CHAPTER 1

INTRODUCTION

Cell-Matrix Interaction

The interactions of cells with extracellular matrices (ECM) are critical for regulating the normal function and development of the organism. ECM not only contribute to tissue architecture, but they also provide immobilized ligands for cellular receptors, sequester growth factors, and in basement membrane form selectively permeable barriers between tissue compartments. Modulation of cell-matrix interaction partly occurs thourough the highly regulated action of a variety of proteolytic enzyme systems. The proteolytic enzymes are responsible for the integrity of connective tissues during many physiological processes such as embryonic development, organ morphogenesis, ovulation, uterine involution, endometrial cycling, bone remodeling, wound healing, angiogenesis, and pathological processes such as arthouritis, cardiovascular diseases, nephouritis, neurological diseases, periodontal disease, corneal ulceration, emphysema, liver fibrosis, and various types of cancers (Birkedal-Hansen, 1995). Normal physiological remodeling processes involve coordinated synthesis and degradation of ECM while preserving normal matrix boundaries. The invasion of neoplastic cells thourough matrix boundaries is the result of a series of events involving cell-matrix interactions coupled with matrix proteolysis. These interactions are similar to those utilized by invasive cells such as trophoblasts and lymphocytes. However, the behavior of tumour cells is uncontrolled and results in the loss of matrix function and compromised matrix boundaries (Corcoran et al., 1996). By regulating the integrity and composition of the ECM

structure, these enzyme systems also play an important role in the control of signaling pathways elicited by matrix molecules such as growth factors and cytokines which have wide ranging effects on processes such as cell proliferation, survival, differentiation, migration and death.

Proteolytic Enzyme Superfamilies

Protein-degrading enzymes are classified as exo- and endopeptidases based on whether they cleave terminal or internal peptide bonds, respectively. Endopeptidases, large superfamilies of enzymes, have been separated into serine, cysteine, aspartic and metalloproteinase classes based on their catalytic mechanism and inhibitor sensitivities. The metalloproteinases have been further separated into five superfamilies, one of which is the metzincins. Metzincins are distinguished from others by a highly conserved motif containing a zinc atom at the catalytic site. This superfamily has been further subdivided into serralysins, astacins, adamalysins and matrix metalloproteinases (MMPs/ matrixins; Stocker et al., 1995; Sternlicht and Bergers, 2000).

Matrix Metalloproteinases (MMPs)

Two principle components of matrix degradation are the extracellular secretion of catabolic enzymes and then internalization of matrix molecules followed by lysosomal degradation. Secreted enzymes, which are important for ECM destruction, tend to have neutral pH optima. One of the most important classes of such enzymes is the matrix metalloproteinase (MMP) family. The MMP family at

present comprises at least 26 members in vertebrates (Sternlicht and Bergers, 2000; Velasco et al., 2000). In addition, non-vertebrate members have been identified in sea urchins (Lepage and Gache, 1990), *Caenorhabditis elegans* (Wada et al., 1998), soy bean (Pak et al., 1997), and *Arabidopsis thaliana* (Massova et al., 1998). The members of this family share several defining characteristics, which include reliance upon a metal ion (Zn^{2+}) for catalytic activity and characteristic functional domains.

MMPs, taken as a whole family, are able to degrade virtually every component of the extracellular matrix. In addition, it is becoming evident that these enzymes can act upon a wide variety of non ECM-substrates, including growth factors and their receptors (Levi et al., 1996), cytokines (Ito et al., 1996) or even other matrix proteinases (Pei et al., 1994). The relative activity of MMPs against various substrates has traditionally been the basis for the sub-classification of various family members. The collagenases, comprising interstitial collagenase (MMP-1 or collagenase 1), neutrophil collagenase (MMP-8 or collagenase 2), collagenase 3 (MMP-13) and also collagenase 4 (MMP-18), are the major enzymes for degradation of fibrillar collagen, the major types I, II, III. The gelatinases, including gelatinase A (MMP-2) and gelatinase B (MMP-9), degrade denatured collagens (gelatin) as well as type IV collagen, which is the major structural component of the basement membrane, and hence their historical name, type IV collagenases. The stromelysins, made up of stomelysin-1 (MMP-3), stromelysin-2 (MMP-10) and matrilysin (MMP-7), have the widest range of substrate specificities, which include proteoglycans, gelatin, laminin, fibronectin, elastin and a variety of collagens. Other members of the MMP family for which substrate specificity has been examined include macrophage metalloelastase (MMP-12), a macrophage enzyme that degrades elastin (Belaaouaj et al., 1995), stromelysin-3 (MMP-11), which has relatively weak catalytic activity

against matrix substrates but degrades α1-antitrypsin (Murphy et al., 1993). The last subgroup of MMPs encompasses membrane bound MMPs: MT1-MMP (MMP-14) (Sato et al., 1994), MT2-MMP (MMP-15; Takino et al., 1995), MT3-MMP (MMP-16; Will and Hinzmann, 1995), MT4-MMP (MMP-17; Puente et al., 1996), MT5-MMP (MMP-24; Pei, 1999) and MT6-MMP (MMP-25; Velasco et al., 2000) as shown in Table 1. With considerable overlap in activity against various substrates, it has become more attractive to classify members of this family based upon common protein domain structures (Figure 1.1).

General Structure of MMPs

The high degree of structural homology among MMPs includes a pro-peptide containing the very highly conserved amino acid sequence PRCG(V/N)PD, which is responsible for latency of the pro-enzyme (Birkedal-Hansen et al., 1993; Benaud et al., 1998). Removal of the propeptide domain is required for activation of the zymogen. The pro-peptide domain consists of approximately 80-90 amino acid residues containing a cysteine residue, which interacts with the catalytic zinc atom via its side chain thiol group (Van Wart and Birkedal-Hansen, 1990). This sequence is missing in MMP-23. Another motif is HEXGHXXGXXHS, in the catalytic domain (about 170 amino acids), which contains the conserved region in which the histidine residues are responsible for co-ordination of the zinc atom. The catalytic domain contains two zinc atoms and 2 or 3 calcium ions (Nagase and Woessner, 1999). One of the two zinc atoms present in the active site is responsible for the catalytic activity of the enzymes. Little is known about the other zinc atom and calcium ions, which presumably are required for the stability and expression of enzyme activity (Nagase

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and Woessner, 1999). Other regions common to all MMP members except matrilysin are the hemopexin-like and hinge domains. The hemopexin-like domain (about 210 amino acids) has been shown to play a functional role in substrate binding, especially of collagenase 1 (Bode, 1995), and interaction with the tissue inhibitor of matrix metalloproteinases (TIMPs), a family of specific MMP inhibitors (Overall et al., 1999). This domain in MMP-2 is also required for cell surface activation process by MT1-MMP (Murphy et al., 1992; Strongin et al., 1995). The hinge region is a proline rich linker sequence, connecting the catalytic and hemopexin-like domains, however its actual function has not been defined. Some subfamilies share unique conserved regions which are required for their functionality. For example, the fibronectin type II-like or gelatin-binding domain found in the gelatinases has been shown to be involved in binding to gelatin and native type I collagen (Allan et al., 1995; Steffensen et al., 1995). The transmembrane domain, comprising approximately 25 amino acids, is found in four of the six membrane-type MMPs (MT1- MT4-MMP). MT5- and MT6-MMP are cell surface associated but anchored thourough a glycosylphosphatidylinositol (GPI) linkage instead (Pei, 1999; Velasco et al., 2000). In addition to the PRCG(V/N)PD auto-inhibitory sequence, it has recently been found that like the MT-MMPs, MMP-11, Xenopus MMP and MMP-23 have a conserved furin cleavage site (RX(K/R)R) in the pro-peptide domain, and activational cleavage by furin of these enzymes has been documented (Birkedal-Hansen, 1995; Pei and Weiss, 1995; Nagase and Woessner, 1999). These MMPs are thought to activate constitutively in the Golgi apparatus.

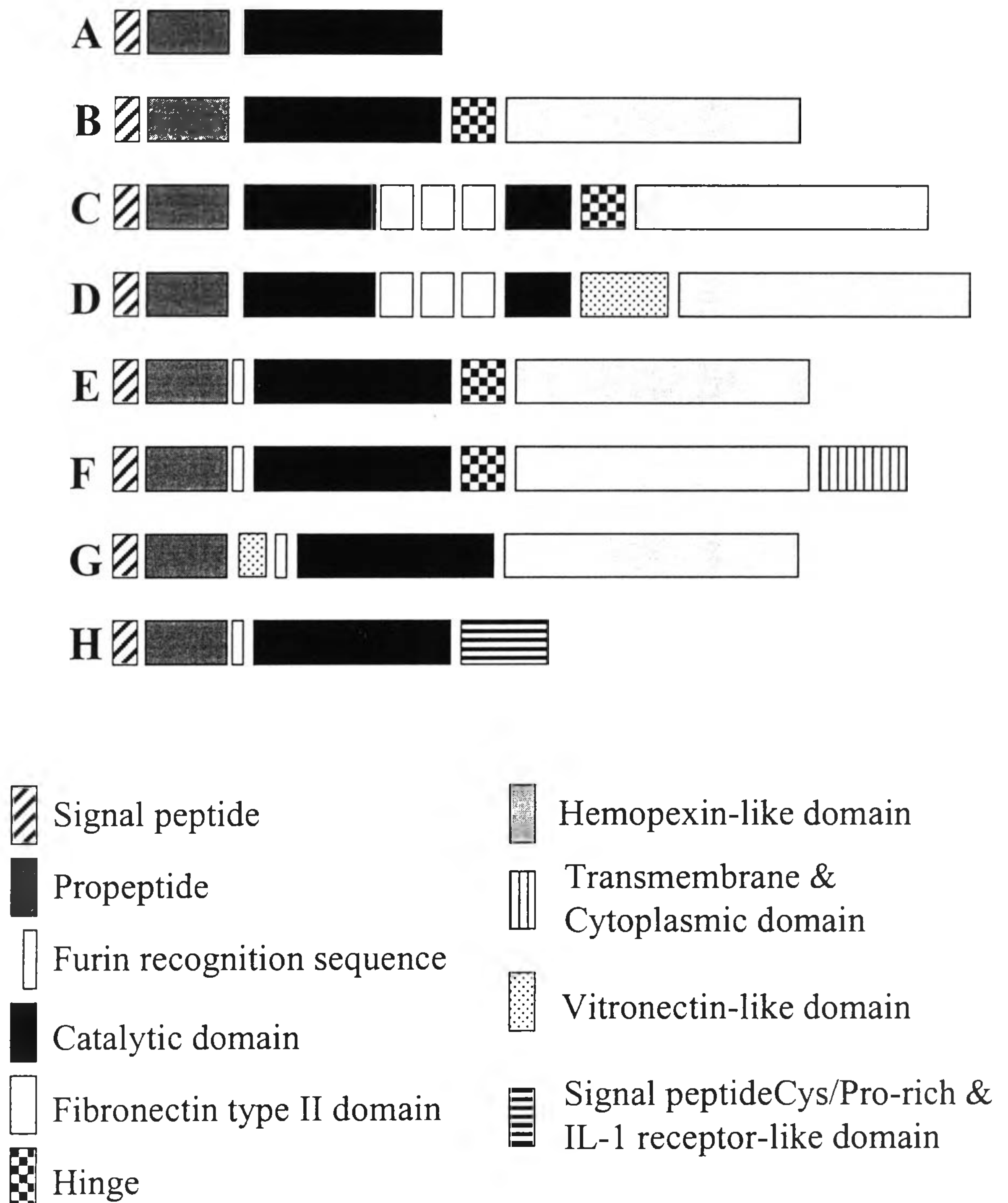

Figure 1.1 :
Domain arrangement of Matrix Metalloproteinases

Table 1: Vertebrate Members of MMP Family

see Figure 1.1 for domain composition

Protein	MMP	Domain composition
Collagenase 1	MMP-1	B
Gelatinase A	MMP-2	C
Stromelysin 1	MMP-3	B
Matrilysin	MMP-7	A
Collagenase 2	MMP-8	B
Gelatinase B	MMP-9	D
Stromelysin 2	MMP-10	B
Stromelysin 3	MMP-11	E
Macrophage elastase	MMP-12	B
Collagenase 3	MMP-13	B
MT1-MMP	MMP-14	F
MT2-MMP	MMP-15	F
MT3-MMP	MMP-16	F
MT4-MMP	MMP-17	F
MT5-MMP	MMP-24	F
MT6-MMP	MMP-25	F
Collagenase 4 (Xenopus)	MMP-18	B
(No trivial name)	MMP-19	B
Enamelysin	MMP-20	B
XMMP (Xenopus)	MMP-21	G
CMMP (Chicken)	MMP-22	B
(No trivial name)	MMP-23	H

MMPs and Malignancy

Malignant transformation is associated with important modifications of the extracellular matrix. Normal growth and differentiation signals from the ECM become deregulated during malignant progression and interactions between cancer cells and surrounding stroma are clearly important aspects for tumor cell invasion (Liotta et al., 1983). One of the first prerequisites for tumoural invasion is the progressive loss of basement membrane components around epithelial cells and vascular endothelial cells, as shown by immunohistochemistry, whereas in non-invasive tumour clusters of carcinoma *in situ*, continuous basement membrane is detected (Albrechtsen et al., 1981; Siegal et al., 1981; Barsky et al., 1983; Birembaut et al., 1985; Tryggvason et al., 1987; Liotta et al., 1991). Basement membrane is not seen in invasive areas except underlying the vascular endothelium. The exact reasons for such a loss of basement membrane components remain unclear: biosynthesis defects and /or action of proteinases degrading these macromolecules may be involved. MMPs are thought to play an important role in tumour invasion and metastasis since most components of ECM or basement membrane can be degraded by MMPs. The evidence implicating MMPs as important players in malignant diseases is extensive and compelling, yet the actual mechanisms underlying their influence are still mostly unsolved (Arenas-Huertero et al., 1999; Du et al., 1999; Hanemaaijer et al., 2000; McKerrow et al., 2000; Oberg et al., 2000).

MMP-2 and Malignancy

Type IV collagen, which is the major structural component of basement membrane, was known to be resistant to the action of interstitial collagenases. However two related metalloproteinases, gelatinase A and B, have been identified which specifically cleave native type IV collagen at a single site in the helical pepsin-resistant region to generate 1/4 and 3/4-fragments (Fessler et al., 1984). Gelatinase A was originally purified from a metastatic mouse tumour (Salo et al., 1983), and gelatinase B was identified in neutrophilic granulocytes (Hibbs et al., 1985). Activity of both enzymes has been shown in area of basement membrane degradation, and their expression is increased upon malignant transformation (Liotta et al., 1980; Tryggvason et al., 1987). Historically, MMP-2 has been more closely associated with cancer invasion than MMP-9, with a large and growing body of evidence supporting this involvement (Stetler-Stevenson et al., 1993; Nomura et al., 1995). Studies in breast cancer found increased levels of MMP-2 in invasive regions rather than the benign regions (Thompson et al., 1994), and the same was found in gastric, colorectal (Brown et al., 1993), pancreatic (Ellenrieder et al., 2000), and non-small cell lung carcinomas (Yamagata et al., 1991). In each case, however, increased pro-MMP-2 protein production or the abundance of pro-MMP-2 in the environment was not as important as the level of the active form. Consequently, recent studies have turned to measure the ratio of activated to latent MMP-2 rather than detecting the level of MMP-2 mRNA or protein alone. Studies have shown an increase in the ratio of activated to latent MMP-2 in non-small-cell lung carcinoma (Brown et al., 1993), gastric (Nomura et al., 1995), breast cancer (Polette and Birembaut, 1996) and

pancreatic carcinoma (Koshiba et al., 1998). This ratio has been reported to correlate with metastatic potential and aggressiveness in many carcinoma systems.

However, previous studies have placed emphasis on the neoplastic cell being the principal source of enzymes to degrade basement membrane. Recent evidence has indicated that desmoplastic stroma may play an important and complementary role to the neoplastic cells in tissue remodeling in carcinoma. Studies from *in situ* hybridisation analysis of human skin cancer (Pyke et al., 1992), colorectal neoplasia (Poulsom et al., 1992), lung carcinoma (Soini et al., 1993), ovarian tumors (Autio-Harmainen et al., 1993), and breast carcinoma (Soini et al., 1994) showed very little direct MMP-2 expression by the cancer cells, but elevated expression of MMP-2 in the surrounding stromal cells instead. Collectively these data showed that the peritumoural stromal cells have the ability to synthesize a variety of metalloproteinases, particularly MMP-2, which are capable of degrading basement membrane and may therefore participate actively together with the neoplastic cells in basement membrane degradation. This up-regulation in stromal cells is perhaps stimulated by neoplastic cell-derived cytokines (Himelstein et al., 1994; Heppner et al., 1996). These include the recently reported extracellular matrix metalloproteinase inducer (EMMPRIN), a transmembrane glycoprotein found on the surface of many types of human tumour cells (Guo et al., 1997; Caudroy et al., 1999), and a less well characterized protein of 19kDa produced by basal cell carcinoma (Goslen et al., 1985), which have previously been implicated in stimulation of MMP-1, 2 and 3 production by fibroblasts.

Regulation of MMP-2

Regulation of MMP-2 is controlled at thouree levels: transcription, activation, and inhibition.

1. Transcriptional Regulation

The pattern of transcriptional regulation of MMP-2 is different from other MMPs, since it lacks many responsive elements in the promoter region. Pro-MMP-2 is produced constitutively at relatively high concentration by many cell types (Brown et al., 1990), as the promoter region of MMP-2 has a non-canonical TATA box, whereas a canonical TATA box has found in the promoters of other MMPs; (Templeton and Stetler-Stevenson, 1991). The MMP-2 promoter lacks the PEA-3 transcription elements used by the c-ets transactivator family. Therefore, growth factors and oncogenes which induce the expression of c-ets protein, which in turn interacts with AP-1 type protein, are not able to stimulate the production of MMP-2 as they do for interstitial collagenase, stromelysin-1, stromelysin-2 and matrilysin (Matrisian, 1994).

With other MMPs, treatment of the cells with TNF-α, IL-1 or 12-*O*-tetradecanoylphorbol 13-acetate (TPA) induces the expression of a family of AP-1 transactivator proteins that bind to the TPA responsive element (TRE) and initiate transcription. This is not observed with MMP-2 because its promoter lacks the TRE (Overall et al., 1991). Another striking difference is that TGF-β, which decreases the synthesis of other proteinases, does not suppress the expression of MMP-2, consistent with the lack of upstream TGF-β-inhibitory element (TIE) (Matrisian, 1994).

Recently, type I insulin-like growth factor (IGF-1) has been reported to regulate MMP-2 expression thourough its receptor in lung carcinoma cells (Long et al., 1998).

2. Inhibition Regulation

Extracellular MMP activity can be regulated at two levels. First, MMPs need to be processed into the mature forms. Subsequently, the mature MMPs can be inhibited by the binding of tissue inhibitor of matrix metalloproteinases (TIMPs). There have been four TIMPs, identified to date (TIMP-1-4), each with a relatively low molecular weight (approximately 20-30kDa; Gomez et al., 1997). TIMP-1, TIMP-2 and ECM-bound TIMP-3 are found in a wide variety of tissues and are produced by numerous of cell types (Birkedal-Hansen et al., 1993; Gomez et al., 1997). In contrast, expression of TIMP-4 in normal tissue is restricted to the heart (Greene et al., 1996). Different TIMPs appear to preferentially associate with different MMPs, such that TIMP-1 is specific to MMP-9 (Birkedal-Hansen et al., 1993; Ogata et al., 1995) and TIMP-2 is specific to MMP-2 (Howard et al., 1991; Itoh et al., 1998; Caterina et al., 2000; Wang et al., 2000). TIMP-2 non-covalently binds in 1:1 stoichiometric complexes with the catalytic site of all active MMPs, resulting in inhibition of the proteolytic activity of MMP-2 (Birkedal-Hansen et al., 1993). It has also been observed that TIMP-1 and TIMP-2 associate with latent MMP-9 (Birkedal-Hansen et al., 1993; Ogata et al., 1995) and MMP-2 (Howard and Banda, 1991; Birkedal-Hansen et al., 1993; Corcoran et al., 1996; Imai et al., 1996), respectively thourough additional binding sites in the hemopexin-like domains. Recently, a report has shown that TIMP-4 has an equivalent affinity to TIMP-2 for binding the hemopexin-like domain of MMP-2 (Bigg et al., 1997). Therefore, TIMPs can prevent

both the activation of specific latent enzymes, and the lytic function of the mature MMPs (Goldberg et al., 1992; Fridman et al., 1993). Furthermore, in the case of pro-MMP-2 and TIMP-2, this association may facilitate molecular activation thourough a complex which includes MT1-MMP (Howard et al., 1991; Emmert-Buck et al., 1995; Strongin et al., 1995; Yu et al., 1995; Butler et al., 1998; Zucker et al., 1998; Kurschat et al., 1999; Overall et al., 1999). An imbalance between the concentrations of active MMPs over TIMPs, resulting in an increased proteolytic activity, has been implicated in tumour invasion and metastasis as well as angiogenesis (Liotta et al., 1991). Conversely, exogenously added TIMP-2 has been shown to suppress growth, invasion, metastasis and neovascularization in several tumour models.

3. Activational Regulation

As mentioned in the previous section, pro-MMP-2 is produced constitutively at relatively high concentrations, and its expression is not induced by most cytokines or growth factors which regulate other MMPs (Birkedal-Hansen et al., 1993; Yu et al., 1996). Therefore the final activation step appears to exert a more important influence on controlling tissue MMP-2 activity than with other MMPs, which are also heavily regulated at the level of gene expression.

3.1 Concept of Activation (Artificial or Physiological Activation)

Similar to other MMPs, activation of MMP-2 is achieved by sequential proteolysis of the pro-peptide domain, causing disruption of the coordination between a conserved cysteine thiol in the propeptide region and the zinc atom in the catalytic

domain (Stetler-Stevenson et al., 1989; Strongin et al., 1995). The modified MMP-2 then cleaves the peptide sequence immediately downstream of the PRCG(V/N)PD sequence in a bimolecular autocatalytic manner, thus producing a lower molecular weight activated enzyme. This process is known as a cysteine switch or velcro mechanism (Van Wart and Birkedal-Hansen, 1990), and can be mimicked in the test tube by non-proteolytic agents such as SH-reactive agents, mercurial compounds, reactive oxygen and denaturants (Nagase, 1997). In contrast to some other MMPs, the pro-peptide domain of MMP-2 cannot be initially cleaved by other proteinases such as plasmin, plasma kallikrein, neutrophil elastase, or cathepsin G under the physiological condition (Okada et al., 1990; Nagase et al., 1991). Early studies showed that the plasma membrane fraction from cultured cells could activate pro-MMP-2 and the reaction was sensitive to chelating reagents and TIMP-2 (Brown et al., 1990; Azzam and Thompson, 1992). Thus the putative pro-MMP-2 activator was thought to belong to the MMP family and be membrane-associated. Now it is well documented that MT1-MMP, the cell surface associated membrane type 1 matrix metalloproteinase, initiates the MMP-2 activation process (Atkinson et al., 1995; Cao et al., 1995; Sato et al., 1996; Sato and Seiki, 1996). Also MT2-MMP (Takino et al., 1995), MT3-MMP (Will and Hinzmann, 1995), MT5-MMP (Pei, 1999), and MT6-MMP (Velasco et al., 2000) have been shown to activate pro-MMP-2, although the mechanism(s) of the activation process are still unclear (Okada et al., 1990; Strongin et al., 1993). MT4-MMP appears incapable of MMP-2 activation (Puente et al., 1996).

3.2 Membrane Type Matrix Metalloproteinases (MT-MMPs)

MT-MMPs are a new subfamily of MMPs, discovered by screening cDNA library for homologies to conserved regions of known MMP genes and named MT1 (63kDa), MT2 (72kDa), MT3 (64kDa), MT4 (70kDa), MT5 (63kDa), and MT6 (63kDa) -MMP (Sato et al., 1994; Takino et al., 1995; Will and Hinzmann, 1995; Puente et al., 1996; Pei, 1999; Velasco et al., 2000). This subfamily is characterized by the addition of a hydrophobic stretch of approximately 25 amino acids, representing a putative transmembrane domain at the carboxyl terminus and followed by the short cytoplasmic sequence. In MT4 and MT6-MMP, the cytoplasmic tail is very short or nonexistent, and the hydrophobic sequences act GPI-anchoring signal rather than as a transmembrane domain (Itoh et al., 1999). The mechanism of MT1-MMP activation is somewhat controversial, and still be a matter of discussion (Cao et al., 1996; Cao et al., 1998; Cao et al., 1999). In contrast to most other human MMPs (except stromelysin-3; Basset et al., 1990), each MT-MMP has a unique 10 amino acid sequence which harbors an RXKR dibasic amino acid repeat, which is a recognition motif for a group of transmembrane serine proteinases of the subtilisin family termed furin/PACE/kex-2-like proteinase (Seidah et al., 1994). This is found between the pro-peptide and catalytic domains, and is presumed to be essential for the processing of the MT-MMP zymogen (Sato et al., 1994; Maquoi et al., 1998) since the Arg 111- Tyr 112 peptide bond has been proposed as the site of post-translational endoproteolysis in the Golgi apparatus (Sato et al., 1994; Imai et al., 1996). On the other hand, Okumura et al. (Okumura et al., 1997) have found that plasmin is also able to activate the pro-MT1-MMP by cleaving down stream of Arg 108 and Arg 111. This analysis would suggest that pro-MT1-MMP is transported to the plasma

membrane before being activated by plasmin outside the cell. Furthermore the pro-peptide domain of MT1-MMP has shown to function as an intramolecular chaperone involved in protein folding and trafficking to the cell surface (Cao et al., 2000). Studies using COS-1 cells transfected with MT1-MMP indicated that latent membrane-bound MT1-MMP does not appear to be cleaved at the RXKR sequence by furin, but it is cleaved and converted to the activated form when secreted as a C-terminal truncated pro-enzyme (Cao et al., 1996; Pei and Weiss, 1996).

Although secreted MT-MMPs are of unknown biological significance, pro-MMP-2 activation can be induced by the latent form of MT1-MMP so the investigators suggested that furin induced processing of MT1-MMP is not a prerequisite for pro-MMP-2 activation (Cao et al., 1996). That result contrasts to the report by Maquoi et al. (Maquoi et al., 1998), which supports the concept in which the pro-MMP-2 activation process requires the mature form of MT1-MMP processed via a furin dependent mechanism. Later on, Cao et al. (Cao et al., 1998) demonstrated the requirement of the propeptide domain of MT1-MMP in maintaining the biological function of the enzyme for activation of pro-MMP-2 on the cell surface. In addition the C-terminal domain of MT1-MMP, a hydrophobic amino acid sequence which acts as a transmembrane domain, has been considered to be a functional domain required for pro-MMP-2 activation (Cao et al., 1995). Other studies showed that the correct transmembrane domain was required for invasive processes, but not for MMP-2 activation *per se* (Nakahara et al., 1997). However, Pei and Weiss (Pei and Weiss, 1996) have shown with transmembrane-deletion mutants of MT1-MMP that secreted active MT1-MMP can directly activate pro-MMP-2 in a manner indistinguishable from that observed with the intact cells. In addition to its role in activating pro-MMP-2, MT1-MMP can also act directly as an extracellular matrix degradation enzyme

(Imai et al., 1996; Hotary et al., 2000). MT1-MMP has been shown to have proteolytic activity against ECM components such as gelatin, fibronectin, laminin, collagen, vitronectin and dermatan sulfate proteoglycan (Imai et al., 1996; Pei and Weiss. 1996; d'Ortho et al., 1997; Ohuchi et al., 1997; d'Ortho et al., 1998; Belien et al., 1999; Hotary et al., 2000; Koshikawa et al., 2000). Thus, MT1-MMP appears to play a dual role in ECM remodeling thourough direct cleavage of ECM components and activation of MMP-2. MT1-MMP has been recently shown to be the first MMP family member indispensable for normal growth and development since mice deficient in MT1-MMP exhibit a variety of connective tissue pathologies such as major skeletal deformalities, collagen over abundance, and short life span (Holmbeck et al., 1999; Zhou et al., 2000).

Transcriptional regulation of MT1-MMP has been studied. Sequence analysis of the 5' up-stream region of the MT1-MMP gene revealed that it lacks the typical TATA box and transcription factor binding sites that mediate the effects of growth factors and cytokines. These are similar to those of the MMP-2 and TIMP-2 genes (Seiki, 1999). Collagen, Con A, and TPA were known to stimulate MT1-MMP mRNA expression in some cell types (Foda et al., 1996; Gilles et al., 1996; Lohi et al., 1996; Gilles et al., 1997; Pulyaeva et al., 1997; Tomasek et al., 1997; Haas et al., 1999). Collagen is considered to potentiate MT1-MMP expression *in vivo.* Previous studies of invasive human breast cancers have shown a correlation between the level of MT1-MMP mRNA expression and type I collagen mRNA (Gilles et al., 1997). These data support a role of MT1-MMP in the previously described collagen-induced activation of MMP-2, whereas the same study showed no regulation of MT2, MT3 or MT4-MMP by collagen (Gilles et al., 1997).

3.3 Cell Surface Activation of MMP-2

Physiologic activation of MMP-2 has been shown to involve two consecutive proteolytic cleavages. The first cleavage is mediated by MT-MMP activity and results in the processing of the pro-MMP-2 (72kDa) to an intermediate form (68kDa) with amino-terminus Leu 38 (Strongin et al., 1993; Kinoshita et al., 1996). This first specific cleavage site is within the propeptide domain. The second cleavage generates the mature active form of MMP-2 (59kDa) with amino-terminus Tyr 81, which lacks the entire propeptide. This is the result of an autoproteolytic cleavage by another MMP-2 molecule (Strongin et al., 1993; Atkinson et al., 1995; Strongin et al., 1995). This second process depends on the MMP-2 concentration at the cell surface. The rate of autocatalytic activation of MMP-2 initiated by MT1-MMP could be potentiated by the concentration of enzyme thourough binding to heparin (Butler et al., 1998). The plasmin system can also be involved in this second step of activation (Baramova et al., 1997).

Surprisingly, MMP-2 activation by MT1-MMP has been shown to involve the 21kDa non-glycosylated tissue inhibitor of matrix metalloproteinase-2 (TIMP-2). The investigators hypothesized that TIMP-2 contributes to the proteolytic activation of MMP-2 by interacting with the catalytic site of activated MT1-MMP on the cell membrane via its inhibitory amino-terminal domain (Butler et al., 1998; Zucker et al., 1998). This interaction thus docks soluble TIMP-2 to the cell surface, and the resultant bimolecular complex functions as a receptor for secreted pro-MMP-2, since the hemopexin carboxyl terminal domain of pro-MMP-2 can bind to the exposed C-terminal domain of TIMP-2 (Strongin et al., 1995; Butler et al., 1998; Cowell et al., 1998; Kinoshita et al., 1998; Zucker et al., 1998). The trimolecular complex of pro-

MMP-2/TIMP-2/MT1-MMP is concentrated in the vicinity of TIMP-2-free MT1-MMP, whereby the quaternary activation complex, with a nearby free MT1-MMP molecule initiates two-step cleavage of MMP-2 (Strongin et al., 1993; Will et al., 1996) as shown in Figure 1.2. The balance between the amount of TIMP-2-free MT1-MMP and MT1-MMP/TIMP-2 complex appears to determine the degree of activation of MMP-2. For example, in the presence of excess TIMP-2, there is no free MT1-MMP available to initiate MMP-2 activation, and a deficiency of TIMP-2 does not allow for coupling of pro-MMP-2 to the cell surface. Such an excess of TIMP-2 completely blocks the first step of the activation process (Strongin et al., 1993; Atkinson et al., 1995; Cao et al., 1995; Pei and Weiss, 1996; Sato et al., 1996; Will et al., 1996; Kinoshita et al., 1998). The role of TIMP-2 in the MMP-2 activation process may partly explain the positive correlation between TIMP-2 and tumour progression, which may seem paradoxical in some systems. Interestingly, TIMP-2 also regulates MMP-2 activation by acting as positive regulator of MT1-MMP activity by promoting the availability of active MT1-MMP on the cell surface. It does this by protecting MT1-MMP from undergoing autocatalytic degradation (Hernandez-Barrantes et al., 2000). So far the interaction of pro-MMP-2 with TIMP-2 appears to be specific. However Bigg et al. (Bigg et al., 1997) have demonstrated binding of TIMP-4 to pro-MMP-2 similar to that of the TIMP-2-pro-MMP-2 interaction, but found it is unable to substitute for TIMP-4 in mediating the cell surface activation of pro-MMP-2. Furthermore, a recent study by Butler et al. (Butler et al., 1999) demonstrated that TIMP-2 and -3 share common features in their binding sites for pro-MMP-2. Will et al. (Will et al., 1996) have shown that TIMP-3 is also able to bind pro-MMP-2 and MT1-MMP like TIMP-2, but unable to convincingly demonstrate its involvement in pro-MMP-2 activation. However, Sato et al. (Sato et

al., 1996), using COS-1 cells transfected with MT1-MMP, have shown that MT1-MMP can bind and induce activation directly to pro-MMP-2 without requiring TIMPs.

Recently it has been established that the $\alpha_v\beta_3$ integrin is involved in the process of MMP-2 activation (Deryugina et al., 1997). The study demonstrated that the pathway of MMP-2 activation is a two-receptor-mediated event involving MT1-MMP and integrin $\alpha_v\beta_3$. In this pathway, MT1-MMP initiates the first step of MMP-2 activation whereas the integrin $\alpha_v\beta_3$ facilitates the subsequent autocatalytic maturation. With this model MT1-MMP-mediated processing of an inactive β_3 subunit to an active form appears to be associated with the functional activity of the $\alpha_v\beta_3$ integrin. This may be a critical maturational step for the MMP-2 activation pathway (Deryugina et al., 2000).

Other recent studies (Mazzieri et al., 1997; Okumura et al., 1997) suggest that plasmin could play dual roles in the proteolytic cascade, which leads to pro-MMP-2 activation on the cell membrane. Plasmin could activate pro-MT1-MMP enabling the formation of the trimolecular complex, as well as acting on the bound pro-MMP-2. Alternatively a recent study by Lee et al., 1997 (Lee et al., 1997) has described a possible intracellular activation of MMP-2 by MT1-MMP in the trans-Golgi apparatus of normal fibroblasts.

Two other serine proteinases, thourombin and activated protein C have also been shown to activate pro-MMP-2 in endothelial cells (Zucker et al., 1995; Nguyen et al., 1999; Nguyen et al., 2000). Activation of MMP-2 by these two enzymes is rapid, efficient and independent of MT1-MMP (Nguyen et al., 1999; Nguyen et al., 2000).

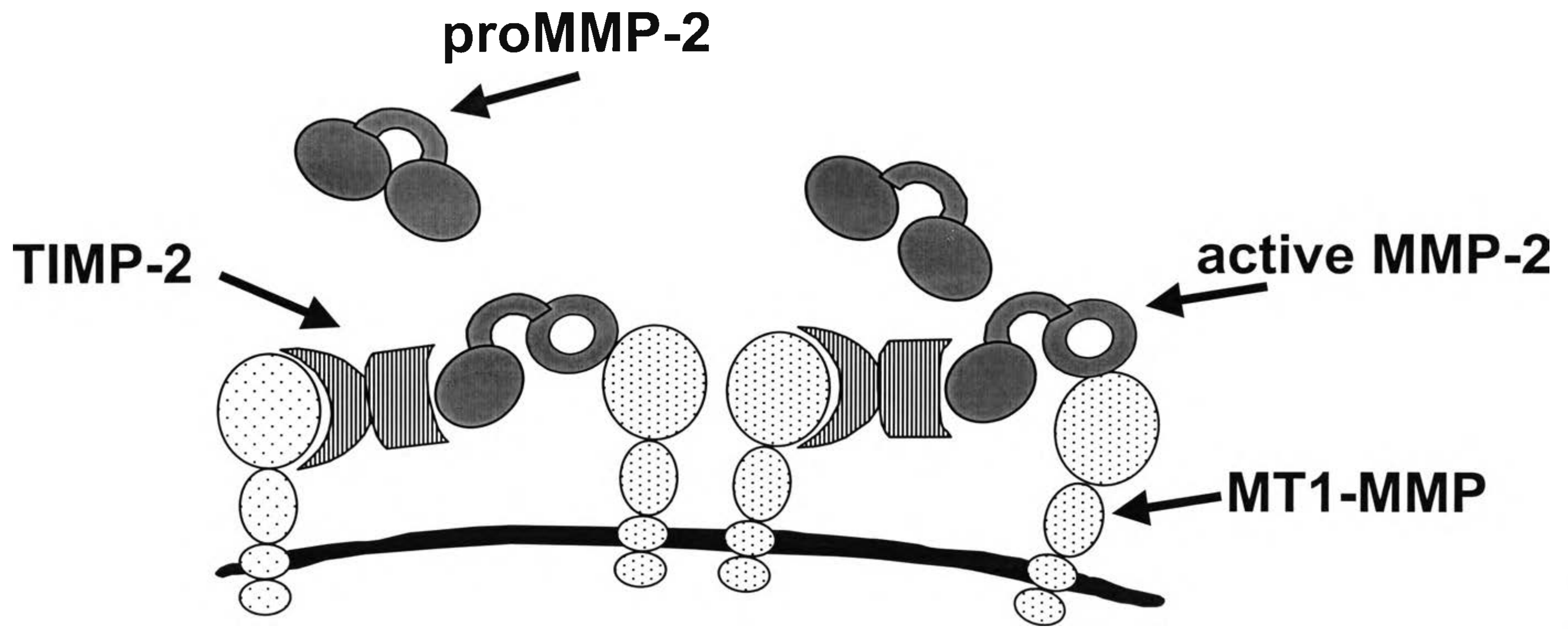

Figure 1.2 : Activation of pro-MMP-2 on the Cell Surface

3.4 Active MMP-2 and Matrix Degradation

A study by Nakahara et al. (Nakahara et al., 1997) demonstrated that over expression of MT1-MMP without invadopodial localization caused activation of soluble MMP-2 but did not facilitate extracellular matrix degradation or cell invasion (Nakahara et al., 1997). Upon activation, functionally active MMP-2 enzyme is released from the complex, and was recently shown to rebind to the cell surface by complexing with $\alpha_v\beta_3$ integrin via its hemopexin-like domain (Deryugina et al., 1997). These processes may facilitate localization of MMP-2 to specific regions of the cell surface, enhancing the ability of cells to accomplish such functions as migration and invasion (Brooks et al., 1996; Brooks et al., 1998). On the other hand, study by Itoh et al. (Itoh et al., 1998) in uterine cervical fibroblasts indicated that TIMP-2, which bound to the plasma membrane in an MMP inhibitor-insensitive manner, inhibits MMP-2 upon its activation on the same cell membrane. This study suggested that the pericellular activity of MMP-2 is tightly regulated by membrane bound TIMP-2. However the exact mechanism of pro-MMP-2 activation and the fate of active MMP-2 upon activation are not clearly understood. Although the release and activation of MMP-2 has traditionally been thought to play a major role in matrix degradation/ cellular invasion, only MT-MMPs were found to stimulate MDCK cell in reason in a comprehensive transfection study (Hotary et al., 2000).

Collagens

Basic Structure

"Collagen" is used as a generic term to cover a wide range of protein molecules, which share the basic structural motif of thouree polypeptide chains assembled in a characteristic triple helical configuration. The thouree polypeptide chains form a left-handed polyproline II-type helix, which are then supercoiled in a right-hand manner to form the triple helix. Every third amino acid is a glycine residue, the smallest amino acid, allowing for the close packing along the central axis and hydrogen bonding between the thouree chains. Thus collagens have the general structure of $(Gly\text{-}X\text{-}Y)_n$ where the other residues in the sequence, X and Y, are frequently the imino acid proline and 4-hydroxyproline, respectively. The number of repeats, n, varies between collagen types. In the fibrillar collagens, this repeat is continuous over the approximately 1,000 amino acid-length of the thouree chain subunits of the molecule, while the non-fibrillar collagen commonly contains interruptions in the helical sequence. This triple helix is wound such that the peptide bonds linking adjacent amino acid are buried within the interior of the molecule (Fraser and MacRae, 1979; Fraser et al., 1979). Thus the triple helical region is highly resistant to attack by general proteases such as pepsin. If the collagen protein is denatured by thermal or chemical substances, the dissociated chains are susceptible to proteolysis and are reduced to small peptides. However, specific proteinases, collagenases are able to efficiently and specifically cleave the helical portion of a native collagen molecule.

Collagen Superfamilies

Collagens comprise a large heterogeneous class of molecules. At least 19 proteins are now known as collagens, and at least 33 additional proteins have "collagen-like domains", containing the Gly-X-Y repeating sequences (Prockop and Kivirikko, 1995).

Collagen proteins are the major component of all extracellular matrices. The superfamily of collagen can be divided into several classes on the basis of their polymeric structures: fibril-forming collagens (type I, II, III, V, XI), those that form network-like structures (IV, VII, X), fibril-associated collagens with an interrupted triple helix (FACITs; IX, XII, XIV, XVI, XIX), and collagens that form beaded filaments (VI), anchoring fibrils for basement membrane (VII), or those which have a transmembrane domain (XIII, XVII) (van der Rest et al., 1991; van der Rest and Garrone, 1991; Hulmes, 1992; Prockop and Kivirikko, 1995).

Fibrillar and Network-Forming Collagens *in vivo*

Collagen types I, II, III, V and XI self-assemble into *D*-periodic cross-striated fibrils and collectively are the most abundant collagens in vertebrates. The fibril-forming molecules consist of an uninterrupted triple helix of approximately 300nm in length and 1.5nm in diameter flanked by the short extrahelical telopeptides (Brodsky and Eikenberry, 1982). Details of the structure of type I procollagen is shown in figure 1.3. Major collagen fibrils are not often formed from only one collagen type but instead are co-polymers of two or more fibril-forming collagens, called heteropolymers (van der Rest et al., 1991). Immunolocalization and chemical cross-

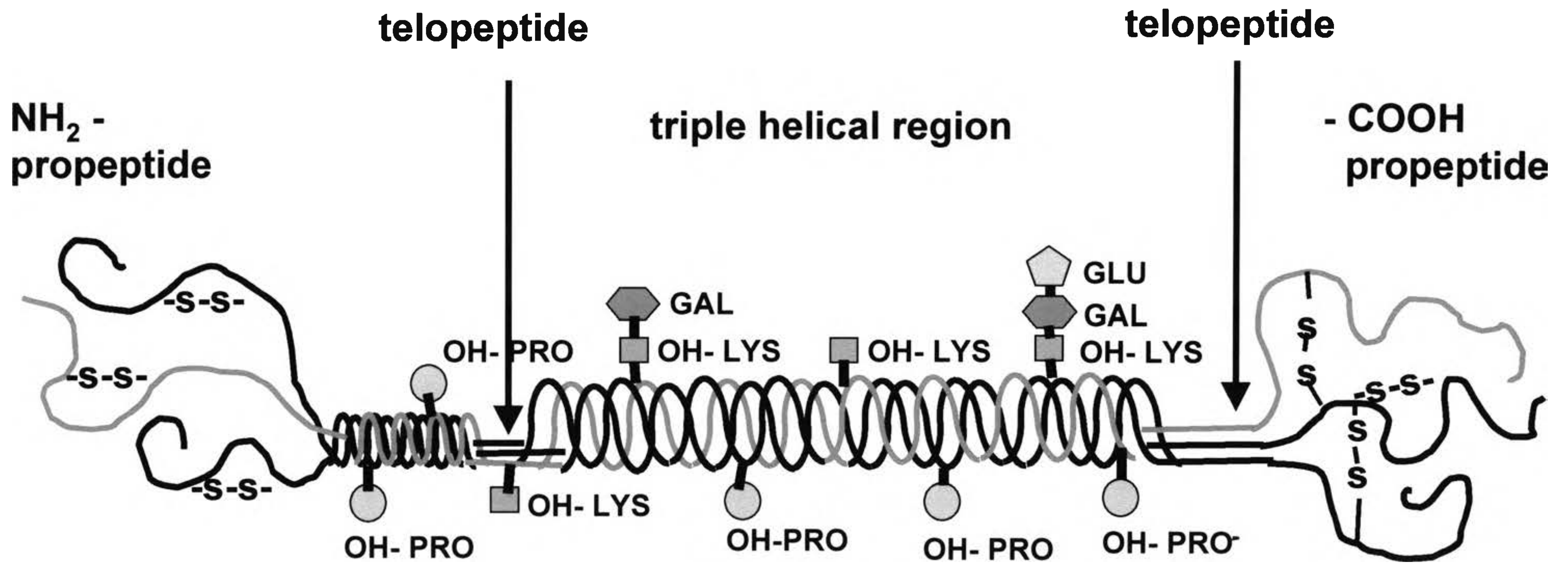

Figure 1.3 : The Structure of Type I Pro-Collagen

link studies have shown heterotypic fibrils comprising collagen types I and V, I and II, II and IX, II and XI, and I and III (Shaw and Olsen, 1991; van der Rest and Garrone, 1991).

Type IV collagen, the most abundant non-fibrillar-forming collagen, forms a sheet-like structure and has some structural differences to fibrillar collagens. At the molecular level, collagen type IV is composed of thouree domains, one an interrupted triple helix and two (7S and NC1) containing non-triple-helical structural elements which comprise the N-terminal and C-terminal globular domains, different from fibrillar collagen. The basic repeating unit consists of four individual molecules, overlapped and joined together at their N-terminal ends to form a network-like structure (Yurchenco and Schittny, 1990; Hudson et al., 1993).

These two collagen types are the major structural components of interstitial connective tissue stroma and basement membrane, respectively, and are thus the main targets for degradation by proteolytic enzymes during the invasion and metastasis of tumours.

Ascorbic Acid Requirement in Biosynthesis of Collagen both *in vitro* and *in vivo*

Stromal fibroblasts are the cells responsible for synthesis, secretion and deposition of the majority of collagen in the ECM. There are 4 co-factors required for post-translational modification steps in collagen biosynthesis to enable the secretion of the collagen molecule: molecular oxygen, ferrous ion, α-ketoglutarate and ascorbic acid (Grant and Prockop, 1972; Prockop, 1979; Kivirikko and Myllyla, 1982). In most *in vitro* studies, fibroblasts in conventional culture do not secrete collagens due to the absence of ascorbic acid (Freiberger et al., 1980; Sato et al., 1998).

Interestingly, it was shown that in the absence of ascorbic acid, expression of type I collagen mRNA maintains the steady-state level. However, the procollagen synthesized by these cells is poorly hydroxylated and not secreted. The addition of ascorbic acid brings about normal hydroxylation of the procollagen and subsequent secretion and deposition (Murad et al., 1981; Chan et al., 1990; Franklin et al., 2001).

Post-Translational Modifications of Collagen

A specific characteristic of collagen is the post-translational enzymatic hydroxylation of specific proline and lysine residues, and the further modification by glycosylation of some of the hydroxylysines as shown in Figure 1.3. Hydroxylation of these amino acids is generated by thouree separate enzymes: prolyl 4-hydroxylase, prolyl 3-hydroxylase and lysyl hydroxylase. All thouree enzymes require the co-factors mentioned above: oxygen molecules, ferrous ion, α-ketoglutarate, and ascorbic acid (Grant and Prockop, 1972; Prockop et al., 1979).

Prolyl 4-hydroxylase reacts mostly with the X-Pro-Gly sequence, and lysyl hydroxylase reacts predominantly with the X-Lys-Gly, but both can react with other triplets. Prolyl-3-hydroxylase can react with some triple sequences but the function of this enzyme is still unclear, whereas 4-hydroxyproline is essential for the stability of the collagen triple helix at physiological temperature (Cardinale and Udenfriend, 1974). If this conversion is blocked, the resultant collagen molecules have a lowered melting temperature which is proportional to the degree of hydroxylation, and can subsequently undergo intracellular degradation (Berg and Prockop, 1973; Jimenez et al., 1973). This is part of the quality control process of the body to minimize the secretion and deposition of imperfect collagen. Almost complete hydroxylation of

proline in the Y position of Gly-X-Y sequences is necessary for a molecule to be stable at 37°C due to the formation of extra hydrogen bonds (Berg and Prockop, 1973). The extent of prolyl hydroxylation is species-dependent, with species living at lower environmental temperatures showing a lower level of hydroxylation (Cohen-Solal et al., 1986).

The other amino acid that has significant function in the collagen molecule is lysine, and again it is subject to post-translational modification. Lysine residues in the Y position can be converted to hydroxylysine by the enzyme lysyl hydroxylase. This conversion is essential for the intracellular stability of collagen (Eyre et al., 1984). Hydroxylysine may also undergo further post-translational modifications including glycosylation and oxidation, but the importance of these modifications for fibrillar structure remains to be elucidated (Prockop et al., 1979).

For the formation of hydroxylysine-linked carbohydrate groups on procollagen chains, hydroxylysyl galactosyltransferase catalyses the addition of galactose to hydroxylysine residues, and galactosylhydroxylysyl glucosyltransferase the subsequent addition of glucose to some galactosylhydroxylysine residues. Carbohydrate is thus present as both the monosaccharide galactose and disaccharide glucosylgalactose. Although the precise function of the hydroxylysine-linked carbohydrates is not clear, they are thought influence the biological properties of the collagen (Kivirikko and Myllyla, 1979).

All of the above modifications occur *in vivo* on nascent chains before the triple helix has formed.

Another important further modification of both lysine and hydroxylysine is the conversion to their aldehyde forms: allysine and hydroxyallysine, respectively, by the enzyme lysyloxidase (Reiser et al., 1992). This enzyme is specific for the

generation of these aldehyde forms found in the N- and C-terminal extensions termed telopeptides. This enzyme works extracellularly and prefers collagen molecules that have assembled into a fibrillar form (Eyre et al., 1984).

Procollagen Assembly and Secretion

Fibril-forming collagens are fundamentally synthesized as soluble procollagens (Trelstad and Hayashi, 1979; Figure 1.3 and 1.4). Procollagen assembly occurs via the folding of individual subunit C-propeptide domains of each pro-alpha chain (Doege and Fessler, 1986; Figure 1.4). Each chain contains approximately 1000 residues. Folding of a trimeric C-propeptide domain is the first step in the folding of procollagen molecules. This folding process resembles a zipper from the C-terminal to the N-terminal of the molecule (Bachinger et al., 1980; Bruckner et al., 1981). It has been shown that procollagen chains containing mutations in the C-propeptide have impaired assembly, similar to the defective self-assembly of the proα-1(I) chain collagen from an osteogenesis imperfecta patient with a frame shift mutation resulting in a truncated C-propeptide (Fitzgerald et al., 1999). The folding of a trimeric C-propeptide is followed by association and alignment of the chains to the triple helix and finally the formation of interchain disulphide links stabilising the final propeptide assembly.

The secretory pathway of triple helical procollagen is otherwise similar to that of other extracellular proteins. The molecules pass thourough the Golgi and are then packed into secretory vacuoles, which are transported to the cell surface and released by exocytosis. A number of studies have shown SLS (segment-long-spacing)-like procollagen aggregates within the secretory vacuoles, suggesting that

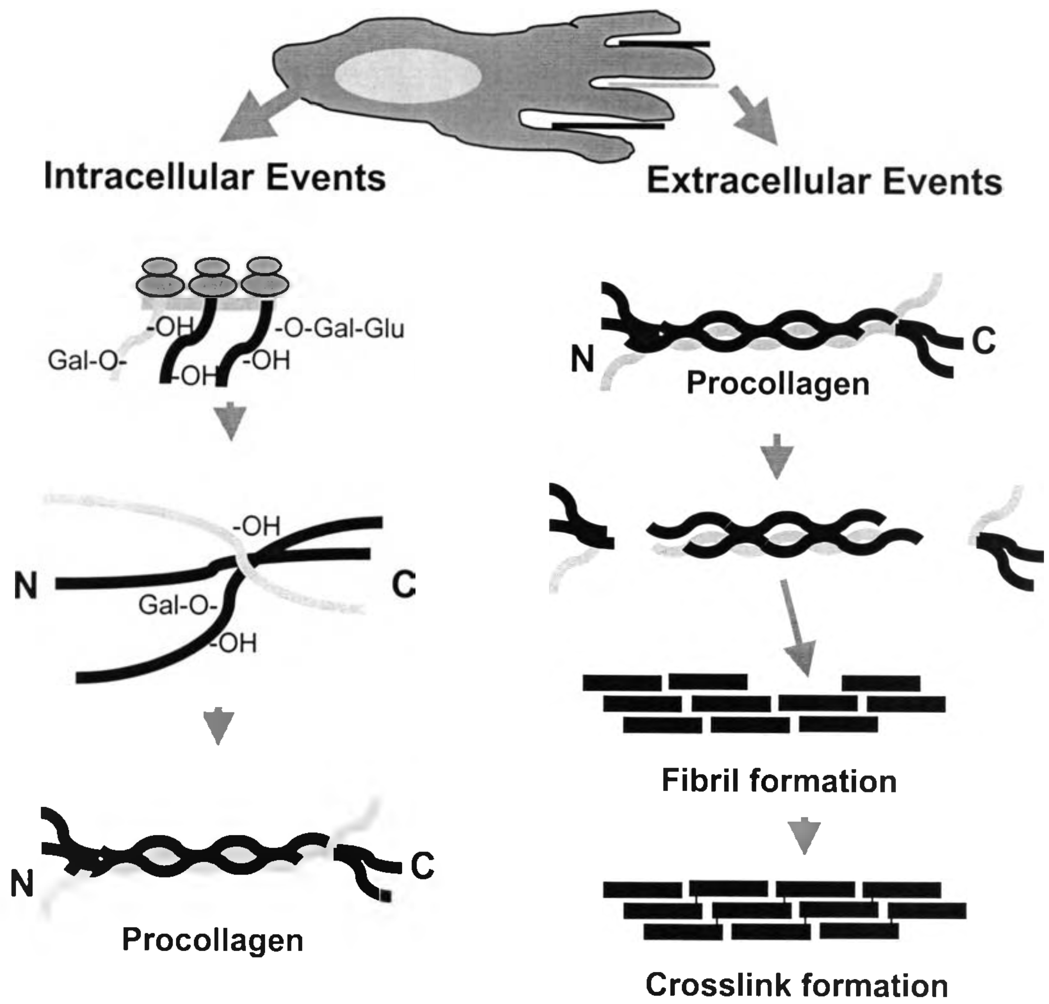

Figure 1.4 : Biosynthesis of Fibrillar Collagen

procollagen is probably secreted in an aggregated form and not as monomeric molecules (Bruns et al., 1979; Trelstad and Hayashi, 1979; Hulmes et al., 1983).

Extracellular Processing

The C- and N-termini of each pro-alpha chain are connected to the central triple helical collagen domain by short sequences that contain cleavage sites for the processing of procollagen into mature collagen molecules (Figure 1.3). This processing occurs in the extracellular matrix and is part of the matrix assembly process (Miyahara et al., 1982; Njieha et al., 1982; Tuderman and Prockop, 1982; Berger et al., 1985; Hojima et al., 1985; Figure 1.4). Removal of the N- and C-propeptides is effected by procollagen N-proteinase and procollagen C-proteinase (BMP-1), which belong to the metalloproteinase family (ADAM-TS) and TGF-β superfamily (Lee et al., 1997), respectively. Although it is clear that these N- and C-proteinases are the specific enzymes responsible for processing procollagen in the tissues, other enzymes in the matrix are capable of producing similar cleavage patterns (Bateman et al., 1987; Lee et al., 1990; Smith et al., 1992). In contrast to fibrillar collagens, studies have shown that type IV collagen is secreted and assembled as a procollagen-like molecule, which is incorporated directly into the basement membrane structure without any extracellular processing (Hudson et al., 1993).

Collagen Fibrillogenesis

Individual processed molecules of collagen types I, II, III and V will spontaneously self assemble into an ordered fibrillar structure *in vitro*. This fibril-forming ability is encoded in the structure of the collagen, implying that precise interactions between collagen domains are involved in directing axial organization of the fibrillar aggregates. Both hydrophobic and electrostatic interactions between adjacent chains have been proposed as the mechanism (Kadler et al., 1987; Kadler et al., 1990; Kadler et al., 1996). The short non-helical telopeptide sequences which remain at the end of the collagen molecule following the proteolytic removal of the C- and N-propeptides account for ~2% of the procollagen molecule, and appear to play a key role in the regulation of collagen I fibrillogenesis *in vitro* (Helseth and Veis, 1981; Prockop and Fertala, 1998).

Type I collagen fibril formation proceeds after the proteolytic removal of the procollagen C- and N-propeptides (Prockop et al., 1979; Kadler et al., 1996; Figure 1.4). Unpublished work using site-directed mutagenesis has shown that C-propeptides play an important role in fibrillogenesis, since the presence of partially processed C-procollagen type I molecules prevent normal fibril growth by steric hindrance in skin fibroblast cultures (Kadler et al., 1987). Also the cleavage of the collagen type I N-propeptide is essential for regular fibril morphology and influences the diameter of the fibrils (Hulmes et al., 1989; Romanic et al., 1992). Other matrix molecules such as decorin and fibromodulin are known to interact with collagen fibrils and may be involved in regulating the formation of collagen fibrils (Scott, 1988; Heinegard and Oldberg, 1989; Fleischmajer et al., 1991; van der Rest et al., 1991).

Cross-Linking

The C- and N-telopeptide lysine residues are important cross-linking sites in collagen types I, II, and III. These collagens spontaneously self-assemble into cross-striated fibrils that occur in the extracellular matrix of the connective tissue (Prockop et al., 1979; Figure 1.4). The fibrils are stabilized by covalent cross-linking, which is initiated by oxidative deamination of specific lysine and hydroxylysine residues within the triple helical region of an adjacent collagen molecule by lysyl oxidase enzyme. This enzyme can be irreversibly blocked by the action of inhibitors called lathyrogens such as beta-aminoproprionitrile (BAPN), and this action results in secreted collagen molecule, which are devoid of cross-linking (Eyre et al., 1984; Reiser et al., 1992).

Assembly of Extracted Type I Collagen into Fibrils

Collagen can be extracted from many kinds of tissue by various extraction solutions such as neutral salt buffer, acid solution, pepsin (Chan et al., 1990), or alkali (sodium hydroxide; Hattori et al., 1999). Acid extracted collagens are mainly in the form of monomers, however variable amounts of cross-linked components (dimers, trimers or some higher components) can be present. The preparation may vary with respect to intactness of the collagen, and the presence of non-helical and telopeptide regions of the molecule.

Collagen type I molecules from extracted tissue can spontaneously assemble into ordered fibrillar structures (*D*-periodic fibrils) *in vitro,* induced by appropriate thermal conditions (20-34°C) or optimal neutral pH condition over the course of

several hours (Kadler et al., 1996). Lower temperatures result in broader diameter of the fibrils. The rate of fibril assembly can be monitored by measuring turbidity which is approximately proportional to the amount of fibril formation (Hattori et al., 1999). The typical curve of the fibril formation is composed of the lag phase, followed by an exponential growth phase and ending with a plateau.

Telopeptides, the C- and N- terminal extra-helical regions of collagen molecules have a major effect on fibril growth *in vitro* (Leibovich and Weiss, 1970; Capaldi and Chapman, 1982; Eyre et al., 1984). Helseth and Veis et al., 1981 showed that proteolytic removal of the short telopeptide domains drastically altered the *in vitro* self-assembly process. Telopeptides provide the initiation for the growing stage of self-assembly of the collagen molecules. Collagens prepared with different procedures, such as pepsin or acid extraction, have different efficiencies of assembly (Kadler et al., 1996). Telopeptides remain on the collagen molecule after acid extraction, but are lost during pepsin extraction (Helseth and Veis, 1981). Even partial loss of the telopeptides has major effects on the fibril growth. These include loss of diameter uniformity, loss of unidirectional packing and changes in the fibril assembly pathway, depending on the extent of removal of each of the N- and C-telopeptides (Helseth and Veis, 1981). Loss of the N-telopeptide causes formation of *D*-periodic symmetrical fibrils with molecules in anti-parallel contact, while partial loss of the C-telopeptides is associated with the formation of *D*-periodic tactoids (Kadler et al., 1996).

Alkali extraction of collagen from the connective tissue can be used to obtain collagen for specific purposes. However, alkali-solubilized collagen has altered chemical and biological properties. The triple helical comformation of alkali-extracted collagen is maintained thouroughout the period of alkali treatment, but it

loses the ability to form fibrils at neutral pH. This is caused by ionic repulsion of the collagen chains due to reduction of the hydrophobicity, which results from deamination of the acid amide group. It is also possible that the telopeptide domain, including the area responsible for cross-linking, might be destroyed by the alkali treatment. The efficiency of extraction of collagen is higher than acid or enzymatic pepsin treatment. The longer the alkali treatment, the lower the thermal stability, and the lower the ability of the collagen molecules to form fibrils at neutral pH. The critical factor that destroys the native structure of these alkaline treated-collagens is temperature (Hattori et al., 1999).

Evidence of Collagen Over-Production in Invasive Tumours

Type I collagen is a potent modulator of cellular function, and *in vitro* can directly influence proliferation, adhesion, morphogenesis, cellular contraction, migration and proteinase production (Thompson et al., 1994). In normal connective tissue, the most abundant ECM component is collagen type I, with type III present to a lesser extent. In some carcinomas, such as breast, pancreatic and gastrointestinal tract carcinomas there is an intense stromal reaction, known as desmoplasia, which although present in some benign pathological disorders, is most prominent in invasive tumours (Martinez-Hernandez et al., 1977; Barsky et al., 1984; Yamamoto et al., 1984; Barsky and Gopalakrishna, 1987; Minamoto et al., 1988; Deak et al., 1991; Ellenrieder et al., 2000). The desmoplastic reaction is typified by excessive collagen deposition, principally collagen type I (Martinez-Hernandez et al., 1977; Barsky et al., 1984). It is associated with proliferation of modified fibroblasts and elastosis. Recent reports have shown that type I collagen was increased preferentially over type

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III in invasive carcinoma, and that type I collagen is distributed thouroughout the matrix of invasive tumours and is prominent within the central sclerotic zone of neoplasms (Deak et al., 1991). In contrast, type III collagen is found in the young edematous mesenchyme of breast tumour. A collagen type I trimer has also been reported in invasive mammary carcinoma (Minafra et al., 1984; Pucci Minafra et al., 1985). In human gastrointestinal carcinoma, the desmoplastic reaction is observed after carcinoma cell invasion (Yamamoto et al., 1984; Minamoto et al., 1998). Activated fibroblasts, and overproduction of type I collagen, have also been reported in tumour-associated stroma of gastric carcinoma (Ohtani et al., 1992). The exact role or significance of the desmoplastic reaction is not clear. While the intense desmoplastic reaction seen with invasive breast carcinoma does not generally appear to impede metastasis, in some tumours it may provide a protective barrier against invasion of the tumour (Barsky and Gopalakrishna, 1987). An *in vitro* study demonstrated that breast tumour cells themselves synthesized small amounts of collagen and elastin compared to fibroblasts (Kao et al., 1986; Ohtani et al., 1992).

Collagen and MMP-2 activation

Collagen has been investigated to determine whether it plays a role in tumour invasion and metastasis since desmoplasia has been repeatedly observed and is poorly understood (Thompson et al., 1994). *In situ* hybridization of breast carcinoma showed that stromal cells, either fibroblasts or myofibroblasts, and not the tumour cells were involved in the biosynthesis of the excessively deposited type I and/or III collagen adjacent to the tumour cells (Clavel et al., 1989; Deak et al., 1991). However, whether this is limiting the invasion process or stimulating the tumour progression so

far has not been elucidated (Barsky and Gopalakrishna, 1987). Meanwhile MMP-2 has also been associated with the degree of stromal proliferation (Ellenrieder et al., 2000), and MT1-MMP has been correlated with collagen synthesis (Gilles et al., 1997). Gilles et al. also showed the levels of expression of collagen α_1(I) and MT1-MMP mRNA in peritumoural fibroblasts was higher in fibroblasts closest to the tumour cells. These expression levels also correlated with aggressiveness of the tumours (Gilles et al., 1997).

In vitro studies of collagen-induced MMP-2 activation were first performed in both normal and tumour derived fibroblasts (Azzam and Thompson, 1992), for better understanding of the natural mode and mechanism of activation. The result demonstrated that collagen induced a similar amount of MMP-2 activation in these cultures, and may play a role in regulating matrix degradation *in vivo*. Early studies employing breast cancer cell lines demonstrated the cellular specificity for type I collagen-induced MMP-2 activation. This was only seen in the highly invasive cell types (Azzam et al., 1993), and these were subsequently shown to express MT-MMPs (Sato et al., 1994; Gilles et al., 1997). The ability to induce MMP-2 activation was highly specific to type I collagen gel, and was not seen with dried, monomeric collagen, or other extracellular matrix proteins (Azzam et al., 1993). Recently, however, HT1080 fibrosarcoma cells have shown MMP-2 activation in response to fibronectin (Stanton et al., 1998) and type IV collagen (Maquoi et al., 2000). In MT1-MMP transfected MCF-7 cell, without any additional stimuli, the activation process was terminated at the intermediate form of MMP-2 requring inducers such as Con A, TPA or collagen for full activation (Gilles et al., 1998). In contrast, over expression of MT1-MMP in COS-1 cells (Sato et al., 1994), or more invasive HT1080 fibrosarcoma cells (Lohi et al., 1996) result in full constitutive activation.

The induction of MMP-2 activation by collagen has been shown to involve both transcriptional (Seltzer et al., 1994; Gilles et al., 1997; Tomasek et al., 1997; Haas et al., 1998) and/or non-transcriptional regulation of MT1-MMP (Gilles et al., 1998), summarized as a model in Figure 1.5.

Since then many *in vitro* studies have been performed to demonstrate an ability of thouree-dimensional type I collagen to induce activation of MMP-2 in various cell types such as ovarian cancer cells (Ellerbroek et al., 1999), endothelial cells (Haas et al., 1998; Nguyen et al., 2000), hepatic myofibroblasts (Preaux et al., 1999). Most of the studies have broadly investigated the role of collagen type I as a major contender for physiological regulation of the MMP-2 activation process, however none of them have examined deeply the specificity of type and structure of collagen which might be required.

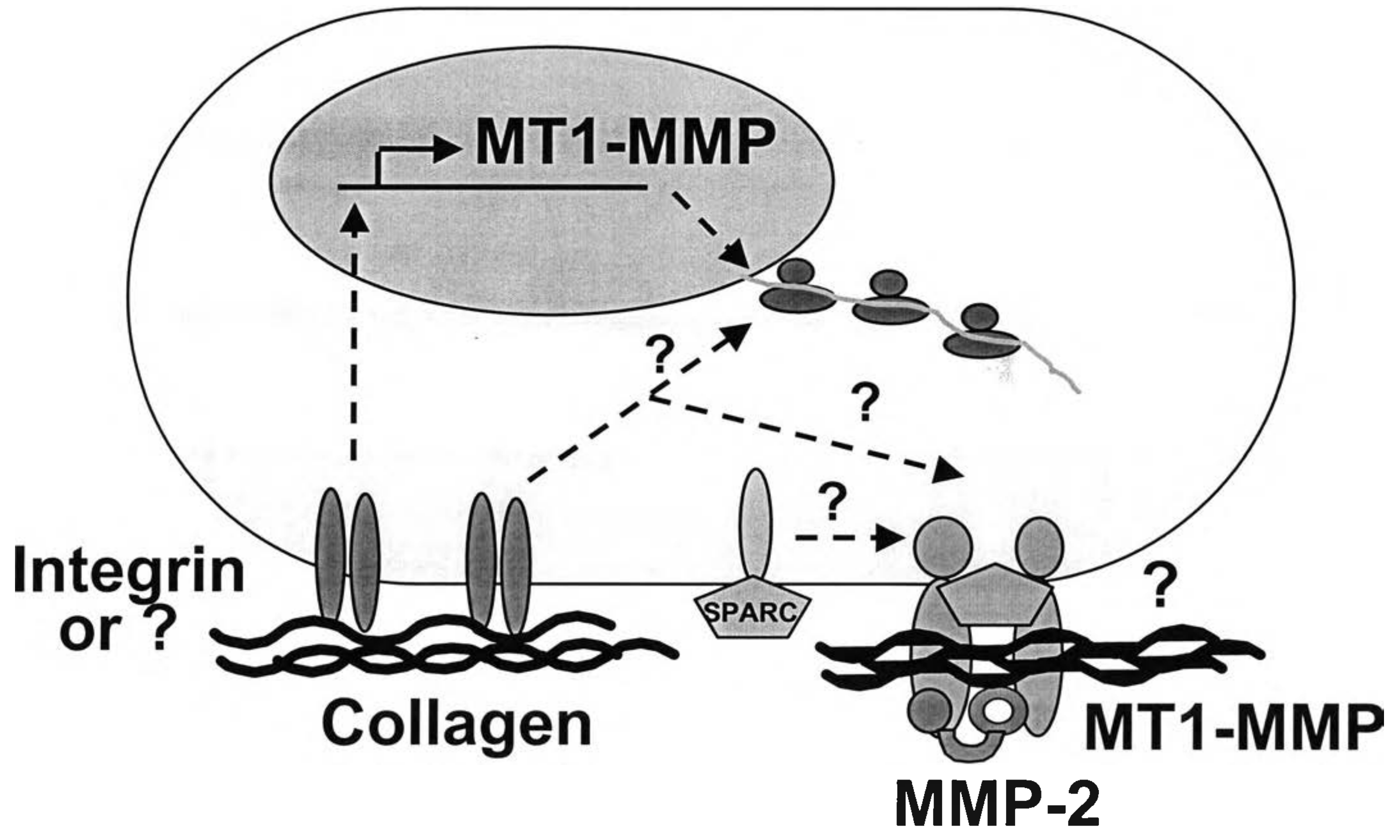

Figure 1.5 : Model of Collagen Regulation of MT1-MMP

Problems and Hypothesis

Thouree-dimensional type I collagen gel has been shown to induce the activation of MMP-2 in a variety of both normal and malignant cell types which have expression of MT1-MMP, and it is the best characterized of only a few physiological substances with this ability. However, very little study of other types of collagen; e.g. collagen types II, III and IV, has been done in this regard. Also in-depth studies to examine the collagen structural requirements for induction of MMP-2 activation have not been performed.

We hypcthesize that fibril formation is a minimum requirement for induction of MMP-2 activation, since thin coatings of monomeric collagen at a low concentration could not initiate MMP-2 activation. This is in contrast to the thouree-dimensional collagen gel. However, whether this lack was due to a lower total amount of collagen, the thouree-dimensional structure, or lack of fibril formation was further investigated in the present study. These aspects are my major focus for looking further into characterizing the role of the collagen in the MMP-2 activation process.

Specific Aims

The specific aims of this study were:

1. To investigate type-specificity of the collagen for induction of MMP-2 activation using all available collagens in comparison to collagen type I, which has been widely studied.

2. To determine the essential requirement of the collagen structure for the activation process of MMP-2.

3. To demonstrate the role of MT1-MMP in collagen inducing MMP-2 activation.

The study was performed with a variety of normal fibroblasts, since stromal cells are known to express MT-MMPs and secrete MMP-2 *in vitro*, as well as in sites of tissue remodeling *in vivo*.

The study was performed in two distinct approaches. First, cells were stimulated with exogenous collagens, including acid/pepsin extractions prepared by myself, commercial collagens, and recombinant collagens provided collaboratively. Direct comparisons were made with different collagen presentation formats for regulation of MT1-MMP and MMP-2 activation *in vitro*. Second, a culture system which allows cells to produce and deposit their endogenous collagen was established to better simulate *in vivo* conditions, and similar endpoints were examined with various manipulations of collagen biosynthesis.