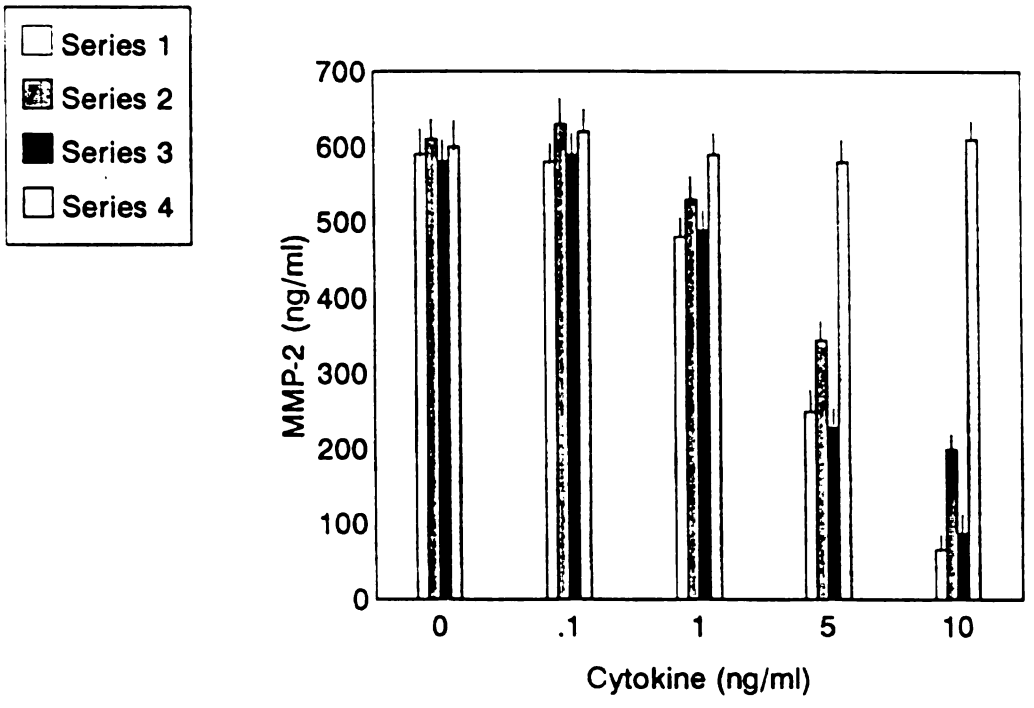


Figure 6.5. Concentration-dependent effect of Bar series (1) IL-10, (2) IL-6, (3) IL-2, and IL-4 on the production of MMP-1 by HPCA-10aHPV18 prostate cancer cell line (Wang 1996).

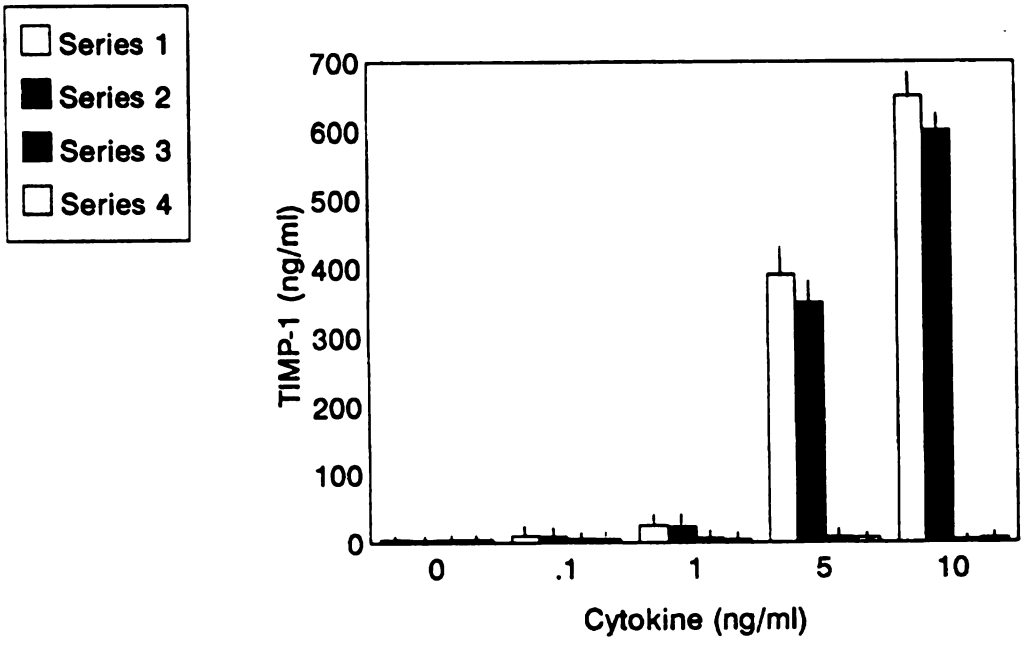


Figure 6.6. Concentration-dependent effect of Bar series (1) IL-10, (2) IL-6, (3) IL-2, and (4) IL-4 on the production of TIMP-1 by HPCA-10aHPV18 prostate cancer cell line (Wang 1996).

contamination makes differentiation of biomarker expression between the basal versus luminal components of the gland or duct impossible. Therefore, an immunohistochemical technique combined with *in situ* hybridization has been suggested (Myers 1996). A recent study used MMP expression to clarify the relationship of PIN to prostatic adenocaricnoma (Myers 1996). The expression of MMP was observed in PIN cells, normal cells, and malignant cells. Enhanced MMP activity was associated with dysplastic PIN cells and malignant cells. This might represent an important event in the development of prostatic adenocarcinoma. It has, therefore, been suggested that the observation of MMP expression via *in situ* hybridization studies may serve as a useful biomarker for prostate adenocarcinoma progression (Myers 1996).

An increase in MMP-2 (gelatinase A) immounostaining was recently demonstrated in the progression of prostate cancer (Montironi 1996). This study showed little or no staining in normal prostate, moderate staining in PIN and heavy staining in invasive prostatic adenocarcinoma. This expression of MMP-2 appeared to be localized to the cells in contact with the stroma or close to the stroma (Figure 6.7, 6.8, & 6.9) (Montironi 1996). These findings further support the use of MMPs as biomarkers for malignant conversion.

Taxol has been used to block MMP-2 (gelatinase A) secretion in PC-3 cell lines (Stearns 1992). Taxol is an alkaloid with great potential in therapeutic treatment of cancer. The main effect of taxol is stabilization of microtubule structures. Taxol binds tubulin to form abnormally stable taxol microtubules this action disrupts the dynamic state required for normal function in the cell (Figure 6.10) (Stearns 1992). It was shown that taxol blocks protease secretion and a dose dependent block of invasion and metastasis *in vitro* and *in vivo* occurred. These results suggest that taxol has a therapeutic potential for the inhibition of invasion, by its ability to block MMP-2 secretion (Stearns 1992). Mechanistically, targeted

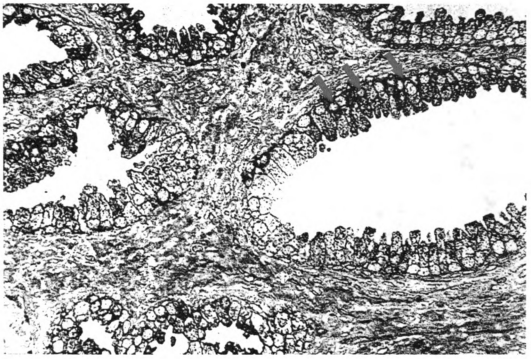


Figure 6.6. Normal prostate gland showing strong immunoreactivity (indicated by arrows) for MMP-2 in the basal cells and little immunoreactivity in the secretory epithelium (Montironi 1996).

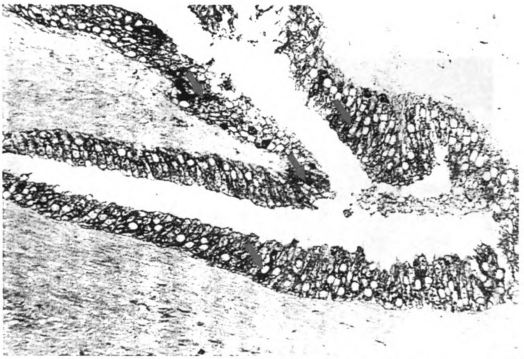


Figure 6.7. High grade prostatic intraepitheial neoplasia shows intense MMP-2 staining (indicated by arrows) in cells mainly in the cell layer adjcent to the stroma (Montironi 1996).

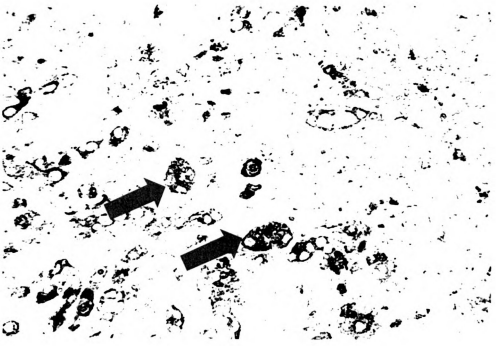


Figure 6.8. Prostatic adenocarcinoma shows intense immunostaining for MMP-2 (arrows) in small clusters of neoplastic cells located in the periphery of the tumor nodules at the level of advancing front of the tumor (Montironi 1996).

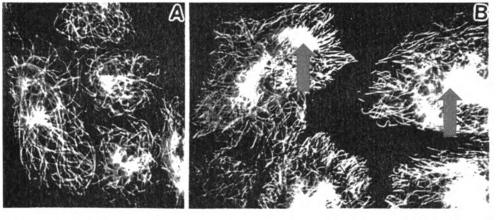


Figure 6.9. Immunofluorescence images of PC-3 ML cells labeled with beta-tubulin anti bodies and a secondary antibodiy conjugated to fluoresein isothiocyanate. Panel A, show untreated cells. Panel B, shows PC-3 ML cells treated with 0.5 mM taxol for 6 hour, the more intense signal is due to the stabilization of microtubule bundles (arrows). This stabilization had an inhibitory effect on MMP-2 secretion (Stearns 1992).

chemotherapy, distinct from cytotoxic agents that can interfere with prolonged invasion, could prove valuable in the treatment of established prostate adenocarcinoma.

All-*trans* Retinoic Acid (ATRA) has been shown to be effective in the inhibition of uPA by increasing PAI-1 levels (Kim 1995, Waghray & Webber 1995) . This effect is similar to the blockage of gelatinase A (MMP-2) and gelatinase B (MMP-9) in that it results in the inhibition of ECM degradation (Waghray & Webber 1995). Plasmin is involved in the degradation of the basement membrane and has been implicated in a proteolytic cascade that result in the activation of several MMPs and the subsequent breakdown of the basement membrane (Kim 1995, Webber 1996). Treatment with ATRA resulted in a decrease in the net activity of uPA, gelatinase A and gelatinase B (Waghary & Webber 1995). Although N-(4-Hydroxyphenyl)-retinamide (4-HPR) another retinoid, increased uPA expression, the net uPA activity was decreased because of a marked increase PAI-1 expression (Kim 1995). Both treatment with ATRA and treatment with 4-HPR decreased invasion and increased in PAI-1 (Kim 1995, Waghray & Webber 1995, Webber 1996).

Dahiya showed that 13-*cis* Retinoic Acid (RA) blocked invasion by the human prostatic carcinoma LNCaP cell line (Dahiya 1994). This blockade might occur in two ways: first 13-*cis* RA significantly inhibits the binding of LNCaP cells to the basement membrane and second 13-*cis* RA inhibits the degradation of type IV collagen. The mechanism was not fully characterized but thought to be caused by the ability if 13-*cis* RA to modulate the expression of various genes both *in vitro* and *in vivo*. These data suggest that retinoids have the potential for use as chemopreventive and chemotherapeutic agents, however, their modulation of invasion is not fully understood. Therefore, more research is indicated.

Cinnamic acid is a naturally occurring fatty acid. This compound is widely distributed in the plant world and is commonly use to add flavor in cooking. This

substance appears to have an antitumor effect against a broad spectrum of human tumors at doses that have no significant effect on normal cells. In a recent study, PC-3, DU-145, and LNCaP prostate cancer cell lines were tested in response to treatment with cinnamic acid. A significant decline in cell growth was observed in all of these cell lines (Liu 1995). These studies showed a decrease in the expression of gelatinase A, as well as an increased expression of TIMP-2 in human melanoma cells (Liu 1995). It is possible that cinnamic acid might also serve as a possible chemopreventive agent in the treatment of prostate cancer.

CONCLUSIONS

Prostate cancer is the second leading cause of death from cancer in American men. Several steps in tumor growth and metastasis require proteolytic degradation of the extracellular matrix. This ECM degradation and subsequent invasion is a recurrent theme throughout the process of cancer metastasis. MMPs and TIMPs are major players in these processes. MMPs and their inhibitors in the normal cell aid during development and wound healing. In contrast, increased MMP expression in transformed cells can lead to progression toward cancer invasion and metastasis. MMP overexpression has been identified in highly invasive prostatic cancer cell lines, and induced MMP overexpression has been shown to increase the invasive potential of non-invasive prostatic cell lines.

The saving grace to this grim picture is the fact that the transformation of normal prostate cells to malignant adenocarcinoma occurs over a long latent period of 20-30 years. This period of latency provides a window of opportunity for both treatment and prevention. With further understanding of the role of MMP in prostatic malignant transformation, important chemotherapeutic and chemopreventive agents can be developed to combat this disease.

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CHAPTER 7

COMMON MECHANISMS OF MATRIX METALLOPROTEINASES IN NORMAL AND PATHOLOGICAL CONDITIONS INVOLVING EXTRACELLULAR MATRIX TURNOVER

ABSTRACT

Several normal and pathological conditions involve the turnover of extracellular matrix. Conditions such as wound healing, regeneration, endometrial breakdown, mammary gland involution, ductal morphogenesis, angiogenesis and apoptosis are normal conditions involving ECM turnover. Major pathological conditions such as cancer invasion and metastasis, chronic wounds, and arthritis, are conditions that require ECM degradation. Previous chapters have identified matrix metalloproteinases as important factors involved in ECM turnover. Since the pioneering work of Gross (Gross 1962) in the identification of collagenase type I, much research has been conducted in an effort to determine the role of MMPs in other conditions characterized by ECM turnover. This chapter highlights some of the common mechanisms of MMP expression and secretion, activation and MMP inhibition as described in previous chapters and compares and contrasts them in normal and pathological conditions.

INTRODUCTION

The extracellular matrix is a polyfunctional mosaic composed of various molecules such as collagens, elastin, proteoglycan, fibronectin and laminin. This intercellular medium affects fundamental cell functions such as cell adhesion, proliferation and differentiation. The early notion that ECM is an inert and stable structure has been dispelled and it is now clear that a dynamic equilibrium between synthesis and degradation of ECM components is required for matrix maintenance.

The active destruction of ECM integrity by members of the matrix metalloproteinase family occurs during processes as diverse as development, wound healing, regeneration, arthritis and cancer metastasis. Since several of these normal and pathological processes require the turnover of extracellular matrix, and MMPs degrade the ECM, it can be deduced that MMP expression in these process is a common mechanism of ECM turnover.

This chapter seeks to identify some common mechanisms involved in ECM turnover by MMPs. It will use development, wound healing and regeneration as models of normal conditions, and arthritis, glomerular nephropathy, myocardial infarction and cancer as models of pathological conditions. By comparing and contrasting regulation versus disregulation of MMPs in these normal and pathological models this review provides valuable information. This information can be applied to further research into the treatment of several life-threatening diseases.

NORMAL CONDITIONS

Matrix metalloproteinases were first described in *Xenopus Leavous*, the African Clawed Frog. Interstitial collagenase (MMP-1) was identified as the active protein involved in the degradation of collagen in tadpole tail tissue explants (Gross 1962). The protein structure of interstitial collagenase was subsequently accepted as the prototypic member of the family of MMPs (Goldberg 1992). In addition to development, there are other normal conditions that are also characterized by ECM turnover.

Development

It has been speculated that normal cell proliferation, and migration of fibroblasts *in vivo* require the selective secretion of matrix degrading enzymes. It is suspected that MMPs and their inhibitors can modulate these processes through

their role in ECM remodeling. Expression of MMPs can affect the release of a cell from its normal matrix interactions to allow for growth and division. MMPs can also facilitate directional degradation required for the migration of daughter cells observed in development.

During amphibian metamorphosis, the intestinal epithelium dramatically changes due to apoptosis of larval cells and the proliferation and differentiation of adult cells. This transition from larval to adult epithelium appears to be dependent on the remodeling of the ECM and is induced by thyroid hormone (TH). In one study, the TH response genes from *Xenopus* small intestinal cells were cloned in the efforts to identify the molecular mechanisms of this induction. One of these early response genes is the *Xenopus* homolog of the mammalian stromelysin-3 (MMP-11) gene (Ishizuya-Oka 1996). Stromelysin-3 has recently been described, and like other MMPs, it has a prodomain, a zinc binding catalytic domain, and a hemopexin-like binding domain. However stromelysin-3 differs markedly from previously described MMPs in that has a furin recognition sequence and can be activated by the Golgi enzyme furin in the secretory pathway and released as a 45 kDa active enzyme. The substrates for stromelysin-3 are also distinct in that stromelysin-3 does not appear to cleave the classic MMP substrates such as gelatin, casein, and elastin. Stromelysin-3 does cleave serine protease inhibitors, α1-proteinase inhibitor and α2-antitplasmin. The degradation of these substrates by stromelysin-3 can facilitate an increase in elastase and plasmin activity. The expression of stromelysin-3 has been associated with epithelial regression as seen in mammary glad involution (Noel 1996), limb bud interdigitation and breast tumor cell invasion (Basset 1993).

A study using *in situ* hybridization was performed to examine the expression of the stromelysin-3 gene in response to TH in the development of *Xenopus* small intestine (Ishizuya-Oka 1996). *in situ* hybridization is a technique that uses a labeled

anti-sense RNA probe to detect the presence or absence of a particular mRNA, in this case the MMP-11 mRNA. The results of these *in situ* hybridization studies are summarized in Figure 7.1 (Ishizuya-Oka 1996). The small intestines of *Xenopus* tadpoles were examined from stage-55 (four weeks after fertilization) to the stage-66 (end of metamorphosis). There are three layers in the small intestine of *Xenopus*, beginning from the lumen; they are the epithelial layer, the connective tissue layer, and the muscular layer. In the larval intestine there is a single fold into the lumenal space, called typhlosole (Ty). The thickest region of connective tissue is located in this region. As normal development proceeds from a larval epithelium, to an adult epithelium more folds are developed leading to a more absorptive organ. In the early stages (55-59), stromelysin-3 mRNA is detected only in a few cells in the connective tissue layer and only in the lower regions near the muscular layer of the small intestine (Figure 7.1 panel A and B). At stage-61 most of the cells in the connective tissue layer stained for stromelysin-3 mRNA, several of these cells are right under the epithelial layer. The increase in the expression of stromelysin-3 coincides not only with a remodeling of the basement membrane but also the degeneration of larval epithelium and the rapid growth of adult epithelial primordial cells (Figure 7.1 panel C). Once the larval epithelium is replaced by the adult epithelium at stage-65, no stromelysin-3 mRNA positive cells are detected (Figure 7.1 panel D). The transitory staining for stromelysin-3 mRNA may represent the migration of stromal elements expressing stromelysin-3 from the muscular layer toward the epithelial layer. These results suggest that the remodeling of the basement membrane, induced by stromelysin-3, promotes the epithelial transformation from larval to adult by inducing regional apoptosis and selective cell survival.

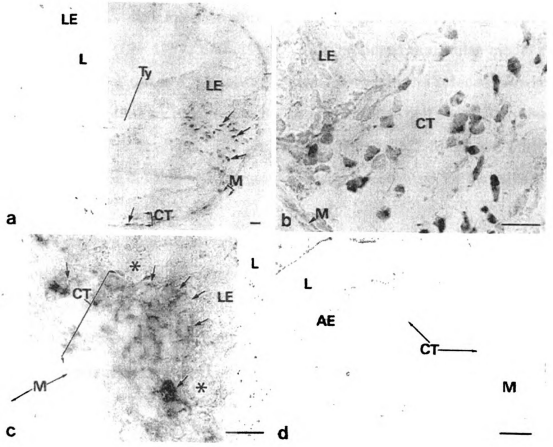

Figure 7.1. Cross-sections of the anterior part of the small intestine hybridized with the antisense stromelysin probe. The small intestine at stage 59 is shown in panel a. The layer of connective tissue (CT) is thin except for the typhlosole (Ty). Signals (arrows) are observed in some cellsof the connective tissue near the muscular layer (M), but are weaker in the upper region of the typhlosole x150. Panel b shows a higher magnification of the bottom region of the typhosole at stage 59. Most of the cells in the connective tissue show a positive reaction x610. The small intstine at stage 61 is shown in panel c. The basal surface of the epithelial primordia (astrisks) into the connective tissue. Most of the connective tissue cells just beneath the epitheilum are positive (arrows) x610. Intestinal fold at stage 65. No positive cells are seen in the connective tissue cells x490. Bars:20 µm. LE larval epithelium; AE adult epithelium; L lumen (Ishizuya-Oka 1996).

The co-expression of membrane-type matrix metalloproteinases (MT-MMP) and tissue inhibitor of metalloproteinase-2 (TIMP-2) has been studied in mouse development. MT-MMP has been shown to activate gelatinase A (MMP-2) at the surfaces of cells (Lee 1997, Sato 1994). This proposed molecular model for activation of progelatinase A is likely to be most relevant *in situ* at locations where MT-MMP and TIMP-2 are produced by the same or adjacent cell populations. Such is the case in mouse development. The co-expression of MT-MMP and TIMP-2 genes during mouse development suggests a possible mechanism for the activation of gelatinase A.

Recent work from a number of laboratories have resulted in the discovery of a subfamily of MMPs, the membrane-type or MT-MMPs (Sato 1994, Takino 1995). These MT-MMPs are distinct from other MMPs in that they are type I membrane proteins (N terminus of catalytic domain is extracellular) and have a single membrane-spanning region. MT-MMP has the potential to be activated by furin or furin-like enzymes. The activation complex is trimolecular and involves MT-MMP, progelatinase A and TIMP-2. A working theory of gelatinase A activation by MT-MMP and TIMP is diagramed in Figure 7.2. TIMP-2 and MT-MMP interact at their NH2 terminal domains (Imai 1996) (Figure 7.2 panel A). The interaction of MT-MMP and TIMP-2 allows each of these molecules to interact with gelatinase A (Figure 7.2 panel B). Once the three molecules come together MT-MMP activates proMMP-2 by cleaving the pro-domain. Gelatinase A is active as long as the trimolecular complex remains intact, when the complex is broken MMP-2 is immediately inactivated by TIMP-2. Since activation of gelatinase A by MT-MMPs appears to be facilitated by TIMP-2, the co-expression of TIMP-2 and MT-MMP would provide the necessary environment for gelatinase A activity.

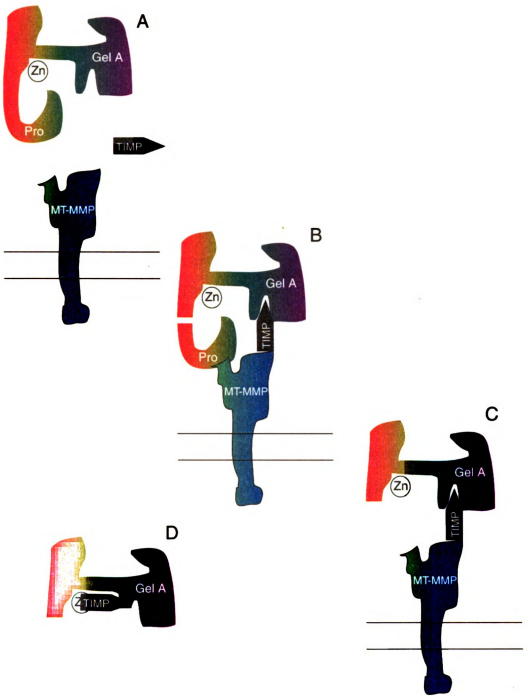

Figure 7.2. MMP-2 Activation by MT-MMP/TIMP complexes with MT-MMP (panel A), pro MMP-2 associates with the MT-MMP/TIMP complex and is activated by MT-MMP (panel B), The MMP-2/TIMP/MT-MMP complex is active as long as the trimolecular complex is together (panel C). Once MMP-2 breaks free it is immediately inactivated by TIMP.

Using *in situ* hybridization antisense probes for MT1-MMP and TIMP-2, the co-expression of MT-MMP and TIMP-2 genes in developing mice was observed (Figure 7.3 through 7.6). MT-MMP and TIMP-2 were prominently expressed in large arteries like the aorta and umbilical arteries (Figure 7.3). In addition, low levels of expression were seen in the myocardium, with more prominent expression in the cardiac outflow tract as it approaches the root of the aorta. The expression appears in smooth muscles of the developing tunica media. Co-expression of MT-MMP and TIMP-2 during bladder development and in specific urogenital organs further demonstrated this association with smooth muscle (Figure 7.4). This co-expression of MT-MMP and TIMP-2, in the areas that comprise smooth muscle cells in these organs may be important for smooth muscle cell migration.

Prominent co-expression of MT-MMP and TIMP-2 was seen in tendons, ligaments, muscle, and joint capsules (Figure 7.5). The ECM of these tissues is principally composed of fibrillar collagens, in particular, types I and III collagens. A recent report demonstrated that MT-MMP has the ability to cleave collagen and gelatin (Ohuchi 1997). Together, these observations suggest that MT-MMP may be involved in the remodeling of dense connective tissues, not just as an activator of proMMP-2 but as a matrix degrading enzyme in its own right, with the potential to deliver both collagenolytic and gelatinolytic activity. This finding may be relevant to the etiology of joint contractures and the repair of ligaments and tendons.

Expression of MMP-14 and TIMP-2 has also been observed at the placental implantation site (Apte 1997). At 12.5 and 14.5 days post coitum, these transcripts are co-localized to the junctional region between the uterine deciduum and placental spongiotrophoblast with minimal labeling in the labyrinth zone of the placenta (Figure 7.6). This expression of MMP may facilitate cellular invasion observed during implantation. The expression of MMP has been associated with several other reproductive mechanisms.

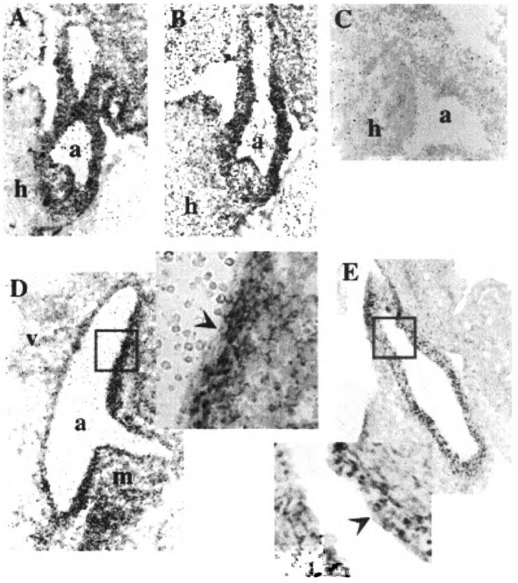

Figure 7.3. Mmp14 (A, D, and E) and Timp2 (B) are expressed in the media of muscular arteries. A-C, show the cardiac outflow tract at 12.5 days p.c. Note hybridization of the aorta (a) but not of the heart (h) in panels A (Mmp14) and B (Timp2). C, shows the cardiac outflow region hybridized to the Mmp14 sense probe. D, abdominal aorta (a) at 12.5 days p.c. hybridized to Mmp14 antisense probe. Note labeling in the media (see enlarged box alongside at upper right) and the absence of labeled cells in the endothelial layer. v, vertebral column; m, posterior abdominal mesenchyme. E, umbilical artery at 17.5 days p.c. hybridized to Mmp14 antisense probe. Boxed area is enlarged alongside (shown at lower left of figure) showing that labeling is restricted to the media. Arrowheads in panels D and E indicate the endothelium (Apte 1997).

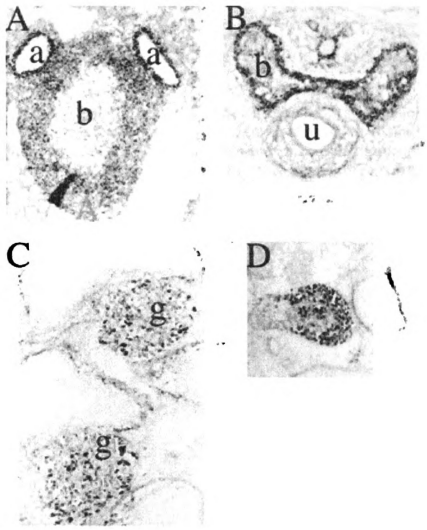

Figure 7.4. Hybridization of Mmp14 probe during urogenital development. A, transverse section through the developing urinary bladder (b) at 14.5 days p.c. showing labeled cells in the muscular wall (the lumen of the urogenital sinus is not seen in this section). Note also labeling of umbilical arteries (a) B, labeled cells are seen in and around the bulbourethral gland (b) adjacent to the bulbar urethra (u). C and D, expression of Timp2 in ganglia of the 17.5 day mouse embryo. C, dorsal root ganglia (g); D, trigeminal ganglion (Apte 1997).

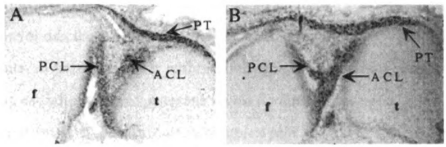

Figure 7.5. MMP-14 and TIMP-2 are expressed in developing synovial joints. Shown are knee joints of 17.5 days p.c. embryos hybridized to an Mmp14 (A) or Timp2 (B) antisense probe. Hybridization is seen with cells of the posterior cruciate ligament (PCL), anterior cruciate ligament (ACL), and patellar tendon (PT). Note labeling of cells in superficial layers of articular cartilage in both femur (f) and tibia (t) and the absence of labeled cells in cartilage elsewhere (Apte 1997).

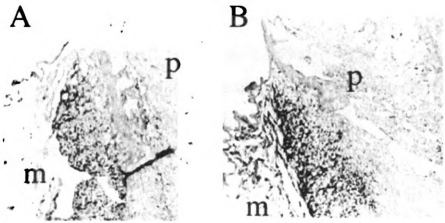

Figure 7.6. Expression of Mmp14 (A) and Timp2 (B) at the placental implantation site (12.5 days p.c.). Similar regions at the periphery of the implantation site are shown. m, myometrium; p, placenta (labyrinthine portion). The labeling appears in the junctional area that includes the decidual reaction of the uterus and spongiotrophoblast of the placenta (Apte 1997).

Endometrial cycling

In normal ovulation cycles the human endometrium undergoes complex changes, involving the proliferation and differentiation of both epithelial and stromal components, in preparation for embryo implantation. In a non-conception cycle, circulating estradiol and progesterone levels decline, in response to the regression of the corpus luteum. This initiates a complex process that leads to the breakdown and shedding of endometrial lining. Traditionally, it has been assumed that this process is a result of the ischemic necrosis caused by vasospasm of the endometrial spiral arteries. There is strong evidence to support the role of proteases in this process.

Studies have shown that MMP expression is responsive to progesterone withdrawal. Progesterone withdrawal is a physiological stimulus for menstruation. MMP-2 (gelatinase A) levels increase in stromal cultures subsequent to progesterone withdrawal and coincident to an upsurge in MMP activity. There is evidence to suggest that steroid hormones modulate MMP-2 expression (Figure 7.7 panel A) (Irwin 1997). Stromal endometrial cells were treated with 10nM estradiol and 1μM progesterone, followed by one of three treatments: 1) total steroid withdrawal; 2) treatment with 0.2 mM progesterone; 3) treatment with 10 nM estradiol, 0.2mM progesterone, and 1 mM RU486 (progesterone antagonist). The expression of MMP-2 increased with total steroid withdrawal and with the estadiol, progesterone and RU486 treatment. The increased gelatinase A activity observed in these treatments was not detected in cells that received the progesterone treatment alone. Integrative densitometery showed a six-fold increase in gelatinase expression after 4 days withdrawal of progesterone (Figure 7.7 panel B) this was followed by a decrease in expression after 6 days. These data indicate that the withdrawal of progesterone is an important modulator in the expression of MMP-2 for the turnover of ECM observed in endometrial cycling.

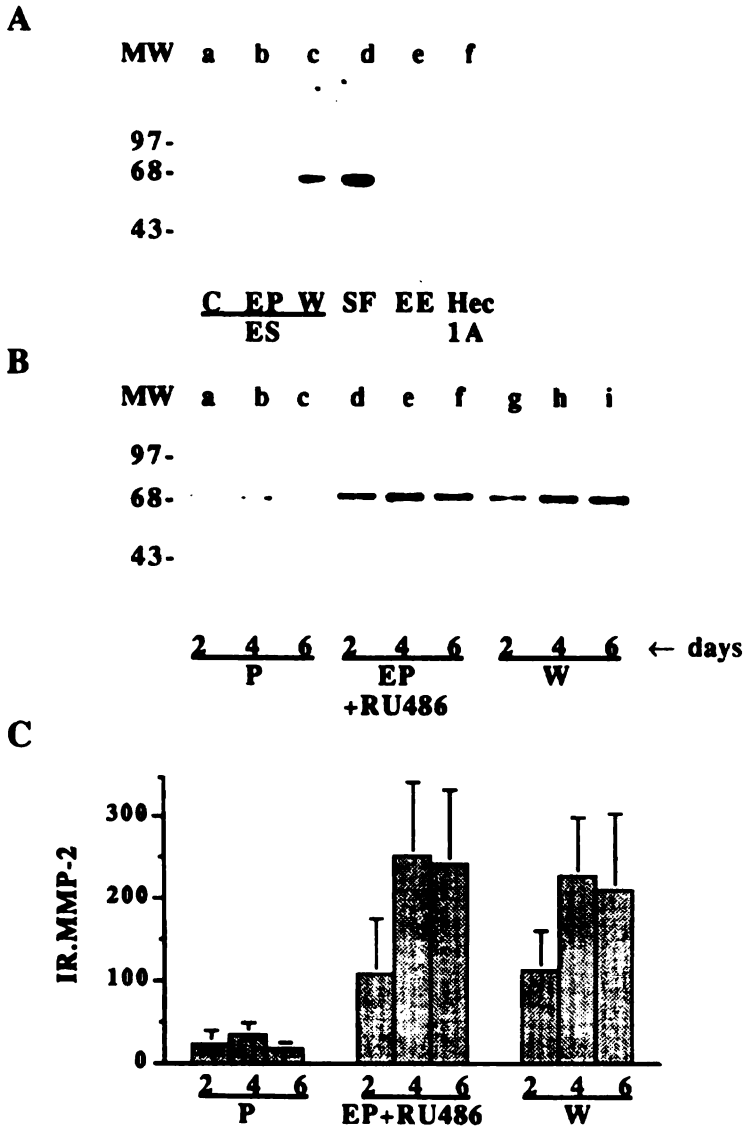

Figure 7.7. Western immunoblot analysis of MMP-2 in stromal cell cultures. Samples of 600 µl (A) or 400 µl (B) of 2 d-conditioned media were concentrated, electrophoresed under nonreducing (A) or reducing (B) conditions, and analyzed by Western immunoblotting with anti-MMP-2. Position of the molecular weight markers (MW) is indicated on the left margin. (A) Stromal cells (ES) were cultured 14 d in serum-free medium as described in Fig. 1, in the absence of steroids (C, lane a), in the presence of 10 nM E + 1 µM P (EP, lane b), or received 10 nM E + 1 µM P for 14 d followed by steroid withdrawal for 4 d (W, lane c). Human skin fibroblasts (SF, lane d), human endometrial epithelial cells (EE, lane e), and HEC-1A cells (lane f), received no steroids. (B) Stromal cells were cultured 14 d inserum-free medium containing 10 nM E + 1 µM P followed by 6 d in the presence of 0.2 µM P (P), 10 nM E + 0.2 µM P + 1 µM RU486 (EP + RU486), or nosteroids (W), and conditioned media collected 2, 4, and 6 d after the time of withdrawal. (C) Integrative densitometry of the 72-kD MMP-2 band on Western blots of 2 d-conditioned media collected 2, 4, and 6 d after the time of withdrawal as described for B. Levels of immunoreactive MMP-2 (IR.MMP-2) in conditionedmedia were estimated running in parallel known amounts (10-500 ng) of purified MMP-2. Values are the mean±SEM (bars) of measurements on two blots (Irwin 1997).

A temporal expression of MMP-2 (gelatinase A) was observed in the human endometrium during the menstrual cycle. Northern blot analysis was performed to examine the expression of MMP-2 (Figure 7.8 panels A and B). The expression of both MMP-2 and β actin were examined, $-actin expression remains consistent throughout the proliferative and secretory phases, and is used as an internal standard to assess the relative amount of mRNA loaded into each lane. The MMP-2 hybridization signal was then normalized to the $-actin hybridization signal to account for any uneven loading of the gels. The expression of MMP-2 was 2.5 to 4.9 times higher than that of β-actin in the late secretory phase (Figure 7.8 panel c) (Irwin 1996). The increased expression of MMP-2 in these late stages further supports the role of MMP in endometrial cycling.

A number of other reproductive events have been associated with the expression of MMPs and their inhibitors. Ovulation and the release of a mature ovum require the coordination of MMPs and their inhibitors (TIMPs). Implantation appears to involve the specific expression of MMP-9 by the embryo and the modulation of invasion with TIMP production by the decidua (Graham 1994). These reproductive events have the common theme of ECM turnover, and it is suspected that modulation of MMPs play an important role in the body's control over these reproductive functions.

Regulation of mammary involution

In normal mammary glands, stromelysin-1 (MMP-3) is synthesized by fibroblast but not by epithelial cells. This expression has been associated with ECM remodeling in the breast, such as, mammary gland ductal branching and mammary gland involution upon the cessation of lactation. The expression of MMPs increases during mammary involution (Talhouk 1992).

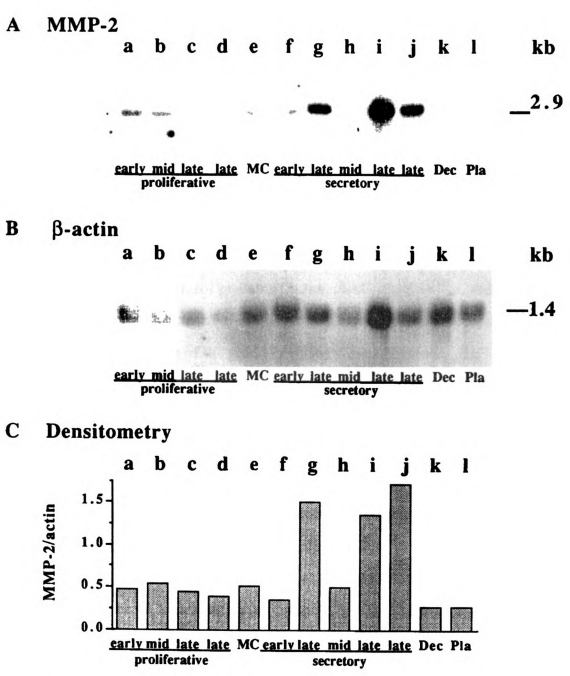

Figure 7.8. Expression of MMP-2 mRNA in human endometrium throughout the menstrual cycle. Samples of total RNA(20 μg) extracted from the following tissues were analyzed by Northern blot hybridization: early, mid, and late proliferative endometrium (lanes a-d); mid cycle endometrium (MC, lane e); early, mid, and late secretory endometrium (lanes f-j); early pregnancy decidua (Dec, lane k); term placenta (Pla, lane l). (A) Autoradiograph of hybridization with the MMP-2 cDNA probe. (B) Autoradiograph of the same membrane hybridized with the -actin cDNA probe. (C) Densitometric analysis of the autoradiographs shown in A and B, using the integrated area under the respective absorbance curves to calculate the MMP-2/-actin hybridization ratio for each sample (Irwin 1996).

During involution, restructuring of the mammary gland occurs as a coordinated process of alveolar apoptosis and lobular-alveolar remodeling. The removal of the suckling triggers this process. With the loss of suckling, milk accumulates within the alveolar lumens, and the levels of systemic lactogenic hormones falls (Feng 1995). Mammary gland involution goes through two distinct phases. In the first phase alveolar cells undergo apoptosis, and there is no remodeling of the lobular-alveolar structure. Also occurring in this phase is expression of TIMP-1 and cell cycle control proteins (*c-Jun*, *JunB*, *JunD*, *c-fos*, and *c-Myc*) and the decreased expression of milk protein genes. In the second phase, the lobular-alveolar structure of the gland is obliterated as proteases degrade basement membrane and ECM components. In phase two, gelatinase A (MMP-2), stromelysin-1 (MMP-3), and stromelysin-2 (MMP-10) and urokinase type plasminogen activator are expressed (Li 1997). The progressive gain of death signals and the loss of surviving factors control the two stages of mammary gland involution. In the first stage, the build up of milk within the alveolar cell triggers apoptosis but also upregulates the expression of survival factors that do not overcome apoptosis but do protect the remodeling of the mammary gland (phase 2). These survival factors persist for 48 hours, and are important for the restoration of lactation in case suckling is renewed. After 48 hours the second phase begins and the activities of MMPs and uPA result in the destruction of the basement membrane and involution of the mammary gland. These studies suggest that a spatial and temporal coordination in the expression of MMPs and TIMPs is important for mammary involution.

Wound healing

Skin wound healing is a complex process characterized by re-epithelization and restoration of underlying connective tissue. During this process, keratinocytes, endothelial cells, and fibroblast proliferate and these cells as well as inflammatory cells migrate to the site of injury where they interact with each other and with the ECM (Okada 1997).

The migration of cells and the remodeling of tissues during wound healing require the controlled turnover (degradation) of the ECM and the release of growth factors. The expression of gelatinase A (MMP-2) has been localized in connective tissue of healing wounds and the expression of gelatinase B (MMP-9) is associated with migrating epithelial sheets during human skin wound healing (Sato 1994).

The expression of MMP RNA during rat skin wound healing has recently been examined (Okada 1997). Several MMPs were discovered in healing wounds by Northern blot analysis, such as, MT-MMP, gelatinase A (MMP-2), gelatinase B (MMP-3), and collagenase 3 (MMP-8), stromelysin-1 (MMP-3) and stromelysin-3 (MMP-11) (Figure 7.9 panel A). The expression of inhibitors such as TIMP-1, TIMP-2 and TIMP-3 were also measure by Northern blot analysis (Okada 1997). The expression patterns of these enzymes and their inhibitors provide a number of very interesting clues to the role of MMPs in healing wounds. The coordinate expression of gelatinase A, MT1-MMP, and TIMP-2 suggest that the trimolecular activation of MMP-2 at the surface of cells is an important mechanism in wound healing. Studies have suggested that stromelysin-1 is the activator of progelatinase B. Data given in Figure 7.9 (panel A) suggest that this mechanism of activation may be relevant in wound healing. These findings were supported by transfection of the African green monkey kidney cell line, COS-1, with plasmids that encode for MMPs that are expressed in the healing wounds of rats (MT1-MMP, MMP-2, MMP-9, MMP-3, MMP-11, and MMP-8) (Okada 1997). COS-1 cells were also

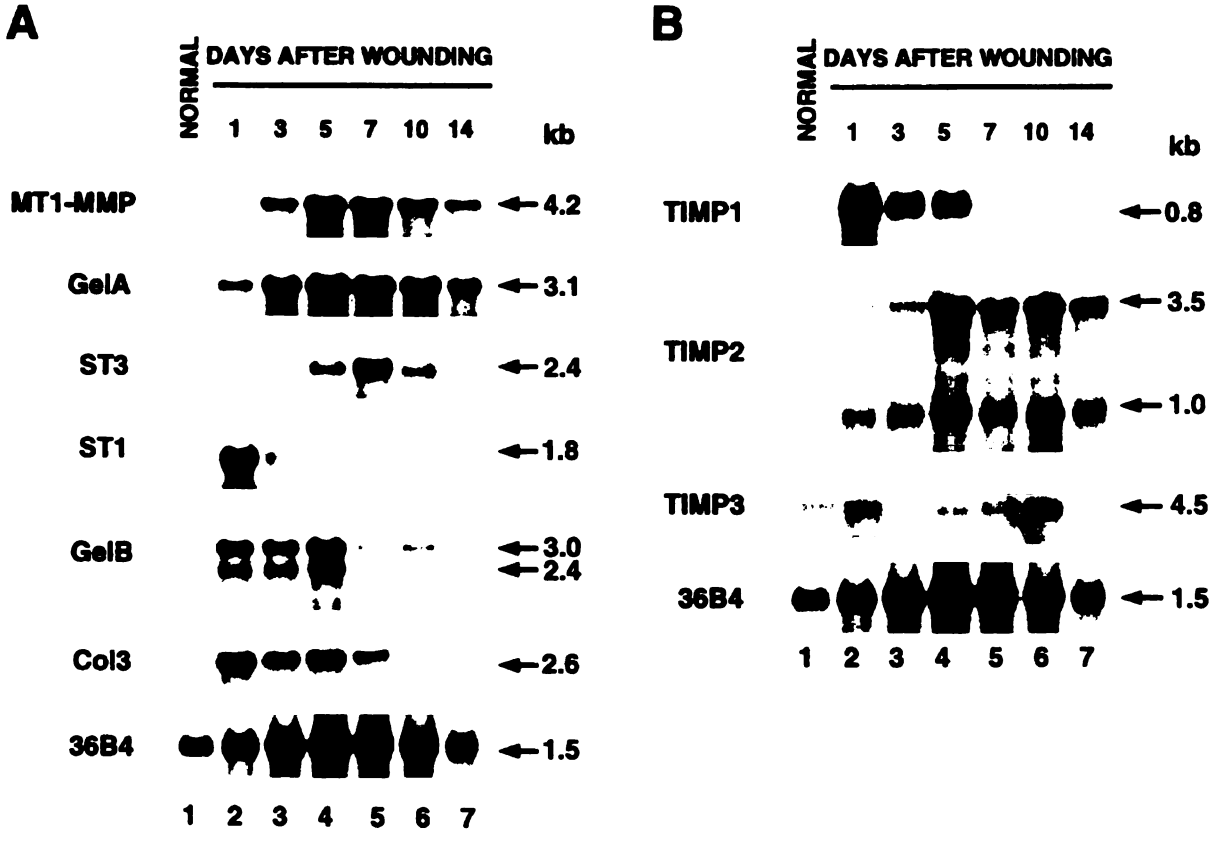

Figure 7.9. Northern blot analysis of MMP and TIMP RNAs during rat skin wound healing. Total RNA (10 μg) from normal skin (lanes 1) and skin wounds on days 1, 3, 5, 7, 10, and 14 after cutaneous incision (lanes 2-7), were electrophoresed, transferred to nylon membranes, and hybridized with 32P-labeled cDNA probes for rat MT1-MMP, GelA, ST3, ST1, GelB, Col3 (A); TIMP1, TIMP2, TIMP3 (B). Blots were reprobed with the 36B4 cDNA used as a loading control (Okada 1997).

transfected with TIMP-1, TIMP-2, or TIMP3 cDNA sequence (Okada 1997). Conditioned medium samples were collected from these transfected cells and analyzed by zymography (Okada 1997). These data indicate that gelatinase A is most efficiently activated by MT1-MMP and most efficiently inhibited by TIMP-3. The most efficient activator of gelatinase B was Stromelysin-1, and all TIMP appeared to be successful at inhibiting the activity of MMP-9 (gelatinase B) (Okada 1997).

The regulation of MMP expression in wound healing requires tight regulation. Any loss in the balance of the MMP to TIMP ratio can lead to uncontrolled degradation of ECM in chronic wounds. There is an association of increasing age with chronic wound disorders. One study recently examined the expression patterns of collagenase-1, gelatinase A, gelatinase B, and Stromelysin-1 in 132 healthy humans between the ages of 19 and 96 years (Ashcroft 1997). These individuals underwent 4-mm thick skin punch biopsies followed by wound excision between day 1 and day 180. The results from this study indicated that the expression of MMP-2 and MMP-9 were increased in older patients. Therefore, age related alteration in the control of MMP expression may be important in the structural changes observed with age in the skin and in rendering old skin susceptible to a variety of pathologies resulting in impaired and chronic wound healing.

Nerve regeneration

Skin and brain have a great deal in common because of their ectodermal origin. Several common mechanisms seem to underlie their normal structuring during development and remodeling following trauma. Several assumptions are required when considering mechanisms of actions in nerve regeneration (Brodkey 1993). Most of the molecules involved in nerve regeneration are the same molecules seen in similar regenerative processes throughout the body, namely MMPs, TIMPs,

cytokines, and growth factors. These molecules are under developmental control in that they are expressed during critical periods of development, and normally downregulated in the adult. These molecules often reappear following injury. Finally, the signals that lead to the expression or reappearance of MMPs, TIMPs, cytokines, and growth factors in nerve regeneration may be similar to the rest of the body.

A remodeling process begins after injury to a peripheral nerve. This process leads to the degeneration and regeneration of the axon (La Fleur 1996). After the injury, axons in the distal segment undergo degeneration, which involves the removal of axonal and myelin debris. Phagocytic cells remove the degenerating axons and myelin, while dividing Schwann cells remain within the basement membrane tube that surrounded the original nerve fiber. When regenerating axons from the proximal segment re-enter the peripheral nerve matrix, Schwann cells ensheath and re-myelinate them. The regenerating axons proceed to grow within the intact Schwann cell-derived tubes.

The recruitment of macrophages appears two to three days after the injury. Macrophages express several MMPs including gelatinase B (MMP-9) and Stromelysin-1 (MMP-3). These MMPs aid in the removal of axonal and myelin debris. These phagocytes also produce TGF-β, TNF-α and IL-1, which induce Schwann cells and fibroblasts to secrete nerve growth factor β (NGF-β) and TIMP-1. These factors increase the rate of regeneration (La Fleur 1996).

During the response to nerve injury, infiltrating macrophages secrete gelatinase B and resident Schwann cells are stimulated to produce TIMP-1. The production of gelatinase B and TIMP-1 in close proximity implies that ECM degradation is restricted to small-defined sites near the cell surface. Gelatinase B (MMP-9) can degrade myelin basic proteins. Increased proteolytic activity may enhance meylin degradation during degenerative stages, or release Schwann cells

from their basement membrane connections during the proliferation stages, and lead to the reestablishment of axons. The expression of TIMP-1 ensures the preservation of the basement membrane, despite the presence of high levels of proteases released after nerve injury. TIMP-1 protects the Shwann cell basement membrane from uncontrolled degradation by gelatinase B (MMP-9) and stromelysin-1 (MMP-3). The basement membrane plays an important role in the maintenance of tissue structure and orderly reconstruction following injury by serving as a scaffold for the cellular migration, arrangement or attachment. Thus, the basement membrane is critical for the promotion and guidance of axonal regrowth. Regenerating axons grow along the inner surface of the Schwann cell basement membrane. The basement membrane also provides the columnar organization of multiplying Schwann cells during the repair process. In addition to the structural support, the basement membrane also provides a favorable substrate for axonal growth.

PATHOLOGICAL CONDITIONS

There are several pathological conditions that involve the turnover of ECM. In many of these conditions, MMPs have been implicated as major contributors to ECM turnover by virtue of their ability to degrade components of the ECM and their expression in diseases such as, arthritis cancer and glomerular nephropathy. This section highlights some interesting pathologies associated with MMPs.

Arthritis

A great deal of research has gone into determining the role of MMPs in the complex cascade of proteolytic events which results in tissue destruction involved in arthritis. There is strong evidence to suggest that cartilage destruction is the result of an imbalance in the MMP to TIMP ratio. The production of MMP and TIMP in cultured synovial explants from rabbits with experimentally induced arthritis

showed that induction of the lesion correlated with a reduction in TIMP and an increase in MMP level (Murphy 1991). Treatment with corticosteroids inhibited the development of arthritis and suppressed the synthesis of MMP in a rabbit model of arthritis (Murphy 1991). Elevated levels of MMP have been extracted from both human osteoarthritic cartilage and the cartilage of dogs with experimentally induced articular changes resembling human arthritis (Martel-Pelletier 1988). Increased MMP expression has also been associated with the appearance and progression of the degenerative changes found in aging cartilage (Martel-Pelletier 1988).

Rheumatoid arthritis is a chronic polyarthritis of unknown etiology affecting 1% of the World's population. Features characteristic of rheumatoid arthritic joint destruction, including bone absorption and synovial overgrowth, can be experimentally reproduced by augmentation of *c-fos* gene expression. A study concerning the role of the *c-fos* proto-oncogene in rheumatoid arthritis was recently preformed in mice (Shiozawa, 1997). This study was of particular interest because of the importance of *c-fos/c-jun* heterodimer (AP-1) in the regulation of the expression of IL-1β, IL-6, TNF-α and most MMPs. To test the requirement of *c-fos* gene expression in arthritic joint destruction, ongoing arthritis was inhibited by blocking the AP-1 signal. Short double stranded DNA oligonucleotides containing AP-1 sequences (AP-1 oligos) and the control DNA oligonucleotides of the same length discordant only at the AP-1 consensus sequences (control oligos) were administered intraperitoneally to mice twice weekly from 14 to 42 days after the first injection until examination of the joints. AP-1 oligos compete for the binding of AP-1 *in vivo* at the promoter consensus binding sites and block the induction of the MMP gene by *c-fos*. Arthritic joint destruction was inhibited in a dose dependent manner in mice treated with AP-1 oligos (Shiozawa 1997). The expression of mRNAs

for IL-1B, IL-6, TNF-α, stromelysin 1 (MMP-3), and the 92-kDa gelatinase (MMP-9) were reduced in mice receiving AP-1 oligos compared to control oligos (Figure 7.10) (Shiozawa 1997). This model helps to identify the pathogenesis of rheumatoid joint destruction. .

Cancer

Matrix metalloproteinases are thought to play an important role in cancer invasion and metastasis since they have the ability to degrade the basement membrane and other components of the ECM (Webber 1995). Degradation of the basement membrane and invasion into the stroma is likely mediated by MMPs and is observed in cancer of the colon, prostate, breast and uterus (Crawford 1995, Lochter 1997, Sterns 1993, Webber 1995). The ECM degradation by MMPs is controlled at the same basic levels previously review in this chapter, expression, activation, and inhibition.

Expression of MMPs in cancer cells can be induced by a variety of growth factors, cytokines, oncogenes, and tumor promoters (Martrisian 1992). MMPs are responsive to growth factors such as EGF, PDGF, bFGF, and TGF-α, which are produced by numerous tumors *in vivo*. TNF-α and IL-1β are produced by infiltrating eosinophils and macrophages and have also been show to induce MMPs in fibroblast cells (Chambers 1995). One recent study in human giant cell tumors (GCT) of the bone found evidence suggesting that these cells secrete cytokines that upregulate the expression of gelatinase A by stromal cells (Rao 1997).

Steroid Hormones appear to down regulate MMP expression in prostate (Schneikert 1996). Several gelatinolytic proteinases have been detected by zymographic analysis of extract of rat ventral prostate. The gelatinolytic activity increased after castration and decreased upon treatment with testosterone (Schneikert 1996). Studies using human prostatatic cell lines, DU145 and LNCaP,

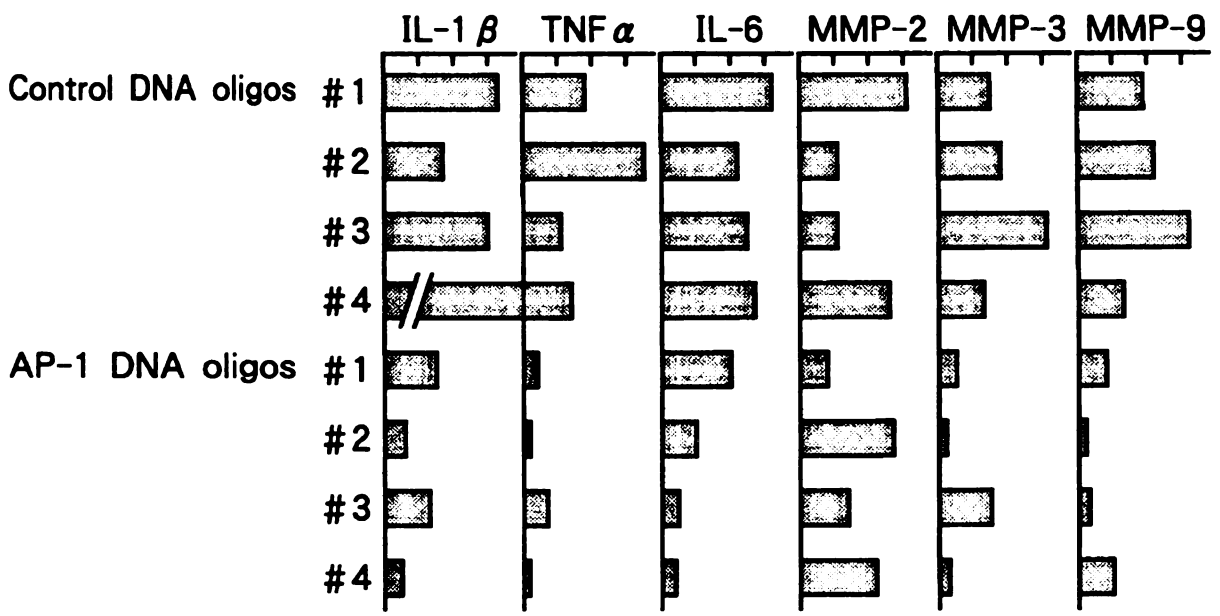

Figure 7.10. Quantification of mRNA for cytokine and matrix metalloproteinase in synovia of mice treated with AP-1 DNA oligos or control DNA oligos. mRNA of mice is reversibly transcribed and coamplified relevant genes are quantified with reference to coamplified G3PDH mRNA using Amplisensor assay. The amount of mRNA is expressed with an arbitrary scale for individual cytokines and MMPs using serial dilution of one representative sample as standard. The differences betwee two groups, except for MMP-2 and IL-1, are < 0.02, and those of IL-1 are < 0.06 by the Student's nonpaired T test (Shiozawa 1997).

show that androgens negatively regulate the expression of interstitial collagenases (MMP-1), stromelysin 1 (MMP-3), and matrilysin (MMP-7) (Schneihert 1996). Androgen receptors appear to exert this effect on the *ets* family of transcription factors. In contrast, the glucocorticoid receptor has no interaction with *ets* transcription factors, and exerts its negative regulation by interaction with the transcription factor AP-1, an important regulator of several MMP genes.

The expression of MMP-2 was examined in patients with benign prostatic hyperplasia (BPH) and varying Gleason grades of malignant prostate cancer (Stearns 1993). The Gleason grade is a tool for rating the aggressiveness of malignant prostate tumor, where scores are given 1-10 with 10 being the most aggressive. These studies showed weak or no staining of gelatinase A in patients with BPH. Patients with malignant prostatic cancer show increasing expression of gelatinase A as Gleason scores increased. These data suggest MMP-2 expression is indicative of an invasive phenotype (Stearns 1993).

The balance of MMP to TIMP is also important in cancer. Upregulation of TIMP-1, by cytokines has been observed (Stearns 1995). The PC-3 human prostatic carcinoma cell line showed low basal expression of TIMP-1. Treatment with varying doses of IL-6 and IL-10 resulted in a dose dependent increase in TIMP expression. Conversely, IL-1 and TNF-α decrease TIMP-1 expression while inducing MMP-2 in fibroblast (Stearns 1995). These results suggest that the balance of TIMP-1 and MMP-2 is important in the progression of prostate cancer and that this balance is somehow mediated by paracrine or autocrine cytokine stimuli.

MT-MMPs can activate progelatinase A in breast adenocarcinomas. *In vivo* observations demonstrate that stromal cells are the main source of MMP expression. More precisely, in breast cancer, MT-MMP and MMP-2 are localized in stromal fibroblast next to the invading fronts of tumor cells. This specific location of expression suggests that tumor cell may induce stromal cells to overexpress

MT-MMPs by various difussable growth factors. An investigation, using two different breast cancer cell lines, one non-invasive (MCF7) and the other invasive (MDA-MB-231), was conducted to compare their ability to induce MT-MMP production in human fibroblasts (Polette 1997). Conditioned medium from MD A-MB-231 was able to induce the fibroblast to express MT-MMP and activate MMP-2. In contrast, MCF-7 condition medium had no effect on the fibroblasts. Gelatinase A and MT-MMP have been associated with invadopodia. As previously described, MT-MMP are highly implicated in the activation of MMP-2 and *in situ* hybridization studies have associated MMP-2 and MT-MMP to the invadopodia of breast cancer cells (Thompson 1994).

Glomerular Nephropathy

The balance of MMP to TIMP is critical to the function of the kidney glomerulus. The glomerular filtration is dependent on the structure provided by the ECM. Abnormal expansion of the ECM can lead to chronic sclerotic states, while increased degradation of ECM can result in acute nephritis. The glomerular mesangial cell (GMC) is centrally involved in both chronic sclerotic and acute inflammatory processes characteristic of many forms of renal disease. Glomerular mesangial cell activation involves augmented proliferation and synthesis of ECM proteins, specifically type IV collagen, laminin and hepran sulfate (Yasuda 1996). Following the activation of GMCs there is an enhanced secretion of gelatinase A by these cells (Turck 1995). This close temporal linkage between GMC activation and GMC secretion of gelatinase A suggests that the turnover of ECM is dependent on the balance between its synthesis and its degradation. Consequently, glomerular sclerosis may be due to increased synthesis of ECM proteins and/or reduced protease

activity, by either decreased expression or increased inhibition. In contrast, glomerular nephritis can be induce by decreased ECM protein synthesis and/or increased protease activity by either increase expression or decreased inhibition.

One recent study examined cultured mesangial cells that overexpressed gelatinase A (Turck 1996). These cells accurately reflected an inflammatory phenotype: they had increased gelatinase A expression, their morphology included lamellopodia, and they did not synthesize IV collagen (Turck 1996). This inflammatory phenotype could be restored to a quiescent phenotype with the suppression of gelatinase A expression by transfection with episomal antisense gelatinase A gene or hammerhead ribosome targeted to gelatinase A mRNA. The genetically altered cells reflected a quiescent phenotype: they did not express gelatinase A, their morphology was rounded, and they synthesized type IV collagen (Turck 1996).

Passive Heymann's nephritis is a glomerular nephritis that can be induced in rats by visceral epithelial injury with injections of 10 :g of a sheep antibody, anti-Fx1A (McMillian 1996). An investigation examined the expression of gelatinase B in normal rats and those injected with experimentally induced Heymann's nephritis at five and fifteen day after injury (injection with sheep antibody) (McMillian 1996). Immunohistochemical staining for gelatinase B antigen was performed on glomerular sections with normal cells and passive Heymann's nephritis cells five and fifteen days after injection of sheep antibody. Normal cells showed very low levels of gelatinase B antigen either within the glomeruli tubules or interstitium. In contrast, passive Heymann's nephritis cells showed a progressively increasing expression pattern. At day five, gelatinase B antigen was primarily expressed in the visceral epithelium. At day fifteen, gelatinase B antigen reactive signal was stronger in the visceral epithelium and was also seen in some of the endothelial in the capillary

lumens (McMillian 1996). These data indicate that gelatinase B is not only expressed by stromal cell, but in the case of nephritis, gelatinase B can also be expressed by visceral epithelium.

In contrast to the increased expression of MMPs, as seen in glomerular nephritis, glomerular sclerosis appears to involve a decrease in MMP activity. Glomerular sclerosis ensues with the accumulation of glomerular ECM. It is possible that an imbalance of the MMP to TIMP ratio favoring TIMP might be involved in this accumulation of ECM. To examine this possible decrease in MMP activity and or increase in TIMP activity, studies have used the Obese Zucker rat model (Schaefer 1997).

The obese Zucker rat is a model of obesity and hyperlipidemia with many similarities to human non-insulin dependent diabetes melitus. This metabolic disease results in the development of albuminuria and mesangial matrix expansion in the glomerulus. The accumulation of matrix components like type IV collagen, fibronectin, laminin and proteoglycans results in glomerulosclerosis. These rats exhibit a decrease in glomerular gelatinolytic activity due to reduced gelatinase B expression and increased expression of TIMP-1. The glomerular gelatinase B mRNA is reduced 5 times, while the expression of TIMP-1, the natural inhibitor of gelatinase B, is enhanced (Scharfe 1997). This rat model shows that glomerular sclerosis can result from the build up of ECM which is meditated by both a loss of gelatinase B expression and increase in the expression of TIMP-1.

APPLICATIONS

Recent epidemiological studies have shown a 40 to 50% reduction in mortality from colorectal cancer in individuals who take non-steroidal anti-inflammatory drugs (NSAIDs) regularly. Most NSAIDs inhibit both cyclooxygenase COX-1 and COX-2. COX-1 is expressed in normal intestine, however, COX-2 is undetectable

in normal intestine, but is elevated in 85% of colorectal adenocarcinomas. Human colon cancer cells, Caco-2, were tranfected to express COX-2 (Tsujii 1997). These cells showed an increased expression of MT-MMP and increased activation of MMP-2 (gelatinase A). The controls were transfected with vector only, and did not show an increase in the expression of MT-MMP or increased in MMP-2 activity. The COX-2 expressing Caco-2 cells showed increased invasion. This invasive activity could be reversed by sulindac sulfide, a known COX inhibitor. The zymogram in Figure 7.11 shows the reversible activation of MMP-2 (Tsujii 1997). These data suggest that the COX-2 induces the production of MT-MMP which activates MMP-2 and result in increased invasion. This action was reversed by treatment by a NSAID.

Human glioblastoma cell lines express gelatinase A and gelatinase B at much higher levels than non-cancerous gliomas and normal brain (Chintala 1997). After treatment with *cis*-diamminedichloroplatinum (cisplatin), glioblastomas cells were examined by gelatin zymography. Gelatinase expression was decreased, however the mechanism for this decrease is not yet known. This study indicates that treatment with cisplatin might reduce invasion by decreasing gelatinase activity, in addition to it cytotoxic effects (Chintala 1997).

Studies examining human tumors and treatment with synthetic inhibitors of matrix metalloproteinases have shown promise (Santos 1997). Batimastat (BB94), one such inhibitor, has been useful in treating patients with breast cancer. Marimastat (BB-2516), another MMP inhibitor, has resulted in reductions of circulating breast tumor antigens, an indication of anti-tumor efficacy. Due to the lack of cytotoxicity of MMP inhibitors and their unique mode of action, this new class of compounds may provide new treatment for cancer patients. New synthetic MMP inhibitors are undergoing laboratory investigation (Santos 1997, An 1997). The MMP synthetic inhibitor, CT1746, has been showed to be effective against gelatinase A, gelatinase

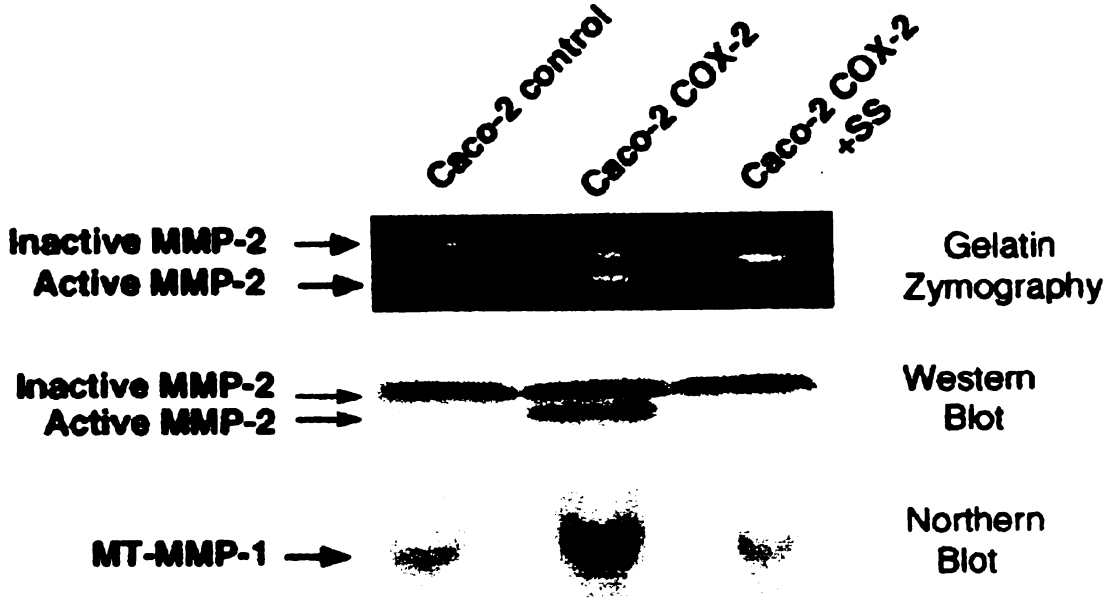

Figure 7.11. Matrix MMP-2 and MT-MMP expression in Caco-2 cells. (Top) Gelatin zymography of proteins secreted into the cell culture medium. Lane 3 demonstrates the result following treatment with sulindac sulfide (SS; 25 micro M) for 24 hr. A 68- and 62-kDa band are seen in the COX-2-expressing Caco-2 cells; whereas, only the inactive 68-kDa band is een in the control Caco-2 cells. (Middle) Immunoblotting results with an anti-MMP-2 antibody in an identical experiment to that shown in the Top panel. This result confirms the gelatin zymography data shown in cells which is inhibited in lane 3 by treatment with 25 microM sulindac sulfide for 6 hr. (Tsujii 1997).

B, and stromelysins (An 1997). Oral administration of CT1746 to nude mice, bearing tumors from the Co-3 human colon cancer cell line, resulted in the reduction of tumor size and a decrease in tumor spread. Treatment with this drug significantly prolonged median survival time in tumor bearing mice from 51 to 78 days (An 1997). These data suggest that synthetic inhibitor of MMP might be useful as non-cytotoxic chemopreventive or chemotherapeutic agents.

CONCLUSIONS

Matrix metalloproteinases are a family of enzymes that have evolved to digest specific extracellular matrix components. The activity of these proteases is regulated at three levels, expression, activation, and inhibition. These points strongly support the role of MMPs and TIMPs as important modulators of ECM turnover. Several condition both normal and pathologic involve this ECM turnover. The evidence discussed in this chapter demonstrates common mechanisms of matrix turnover. These include matrix metalloproteinases, their inhibitors and their ECM substrates, in normal development, wound healing and regeneration, as well as pathological conditions, such as, arthritis, glomerular nephropathy and cancer.

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CHAPTER 8

SIGNIFICANCE AND APPLICATION OF MMPs AND TIMPs

SIGNIFICANCE

The previous chapters have discussed the matrix metalloproteinases, their substrates, and their inhibitors. The functional role of matrix metalloproteinases (MMPs) and tissue inhibitors of MMP (TIMPs) is to modulate extracellular matrix (ECM) turnover in several normal and pathological processes. An intricate balance between MMP and TIMP is seen in normal processes such as development and wound healing. However, a disruption in this balance of MMP and TIMP is observed in important human diseases such as arthritis and cancer. As we approach the new millenium, the percentage of individuals affected by these diseases is increasing (Powell 1993). The knowledge compiled in this thesis can be applied to future research and treatment of these diseases.

ARTHRITIS

Much recent research has been directed towards determining the role of MMPs in the arthritis. A central role for MMPs produced by endogenous tissue cells as well as those of inflammatory cells is now evident. Experimental models have shown that cartilage destruction is the result of an imbalance in favor of MMPs over TIMPs. Increased expression of MMPs and decreased expression of TIMPs has been observed in cultured human tissue cells from rheumatoid joints (Murphy 1991). These studies suggest that tissue destruction, associated with arthritis, is due to an insufficient amount of TIMPs to counteract the destructive effects of

MMPs. Current corticosteroid treatment for arthritis not only blocks inflammation but also appears to inhibit the expression of MMPs (Murphy 1995). Tetracycline inhibits the activity of MMPs, and can reduce the severity of osteoarthritis in dogs (Yu 1992). This inhibition of MMP by tetracycline is independent of its antimicrobial activity and may be mediated by the chelation of zinc and calcium (Suomalainen 1992). New synthetic inhibitors of MMP are being developed as potential therapeutic agents for arthritis (Docherty 1992, Murphy 1991, 1995).

CANCER

Metastasis or the formation of secondary tumors at distant sites from the primary tumor is a major cause of cancer mortality. The failure of most current therapies to successfully treat metastases stresses the urgency of understanding the underlying mechanisms of cancer metastasis. There are several steps in the progression of a primary tumor cell toward a metastatic phenotype (Chambers 1997). Genetic manipulation of MMP and TIMP expression in several tumor cell lines has demonstrated the involvement of these enzymes in tumor invasion and metastasis (An 1997, Aoudjit 1998, Noel 1996). The imbalance of MMP to TIMP in favor of MMP is a major factor that leads to metastasis (Matrisian 1992, Montironi 1996). It has been suggested that retinoic acid can be useful in cancer prevention (Webber 1995, 1996). Considerable scientific evidence has demonstrated that retinoids are effective in the prevention of a variety of cancers and in the inhibition of cancer progression and invasion (Kim 1995, Waghary & Webber 1995). Treatment of prostate cancer cells with retinoids decreases the net proteolytic activity of urokinase-type plasminogen activators as well as MMPs (Waghray & Webber 1995, Webber 1995, 1996). Urokinase is implicated in the activation of MMPs, therefore, treatment with retinoids may decrease MMP activity. There is also recent evidence to suggest that retinoids may modulate the expression of some MMP genes. These

studies indicate the need for further research on the effects of retinoids on cancer metastasis. There are several synthetic inhibitors of MMPs currently under clinical investigation, and include Batimastat and Marimastat (Santos 1997). These drugs show excellent promise for the treatment of human breast cancers and pharmaceutical companies are rushing to develop similar compounds for use as anticancer agents (An 1997). Indeed, the study of MMPs and TIMPs will provide valuable tools by which we can combat cancer.

CONCLUSIONS

Matrix metalloprotinase and TIMPs are important modulators of extracellular matrix turnover. The process of ECM turnover is critical to a number of human physiological conditions including normal processes such as development and wound healing, and pathological processes, such as, arthritis and cancer. It is apparent that the balance of MMP to TIMP is important for these processes, however the regulation of MMP to TIMP balance is not yet fully understood. There are many great challenges to be faced in the elucidation of the mechanisms that control the balance of MMPs and TIMPs. Considering the application of this knowledge toward the treatment of human disease, the rewards for meeting these challenges are equally great.

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