MT1-MMP IN NEUTROPHILS: POTENTIAL MECHANISM FOR COLLAGENASE ACTIVATION

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science (Periodontology) Graduate Department of Dentistry University of Toronto

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M.Sc. 2000

ABSTRACT
Matrix metalloproteinases (MMPs) are important enzymes in the destruction of extracellular matrices in periodontal diseases. One of these enzymes, MMP-8, is derived largely from neutrophils. The active form of MMP-8 is found in the gingival crevicular fluid of progressive periodontitis lesions but the mechanism by which the latent enzyme is converted to active forms in vivo is poorly understood. As activation of MMP-2 can be mediated by a membrane-bound MMP, membrane-type-1 MMP (MT1-MMP), I tested the hypothesis that MT-MMPs are expressed in neutrophils and can activate latent MMP-8. My objectives were: 1) to assess the presence and location of MT-MMP in peripheral blood neutrophils; 2) to determine if MT-MMPs can activate MMP-8. By RT-PCR, I found that human peripheral blood neutrophils expressed MT1-MMP mRNA. The plasma membrane and specific granule fractions of neutrophils were isolated by discontinuous Percoll gradients and from these fractions I found MT1-MMP in the plasma membrane fraction. Soluble biotinylated collagen assays showed some collagenase activity (~28% collagen digestion) in the membrane fraction. The specific granule fraction contained latent MMP-8 that could be activated by APMA. The collagenase activity of a plasma membrane fraction combined with specific granules of neutrophils (54% digestion) was higher than the collagenase activity of the individual fractions (28% for plasma membrane, 1% for specific granules). These results are consistent with the notion that MT1-MMP may contribute to the activation of MMP-8 on the cell surface of peripheral blood neutrophils.
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<table>
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<th>Description</th>
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<tbody>
<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
</tr>
<tr>
<td>MT-MMP</td>
<td>membrane-type matrix metalloproteinase</td>
</tr>
<tr>
<td>TIMP</td>
<td>tissue inhibitor of matrix metalloproteinase</td>
</tr>
<tr>
<td>PMN</td>
<td>polymorphonuclear leukocyte</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrices</td>
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<tr>
<td>SBA</td>
<td>soluble biotinylated collagen assay</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcription-polymerase chain reaction</td>
</tr>
<tr>
<td>PMA</td>
<td>phorbol myristate acetate</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>APMA</td>
<td>P-aminophenylmercuric acetate</td>
</tr>
<tr>
<td>DAPI</td>
<td>4, 6-Diamidino 2-phenylindole</td>
</tr>
<tr>
<td>NHS-LC-Biotin</td>
<td>N-hydroxysulfosuccinimide- long chain- Biotin</td>
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REVIEW OF THE LITERATURE

I. Periodontal Diseases

A. Classification, Natural History and Clinical Course

There are two major categories of inflammatory periodontal diseases, gingivitis and periodontitis, the division of which is based on destruction of alveolar bone. Gingivitis is a reversible inflammatory disease of only the gingiva while periodontitis involves the destruction of the marginal supporting structures of the tooth including the alveolar bone. The hallmark of gingivitis is gingival inflammation which is caused by adherent subgingival bacterial plaques (Loe et al., 1965). Based on a sequence of retrospectively analyzed histopathologic events, inflammatory processes of the periodontium have been separated into three hypothetical stages: the initial, early and established lesions (Page and Schroeder 1976). The initial lesion is an acute inflammatory reaction that occurs 2 to 4 days after the onset of subgingival plaque formation. This lesion is characterized by the loss of perivascular collagen and increased migration of neutrophils through high endothelial venules, across the junctional epithelium and into the gingival sulcus. From 4 to 7 days, the initial lesion progresses to the early lesion in which 60-70% of the collagen in the marginal gingival connective tissue may be destroyed and the inflammatory cell infiltrate is dominated by lymphocytes and macrophages. After 2 to 3 weeks, the established lesion is formed which is synonymous with chronic adult gingivitis. The key histological feature of the established lesion is the presence of plasma cells in the inflammatory infiltrate. This lesion can persist for months or years without progression to periodontitis.
In a small proportion of individuals with gingivitis, some of the affected sites progress to periodontitis (Loe et al. 1986). Several classifications of periodontitis have been suggested but a recent review by Armitage (1999) suggests that there are four major types of periodontitis: i) chronic periodontitis; ii) aggressive periodontitis; iii) periodontitis as a manifestation of systemic diseases; and iv) necrotizing ulcerative periodontitis. It has been suggested that each disease type has a different etiology, a different rate of disease progression and response to treatment, but a common pathway of extracellular matrix (ECM) destruction. Indeed the degradation of ECM macromolecules including collagen fibers, elastic fibers, proteoglycans and glycoproteins is the structural basis for the destruction of the periodontal ligament, the disruption of its attachment to cementum, and ultimately the resorption of alveolar bone (Page and Schroeder 1976, Page et al. 1997).

B. Inflammation and Destruction of ECM

In periodontitis, there are several different mechanisms that may lead to degradation of ECM macromolecules (Birkedal-Hansen 1993). One proposed mechanism is that proteolytic enzymes from putative pathogenic bacteria can directly degrade the ECM. For example, Porphyromonas gingivalis and Actinobacillus actinomycetemcomitans produce hydrolytic enzymes that cleave native type I and III collagen fibrils at physiological pH and temperature (Birkedal-Hansen et al. 1988; Robertson et al. 1984). However, the relative abundance of these bacterial enzymes in the lamina propria of the gingiva and the periodontal ligament may not be sufficient to account for the amount of collagen degradation seen in periodontitis.
A second complementary mechanism is the release of bacterial virulence factors that may act directly on host cells to perturb homeostasis. For example, in response to the virulence factor lipopolysaccharide (LPS) that is present in the envelope of several gram negative anaerobic pathogens, macrophages undergo major shifts in their expressed gene repertoire which can result in the induction, for example, of matrix metalloproteinases (Wahl et al. 1974; Welgus et al. 1990). Other virulence factors such as proteinases from the pathogen P. gingivalis have been demonstrated to induce the expression and mediate the activation of MMPs from mucosal keratinocytes and fibroblasts (Birkedal-Hansen et al. 1984).

The third and most studied mechanism is a cytokine-dependent, host immune, inflammatory response to the antigenic challenge of subgingival pathogens such as Porphyromonas gingivalis, Bacteroides forsythus, and Actinobacillus actinomycetemcomitans. These pathogens colonize teeth and grow to form a biofilm (Darveau et al. 1997) which eventually extends subgingivally if left undisturbed (Haffajee and Socransky 1994). The biofilm and its metabolites disrupt the attachment of the junctional epithelium to the tooth. Subsequently, in a susceptible host, the presence of virulence factors from periodontal pathogens can trigger the synthesis and release of proinflammatory cytokines such as IL-1β, TNF-α and IFN-γ. These cytokines, in turn, induce and enhance host cell pathways for the degradation of the ECM (Page 1998).
C. Role of Neutrophils in Inflammation and Connective Tissue Destruction

One of the key inflammatory cells involved in the degradation of the ECM is the neutrophil. Neutrophils comprise 40-60% of the circulating leukocytes in the peripheral blood and are a first line of defense against microbial attack. Neutrophils are produced in the bone marrow and after maturation, are released into the bloodstream with a circulatory half life of 6 to 7 hours (Gordon 1994).

In the blood flow through capillaries, there are continuous reversible rolling contacts between neutrophils and uninflamed endothelium. These transient, adhesive rolling contacts are mediated by L-selectin, a cell surface protein that is constitutively expressed on the surface of the neutrophil (Smith et al. 1991). However, in the presence of proinflammatory cytokines such as IL-1β and TNF-α, endothelial cells rapidly translocate P-selectins from Weibel-Palade bodies (McEver et al. 1989) to the cell surface and induce biosynthesis of E-selectins (Bevilacqua et al. 1987). The carbohydrate-bearing moieties on the neutrophil surface, gp150-Lewis x and sialyl Lewis-X, will respectively bind to P- and E-selectins (Fukuda et al. 1984; Symington et al. 1985). This consequently strengthens the adhesive interactions between the neutrophils and endothelial cells and increases the number of neutrophils rolling and attaching to inflamed postcapillary high endothelial venules. Subsequently, interleukin-8 (a product of endothelial cells) induces neutrophils to shed L-selectin and express β2-integrins (LFA-1, Mac-1, CR4) that are stored in the specific granules (Miyasaki 1996). LFA-1 and Mac-1 bind tightly to the endothelial adhesion molecules (ICAM-1 and -2) and this binding
initiates the transendothelial migration of the neutrophil through the endothelial cell junctions into the extravascular compartment, a process known as diapedesis.

In periodontal tissues, neutrophils infiltrate the perivascular connective tissue, migrate along a gradient of chemoattractants generated by subgingival bacteria and pass through the junctional epithelium to form a defensive barrier between the subgingival plaque and the gingival tissues (Theilade et al. 1985). Upon encountering bacteria the neutrophil utilizes various bacterial killing mechanisms which can be broadly classified into oxidative and nonoxidative system. Oxidative killing involves the production of toxic superoxide anions ($O_2^-$), hydrogen peroxide ($H_2O_2$), and hydroxyl radicals (OH). However, no studies have conclusively shown that these metabolites alone are capable of producing toxic effects under pathophysiological conditions. Nonoxidative mechanisms involve secretion of antimicrobial components stored in granules. For example, the primary or azurophilic granules contain myeloperoxidase, defensins, and lysozyme while secondary or specific granules contain lactoferrin and lysozyme. (Van Dyke 1994).

In the presence of bacterial factors or complement factors (e.g. C5a), the granule contents are released extracellularly either by secretion or when the neutrophil undergoes apoptosis or cytolysis (Miyasaki 1991). Although the primary function of the neutrophil is host protection, the extracellular release of the granule contents may lead to excessive tissue breakdown because proteolytic enzymes such as elastase, collagenase and gelatinase are also found within these granules and are able to attack key components of the extracellular matrix (Weiss 1989). Notably, the neutrophil collagenase (MMP-8)
found in the specific granules appears to have a direct role in the tissue destruction of periodontitis. In periodontitis, much of the collagenase activity is derived from neutrophils and not from bacteria or other host cells, as based on the pattern of collagen substrate degradation (Gangbar et al. 1990, Lee et al. 1991 & 1995, Overall et al. 1991). A longitudinal cohort study that examined gingival crevicular fluid in patients demonstrated that active collagenase activity was 5 – 6 fold higher in groups with active periodontitis than groups with gingivitis (Lee et al. 1995). Large increases of active collagenase were detected concurrent with connective tissue attachment loss. In contrast, latent collagenase activity was 2-fold higher in patients with inflammation but no destruction.

II. Destruction of Extracellular Matrices

Birkedal-Hansen (1993) identified five distinct pathways that lead to degradation of the extracellular matrices of the periodontium. The first pathway involves the conversion of plasminogen into plasmin, a serine protease that cleaves fibrin and fibronectin (Dano et al. 1985). The second pathway is the neutrophil-serine proteinase pathway. When released, the neutrophil serine proteinases, elastase and cathepsin G, cleave a variety of ECM molecules such as type IV collagen, laminin, fibronectin and proteoglycans (Weiss 1989). The third pathway involves intracellular degradation by fibroblasts and macrophages that phagocytize collagen fibrils and degrade them within phagolysosomes. This intracellular pathway is particularly important in sites of rapid collagen turnover such as the healthy gingiva or periodontal ligament (Melcher and Chan 1981). The
fourth, osteoclastic bone resorption pathway is unique in that removal of mineral (i.e. hydroxyapatite) is required before degradation of matrix proteins can be initiated (Vaes 1988). The fifth pathway is the matrix metalloproteinase pathway. This pathway will be discussed in detail in the following section as it is central to the objectives of this thesis.

A. General Overview of the MMP family

The MMP family is a group of metal-dependent endopeptidases which are capable of degrading most extracellular matrix macromolecules. MMPs are involved in connective tissue remodeling and degradation, embryonic growth and development, and diseases such as rheumatoid arthritis, periodontitis, tumour growth and metastasis (Birkedal-Hansen et al. 1993). The MMPs share sequence homology but differ in terms of substrate specificity and transcriptional regulation.

Based on substrate specificity, the MMPs are classified into four broad categories: i) collagenases ii) gelatinases iii) stromelysins including matrilysin and metalloelastase and iv) membrane-type MMPs (Polette et al. 1998). The collagenases (MMP-1, MMP-8, MMP-13) cleave type I, II, III, VII and X collagen. Neutrophil collagenase (MMP-8) is produced largely by neutrophils whereas interstitial collagenase (MMP-1) is produced by many cell types such as fibroblasts, keratinocytes, endothelial cells, macrophages, chondrocytes and osteoblasts (Birkedal-Hansen et al. 1993). A recent paper (Hanemaaijer et al. 1997) has shown that other cells can produce MMP-8 but the pathophysiological significance of this is unclear. MMP-13 is produced by both epithelial and mesenchymal cells in inflamed and remodelling connecting tissues. Its ability to cleave type II collagen,
type X collagen and cartilage aggrecan suggests that MMP-13 plays a significant role in cartilage collagen degradation (Freije et al. 1994, Mitchell et al. 1996, Knauper et al. 1976). MMP-13 has also been identified in gingival crevicular fluid (Mancini et al. 1999) but the degradative impact of MMP-13 on periodontal connective tissues has not been assessed. Gelatinases (MMP-2, MMP-9) are also synthesized by a wide variety of cells. In addition to gelatin, gelatinases also cleave type IV collagen found in basement membranes. The expression of gelatinases is often associated with invasive and metastatic tumours (Liotta 1980). Stromelysins degrade a wide variety of collagenous and noncollagenous ECM substrates which include collagen, gelatin, laminin and proteoglycans. The final group, membrane-type MMPs, often contain a transmembrane domain. This hydrophobic stretch of amino acids anchors the protein to the plasma membrane and leaves the catalytic domain exposed extracellularly. In contrast, the other MMPs are released extracellularly as soluble proteinases.

B. Domain Structure and Function of Collagenase

Collagenases have a five-domain modular structure that is shared by the other MMPs (Fig. A) (Birkedal-Hansen et al. 1993). The hydrophobic signal sequence of about 17-29 residues is followed by a 77-87 residue propeptide domain that constitutes, for example, the NH₂-terminal domain of the secreted MMP-8 precursor. This propeptide contains a highly conserved sequence (PRCGVPD) which maintains the latency of the enzyme extracellularly until it is removed in the process of enzyme activation (Springman et al. 1990). The catalytic domain of about 160 residues contains a highly conserved Zn²⁺ binding active site (Lovejoy et al. 1994). A 5-50 residue proline-rich hinge region
connects the catalytic domain with a 200 residue pexin-like COOH domain which plays a role in substrate specificity.

![Diagram of Collagenase domains](image)

**Figure A:** Domain structure of Collagenase a) hydrophobic signal sequence; b) NH₂-terminal propeptide; c) catalytic domain with zinc molecule; d) proline-rich hinge region; e) hemopexin-like COOH terminal domain

While the MMPs share certain structural motifs, there are some notable differences between closely related enzymes such as the neutrophil collagenase (MMP-8) and the fibroblast collagenase (MMP-1). MMP-8 has a higher molecular mass than MMP-1 (80kDa/75kDa vs. 57 kDa/52kDa) because of the greater glycosylation of MMP-8. It is speculated that the carbohydrate moieties of MMP-8 encode targeting signals that direct the enzyme to specific granule storage sites (Birkedal-Hansen et al. 1993). Another significant difference between the two enzymes is the mode of transcriptional regulation. MMP-8 is synthesized during neutrophil maturation and is rapidly released from the specific granules after neutrophil activation whereas MMP-1 is not stored in granules but instead is synthesized in response to a wide variety of stimuli including IL-1 and TNF-α.

**C. Substrate Specificity of Collagenase**

Collagenases can cleave the native triple helix of type I, II, and III collagens at a single site in each polypeptide chain at physiological temperature and pH. In type I collagen, the cleavage occurs at the glycine⁷⁷⁵-isoleucine⁷⁷⁶ bond in the alpha 1 chain and at the
glycine\textsuperscript{775}-leucine bond\textsuperscript{776} in the alpha 2 chain. This single cut produces a N-terminal $\frac{1}{4}$ and a C-terminal $\frac{1}{4}$ collagen fragment. Subsequent degradation of the collagen may be mediated by the gelatinases. Wu et al. (1990) demonstrated by site-directed mutagenesis that the primary structure of the collagenase-sensitive site in collagen is an important factor in determining the susceptibility to collagenase. However, the susceptibility of this site cannot be accounted entirely by the amino acid sequence of collagen alone. The collagenase-sensitive region may also be a locus that at 37°C more readily unfolds and relaxes its triple helical structure than other regions (Birkedal-Hansen 1987). In comparison to the fibroblast collagenase, the neutrophil collagenase exhibits 10 to 30 fold higher catalytic efficiency on all substrates except for type III collagen (Netzell-Arnett et al. 1991).

D. Activation Mechanisms of Collagenase
The activation of the latent collagenase is a critical regulatory step especially with MMP-8. As mentioned above, latent forms of MMP-8 are stored in specific granules of neutrophils and upon neutrophil activation, the granule contents are released extracellularly (Weiss et al. 1985, Desrochers et al. 1992). However, collagen degradation cannot occur until MMP-8 is activated. The latency of the collagenase is maintained by the formation of a cysteine-Zn\textsuperscript{2+} bond that links the unpaired propeptide cysteine residue to the active site Zn\textsuperscript{2+} (Van Wart et al. 1990). In enzyme activation, dissociation of the bond between the cysteine thiolate moiety and zinc atom is assumed to be a crucial step. This process is described by Van Wart (1990) as the cysteine switch
activation mechanism. *In vitro* studies have demonstrated that there are different ways in how this activation can be achieved.

Organmercurials, metal ions, thiol reagents and oxidants can directly interact with the cysteine residue to disrupt the cysteine-Zn$^{2+}$ bond *in vitro*. This interaction results in a confirmation shift that triggers a series of autolytic cleavages to occur. The first cleavage leads to a reduction in molecular mass but not activation. The second cleavage, which occurs at Asp$^{64}$-Met$^{65}$, results in 40% increase of the maximum enzymatic activity. The final cleavage occurs at Phe$^{79}$-Met$^{80}$ or Met$^{80}$-Leu$^{81}$ after prolonged incubation with the activator (Knauper *et al.* 1990, Blaser *et al.* 1991).

Another activation system is based on *in vitro* studies using chaotropic agents (KI, NaSCN) or detergents (SDS) to disrupt the cysteine-Zn$^{2+}$ bond, a procedure which induced conformational changes in the polypeptide backbone. This conformational shift also leads to several autolytic cleavages in which a fully processed active form of the enzyme is generated (Nagase 1997).

A third activation process involves proteolytic enzymes (trypsin, plasmin, chymotrypsin, neutrophil elastase, cathepsin B, plasma kallikrein). These enzymes excise a portion of the propeptide which causes the cysteine switch to open. Activation requires the cleavage of peptide bonds probably between residues 70 and 82. specifically, Phe$^{79}$, Met$^{80}$ and Leu$^{81}$ (Nagase 1990).
However, despite these advances based on MMP-8 activation systems studied in vitro, the biological mechanism by which MMP-8 activation actually occurs in vivo is poorly understood. The pathway involving proteolytic enzymes may be the closest surrogate to the in vivo situation. Activation in vivo is frequently associated with a decrease in molecular mass due to the removal of the propeptide. For example, latent MMP-8 in gingival crevicular fluid is 78 kDa while the active form is 60 kDa (Romanelli et al. 1999). Since activation of collagenase is a critical regulatory step, there have been continuing searches for an activation mechanism that truly reflects the in vivo situation. The recently discovered membrane-type MMP was identified as the first physiological activator of gelatinase A (Sato 1994) but little is known about the role of membrane-type MMPs in the activation of MMP-8.

III. Membrane-type Matrix Metalloproteinase

A. Domain structure and Function of MT-MMP

The majority of the MMPs are in a soluble form. However, by the use of RT-PCR and screening a human placenta cDNA library, Sato (1994) discovered a protein of 582 amino acids that had the common MMP five domain structure with three unique insertions. First, there is an insertion of 11 amino acids between the propeptide and the catalytic domain. This insertion contains a stromelysin-3-like RXKR furin cleavage motif. The second insertion is an 8 amino acid residue within the catalytic domain whose function remains undefined while the third insertion of 24 hydrophobic amino acids in the C-terminus represents the transmembrane domain. The transmembrane domain allows the
protein to be anchored to the plasma membrane with its catalytic domain exposed to the extracellular space. Hence, this protein is called membrane-type MMP. Currently, the family of MT-MMP has been expanded to include MT1-, MT2-, MT3-, MT4-, MT5-, MT6-MMPs (Sato 1994, Will 1995, Takino 1995, Puente 1996, Pei 1999 & 1999a).

Many tissues and cells express MT1-MMP. For example, during mouse embryogenesis, MT1-MMP is expressed at high levels in developing blood vessels, kidney, osteocartilaginous and musculotendinous tissues (Kinoh et al. 1996, Apte et al. 1997, Sato & Seiki 1996). MT1-MMP is also found in lung tissue, kidneys, microglia in the brain, ocular tissues, enamel and the pulp organ of teeth (Takino et al. 1995a & b, Sato et al. 1994, Caron et al. 1998). At sites of vascular injury during wound healing, dermal fibroblasts express MT1-MMP. Other cells that express MT1-MMP include smooth muscle cells, endothelial cells, ameloblasts, odontoblasts, chondrocytes, and osteoclasts (Imai et al. 1997, Sato & Seiki 1996, Sato et al. 1997).

In various tumours of lung, stomach, colon, breast, ovary, cervix, urethra, bladder and pancreas, stromal and cancer cells have elevated MT1-MMP expression (Sato & Seiki 1996). Notably, MT1-MMP expression levels correlate with gelatinase A activation and with the malignant potential and invasiveness of the tumour. In an experimental metastasis assay, MT1-MMP expression has been shown to enhance metastatic activity (Tsunezuka et al. 1996). In the context of cancer biology, gelatinase A plays a critical role in the invasion of tumour cells through the basement membrane (Stetler-Stevenson et al. 1993). Because progelatinase A is produced constitutively in high concentration by
many cell types, its activity is regulated mainly at the level of proenzyme activation whereas the expression of other MMPs such as fibroblast collagenase, stromelysin and gelatinase B is enhanced by various growth factors (Birkedal-Hansen et al. 1993). By gelatin zymography, MT1-MMP has been demonstrated to induce progelatinase A activation (Sato et al. 1994). This mechanism will be discussed below.

There is growing evidence that MT-MMP may be a key regulator of ECM turnover. MT1-MMP deficient mice exhibit inadequate collagen turnover which leads to dwarfism, osteopenia, arthritis, and connective tissue disease (Holmbeck et al. 1999). MT1-MMP deficiency greatly affects the skeleton, seen in reduced longitudinal growth, cranial dysmorphism, osteoclasia and osteopenia. MT1-MMP is also essential for growth plate function and secondary ossification. As bone formation requires remodeling of unmineralized connective tissue, when MT1-MMP is deficient, the remodeling of the periskeletal soft tissues is impaired. The block of remodelling leads to increased bone resorption and osteoclastic activity. Collectively, these finding demonstrate the importance of MT1-MMP since this enzyme cannot be adequately compensated by other MMPs or by other collagen degrading mechanisms (Holmbeck et al. 1999). In corroboration of these findings, Zhou et al. (2000) have also demonstrated that MT1-MMP deficient mice have impaired endochondral ossification in skeletal development and impaired angiogenesis. However, neither of these studies examined perturbations of inflammation, so the relative importance of MT1-MMP in the inflammatory response is unknown.
B. Regulation & Activation of MT-MMP

The processes that regulate the synthesis and activation of MT1-MMP are not clearly understood. In some papers (Lehti et al. 1998, Stanton et al. 1988, Hernandez-Barrantes et al. 2000), the latent form of MT1-MMP is reported to be 60 kDa and the active form is 57 kDa. However, the active MT1-MMP can be further processed to a functionally inactive form (44 kDa) that lacks the entire catalytic domain but maintains the hemopexin-like domain and hinge region (Hernandez-Barrantes et al. 2000). MT1-MMP expression is regulated in part by cytoskeleton-ECM interactions. It is induced in fibroblasts by culturing in three dimensional collagen matrices, by mechanical stretching and by treatment of cells with the cytoskeleton disrupting agent cytochalasin D (Gilles et al. 1997, Tyagi et al., 1998, Ailenberge & Silverman 1996).

MT1-MMP is unlike most other MMP genes. The majority of MMP genes are either not expressed in unstimulated cells or are expressed at low levels; their gene expression is increased in the presence of proinflammatory cytokines. On the other hand, in vitro, MT1-MMP is constitutively expressed by different cell types including fibroblasts, endothelial cells and smooth muscle cells. Its expression is not affected by TGF-β but is decreased by dexamethasone and only modestly enhanced by PMA, TNF-α, and the lectin concanavalin A (Lohi et al. 1996). Thus, other aspects of MT-MMP regulation need to be considered such as its activation mechanism.

Like the other MMPs, MT1-MMP contains a propeptide domain that maintains latency. As mentioned before, an 11 amino acid sequence that contains a stromelysin-3-like
RXKR furin cleavage motif precedes the catalytic domain. This motif has been shown to facilitate constitutive intracellular processing and activation of stomelysin-3 (Pei and Weiss 1995) and MT1-MMP itself. The sequence is recognized by furin, a proprotein convertase present in the Golgi apparatus that is able to activate recombinant MT1-MMP (Sato et al. 1996). When furin specifically cleaves MT1-MMP in vitro between Arg$^{111}$-Tyr$^{112}$, MT1-MMP is activated, which in turn enables MT1-MMP to activate progelatinase A. This contention is supported by Pei and Weiss (1996) who reported activation of MT1-MMP using a mutant protein lacking the transmembrane domain purified from MDCK cells. However, Cao et al. (1996) demonstrated that furin-induced activation of MT1-MMP is not a prerequisite for progelatinase activation. Activation of gelatinase continued despite the presence of $\alpha_1$PI$_{PTT}$, a furin inhibitor, and within COS-1 cells cotransfected with furin and mutant forms of MT1-MMP in the RRKR$^{111}$ site. The discrepancy between these studies is explained by Yana & Weiss (2000). First, they reported that $\alpha_1$PI$_{PTT}$ furin inhibitor is unable to efficiently inhibit the proprotein convertase-dependent pathways. Second, it was discovered that the MT1-MMP contains a secondary furin cleavage site of KXXR$^{89}$, which was only used when RRKR$^{111}$ was mutated. Thus in Cao’s study, furin converted mutant MT1-MMP to an active form through cleavage at the secondary site. The activated MT1-MMP was then able to active progelatinase A.

Another possible activation mechanism suggests that plasmin can activate the pro-MT1-MMP by cleavage of Arg$^{108}$ and Arg$^{111}$ (Okumura 1997). These authors suggested that the pro-MT1-MMP is activated by plasmin extracellularly.
C. Substrate Specificity of MT-MMP

MT1-MMP's substrates include a variety of ECM proteins such as type I, II and III collagens, gelatin, fibronectin, vitronectin, tenascin, entactin and laminin-1 (Ochuchi et al. 1997, d'Ortho et al. 1997). In comparison to MMP-1, MT1-MMP is 5-7.1 fold less efficient at cleaving type I collagen while its gelatinolytic activity is 8-fold higher. MT1-MMP is also an efficient fibrinolytic proteinase: overexpression of MT1-MMP enables non-fibrinolytic cells to invade fibrin gels (Hiraoka et al. 1998). However, more significantly, other MMPs are substrates for MT-MMPs. MT1-MMP has been implicated in the activation of pro-MMP2 and pro MMP13 (Sato et al. 1994, Murphy et al. 1999, Knauper et al. 1996a).

The first MMP substrate discovered for a MT-MMP was progelatinase A. Sato et al. (1994) transfected MT1-MMP plasmid into human fibrosarcoma HT1080 cells which constitutively secrete pro-gelatinase A into the culture medium. MT1-MMP generated two new gelatinolytic bands which represented the intermediate and active forms of gelatinase A. MT1-MMP recognizes the Asn^{66}-Leu bond (Kinoshita et al. 1996). After cleavage, the progelatinase A is subsequently processed to an intermediate form. Then, an autoproteolytic reaction occurs to produce the fully active form which is dependent on the presence of gelatinase A at the cell surface (Sato et al. 1996a). This enzymatic activity is inhibited by TIMP-2 and TIMP3, but not by TIMP1 (Will et al. 1996). TIMP-2 has a complex and critical regulatory role in the activity of MT1-MMP. In vitro experiments revealed that both MT1-MMP and TIMP-2 are required for the binding of gelatinase A to
the MT1-MMP at the cell surface and for subsequent membrane activation of gelatinase A (Strongin et al. 1995, Atkinson et al. 1995).

D. Relationship to TIMP-2

TIMP-2, a 21 kDa non-glycosylated protein, belongs to a family of endogenous inhibitors, the tissue inhibitors of metalloproteinases. These proteins form non-covalent stoichiometric complexes with both latent and active MMPs. TIMPs regulate matrix degradation mainly by blockage of autolytic MMP activation (DeClerck et al. 1991).

Previous studies have shown that at low concentrations, TIMP-2 stimulates progelatinase A activation whereas at high concentrations, it inhibits activation (Strongin et al. 1995, Kinoshita et al. 1998). Strongin (1995) also demonstrated in cross-linking experiments that TIMP-2 binds to MT1-MMP and to the hemopexin-like domain of progelatinase A. Based on these observations, a model for activation of progelatinase A was proposed in which the catalytic domain of MT1-MMP binds to the N-terminus of TIMP-2 while the C-terminus of TIMP-2 binds the C-terminus of progelatinase A (Strongin et al. 1995, Butler et al. 1998). This complex enables progelatinase A to cluster at the cell surface near a second active MT1-MMP molecule (Fig. B). If not bound by TIMP-2, this second MT1-MMP will then cleave the propeptide of progelatinase A to initiate activation of gelatinase A.
Because TIMP-2 is a potent and specific inhibitor of MT1-MMP and gelatinase A, the optimal concentration of TIMP-2 to enable gelatinase A activation is quite low and is within a narrow range. This mechanism permits active MT1-MMP to activate the progelatinase A bound onto the complex. An excess of TIMP-2 will lead to the inhibition of the activation reaction (Fig. C).

In addition to regulation of progelatinase A activation, TIMP-2 has an unique interaction with MT1-MMP in terms of the activity of the enzyme on the cell surface. With recombinant vaccinia viruses encoding full length MT1-MMP or TIMP-2, Hernandez-Barrantes (2000) was able to control various expression levels of TIMP-2 in mammalian cells. The results demonstrated that TIMP-2 directly and positively regulated the concentration of active MT1-MMP. In the absence of TIMP-2, there is a significant decrease in the amount of active MT1-MMP on the cell surface due to the generation of
membrane-bound inactive 44 kDa species. However, in cells that co-express MT1-MMP and TIMP-2, cleavage at the Gly-Gly\textsuperscript{285} and the subsequent generation of the inactive form is significantly inhibited by TIMP-2. The authors of this study concluded that because TIMP-2 and not TIMP-1 inhibits the generation of 44 kDa species, the MT1-MMP processing is an autocatalytic event. Thus, by binding and inhibiting a fraction of active MT1-MMP, TIMP-2 reduces the extent of autocatalytic processing of free active 57 kDa species and therefore promotes its accumulation on the cell surface. This in turn enhances progelatinase A activation.

E. Membrane Fixation

The complex of the MT1-MMP•TIMP-2 with progelatinase A illustrates the concept of pericellular proteolysis (Werb 1997). As mentioned before, MMPs are the major enzymes that degrade the ECM. The majority of these enzymes are soluble and are released extracellularly. However, previous studies have shown that ECM degradation in vivo is confined to the immediate pericellular environment of the cell (Andreasen \textit{et al.} 1997, Nakahara \textit{et al.} 1997). This leads to the question of how the proteinases that degrade the ECM can operate in a spatially confined manner. One possible mechanism is the involvement of membrane-bound enzymes such as MT-MMP. Not only is MT1-MMP capable of degrading ECM molecules, it is also capable of localizing and activating other MMPs at the cell surface such as gelatinase A. This activation and localization mechanism may be applied to other MMPs and other disease processes but this has not yet been investigated, most notably for MMP-8.
IV. Introduction and Statement of the problem

Periodontal diseases are a group of chronic inflammatory disorders which involve the destruction of extracellular matrices (ECM). One of the destructive enzymes that degrades collagen in the ECM is the neutrophil collagenase or matrix metalloproteinase-8 (MMP-8). MMP-8 is released as an inactive zymogen which is subsequently activated extracellularly by cleavage of the propeptide. Activation of MMP-2 (gelatinase) by a membrane bound MMP, membrane-type-1 MMP (MT1-MMP) has been demonstrated in fibroblasts, chondrocytes, and various tumour tissue cell lines (Sato et al. 1994). There is growing evidence that MT-MMPs may be key regulators of ECM turnover. For example, MT1-MMP deficient mice exhibits inadequate collagen turnover which leads to dwarfism, osteopenia, arthritis, and connective tissue disease (Holmbeck et al. 1999). However, there is limited information on activation of MMP-8 by MT-MMPs in neutrophils or indeed whether MT-MMPs are expressed on the neutrophil plasma membrane at all. My hypothesis is that MT-MMPs are expressed in neutrophils and can activate latent MMP-8. To address this hypothesis, my objectives were to:

1) Determine if MT1-MMP mRNA is expressed in peripheral blood neutrophils by RT-PCR
2) Determine if MT1-MMP protein is found in the plasma membrane or in granules of peripheral blood neutrophils
3) Compare by SBA the amount of MMP-8 activation by neutrophil membrane fractions that may contain MT-MMPs.
MATERIALS AND METHODS

A. Reagents

ImmunoPure® NHS-LC-Biotin for collagen labeling was purchased from Pierce (Rockford, Illinois). Anti-MT1-MMP rabbit polyclonal antibody used for immunofluorescence and Western blots was from Sigma (St. Louis, Missouri). Anti-nebulin monoclonal antibody used for immunofluorescence was from Sigma. Rhodamine (TRITC)-conjugated affinipure F(ab’)_2 fragment donkey anti-rabbit IgG (H+L) used for immunofluorescence was from Jackson Immunoresearch Lab, Inc. (West Grove, Pennsylvania). Anti-MMP-8 mouse monoclonal antibody (IgG) used for Western blots was from Calbiochem (San Diego, California). ECL reagents were from Amersham International (Buckinghamshire, UK). Anti-mouse IgG1 HRP conjugates (Caltag Laboratories, Burlingame, CA) and anti-rabbit IgG HRP conjugates used for Western blots were from Amersham Life Science (Buckinghamshire, UK.) Purified MMP-8 was from Calbiochem (San Diego, California). P-aminophenylnmercuric acetate (APMA) was from Sigma. Pefabloc was from Boehringer Mannheim (Laval, Quebec, Canada). MT1-MMP PCR primers were kindly donated by Dr. C. Overall (UBC, Vancouver, BC).

B. Isolation of Polymorphonuclear Neutrophils by Human Plasma/Percoll Blood Cell Separation

Blood (80 ml) was drawn from male donors with a 19 gauge butterfly needle and collected into 3.8% sodium citrate. The blood was centrifuged at room temperature for 20 minutes at 175 x g (1080 rpm, Beckman GPR centrifuge) to separate the platelet rich
plasma (PRP) layer from red cell fraction. The PRP layer was centrifuged at 1000 x g (3400 rpm) at room temperature for 15 minutes to obtain the platelet poor plasma (PPP) layer. Dextran sedimentation was performed on the red cell fraction for 30 minutes to facilitate sedimentation of erythrocytes and the leukocyte-rich supernatant was aspirated. Percoll in PPP (2 ml of 42%; v/v) was layered under the leukocyte-rich supernatant with a baked pasteur pipette and then, 2 ml of 51% (v/v) Percoll in PPP was layered under the 42% layer to form a plasma/Percoll gradient. Centrifugation was carried out at 180 x g (1180rpm) at room temperature for 10 minutes to separate the mononuclear, neutrophil and erythrocyte layers. The neutrophil layer was collected and resuspended in Kreb’s Ringer Phosphate with Dextrose (KRPD) buffer at a concentration of 8x10⁶ cells/ml. Under light microscopy, the number and purity of neutrophils were determined. The yield was approximately 1.5x10⁸ cells containing 90-95% neutrophils, 1-2% erythrocytes, 3-5% eosinophils and < 0.5% mononuclear cells.

C. Isolation of PMN Plasma Membranes (γ-fraction) and Specific Granules (β-fraction) by Discontinuous Percoll Gradients

The protocol used to isolate the plasma membrane and specific granules was based on Borregaard (1983) with some modifications. Isolated neutrophils were resuspended in 1X PIPES with Pefabloc at 5x10⁷ cells/ml. Nitrogen cavitation was performed at 4°C for 8 minutes at 700 psi to disrupt the cells. The cavitate was collected in 10X EGTA and centrifuged at 4°C for 10 minutes at 1600 rpm to remove the nuclei and whole cells. For discontinuous Percoll gradients, 4.5 ml of Percoll with a density of 1.120 g/ml, was layered under 4.5 ml of Percoll (density of 1.050 g/ml). The sample (1.5-2.0 ml) was
applied on top and centrifugation was carried out at 12,000 rpm at 4°C for 45 minutes. The density of the gradient was estimated from the migration of calibration beads of known density (Pharmacia Fine Chemicals) in gradients run in parallel. The mean density of the α, β, and γ fractions were 1.135 g/ml, 1.084 g/ml, and 1.026 g/ml respectively. The three fractions were removed and ultracentrifuged at 4°C for 90 minutes at 35,000 rpm to remove the Percoll.

D. Identification of MT1-MMP mRNA Expression by RT-PCR

Total RNA was isolated from cells by the QIAGEN RNAeasy Total RNA kit according to the manufacturer’s instructions and quantified by spectrophotometry (Ultrospec 3000; Pharmacia Biotech; Montreal, Quebec). cDNA was produced from 5 µg of total RNA per sample, using SuperScript II reverse transcriptase (GIBCO) primed with random hexamer primers. RNA (5 µg) was incubated with random hexamers (5.0 µl of 100 ng/µl in DEPC-water) to a final volume of 27 µl at 70°C for 10 minutes. The buffer contained 6 µl of 0.1 M DTT, 8 µl of 5 mM mixed dNTP, 12 µl of 5X first strand buffer and 3 µl of RNA guard and the reaction was initiated at 25°C for 5 minutes. Superscript II (3 µl) was added and incubated at 25°C for an additional 10 minutes and then heated to 42°C for 1 hour. The terminal reaction was conducted at 70°C for 15 minutes. As a negative control, a separate reaction was performed without reverse transcriptase to ensure no genomic DNA were present. PCR primers were as follows:

MT1-MMP: 5'-GGGCCCAACATCTGTGAC-3' and 5'-CCCATCCAGTCC-3'

GADPH: 5'-GGCATGGACTGTGGTCATGA-3' and 5'-TCACCACCATGGAGAAGGC-3'
PCR reactions were started at 94°C for 1 minutes, continued for 35 cycles at 94°C for 30 seconds, 50°C for 30 seconds, and 72°C for 30 seconds followed by a 10 minute extension at 72°C. PCR reactions were carried out in separate reaction vessels in a volume of 50 μl with 0.5 μM primers. 1.5 mM MgCl₂, 3 μl cDNA, 200 μM dNTPs and 2.5 U Taq DNA polymerase. Products were run on 1.5% agarose gels at 120 V for 1 hour and visualized under UV light.

E. Immunolocalization of MT1-MMP

In 8-chamber well glass slides (Lab-Tek®), neutrophils (5 x 10⁵ per well) were incubated at 37°C for 5 minutes to allow neutrophil adherence to the slides. The cells were fixed with ice-cold methanol for 6 minutes at -20°C, washed twice with PBS and non-specific binding sites were blocked with 0.2% BSA at room temperature for 10 minutes. After washing two times with PBS, the cells were incubated with anti-MT1-MMP antibody (10 μg/ml) in a humidor at 4°C overnight. The cells were washed twice with PBS and blocked with 0.2% BSA at room temperature for 10 minutes. TRITC goat anti-rabbit F(ab')₂ fragment (1.5 mg/ml at 1:100) was added for 2 hours at 4°C. Cells were washed twice with PBS, stained with DAPI (1 μg/ml in 0.01% Nonidet and PBS) for 2 minutes and washed again with PBS. The cells were covered with Immunofloure (ICN Biomedicals, Aurora, OH) mounted and examined immediately by epifluorescence microscopy. Fluorescence micrographs were recorded on Kodak High-Speed TMX P3200 film. The positive controls included human gingival fibroblasts that were grown to subconfluence in wells coated with fibronectin (10 μg/ml). Negative controls were
neutrophils treated without primary antibody or neutrophils treated with anti-nebulin antibody (76 μg/ml) as the primary antibody.

**F. Western Blot Analysis**

Human gingival fibroblast lysates, neutrophil lysates and neutrophil cellular fractions (α, β, γ) were incubated in electrophoresis sample buffer (0.05 M Tris-HCl pH 6.8, 8 M urea, 2% (w/v) SDS, 8% (v/v) Bromphenol blue) and 0.15 M of dithiothreitol (DTT) (SB/DTT), boiled for 5 minutes and separated by SDS-PAGE on 10% cross-linked minigels at 120 V for 1½ hours and transferred for 2 hours (64 mA/gel) onto nitrocellulose membranes. After blocking with 5% (w/v) Carnation milk, membranes were incubated with anti-MT1-MMP rabbit antibody (2 μg/ml) or with anti-MMP-8 mouse antibody (5 μg/ml) for 3 hours at room temperature, washed and incubated with either anti-rabbit IgG HRP (1:3000 dilution) or anti-mouse IgG1 HRP (1:2000 dilution) for 1 hour at room temperature. The bands were visualized with ECL reagents and molecular weights of the fragments were determined by comparison with prestained molecular weight standards. Treatment of blots with secondary antibody alone showed no reactivity when the secondary antibody was incubated in 5% (w/v) Carnation milk in TBS-Tween.

**G. Soluble Biotinylated Collagen Assay (SBA)**

Aliquots of samples were incubated with biotinylated collagen at ratios of 10 ng biotinylated collagen/μl sample, for 21 hours at room temperature in the presence of collagenase assay buffer (CAB – 0.05M Tris, 0.2 M NaCl containing 5 mM CaCl₂, 0.5
μl/ml BRIJ 35 and 0.2 μg/ml NaN₃). Biotinylated collagen was prepared as described in Mancini et al. (1999). Reactions were terminated by the addition of SB/DTT and boiled for 5 minutes. Collagen fragments were separated on 7.5% cross-linked SDS-PAGE gels and transferred to a nitrocellulose membrane using the PHAST system (Pharmacia). After blocking with 5% (w/v) Carnation milk in TBS-Tween, the membranes were rinsed with TBS (0.02 M Tris-HCl, 0.14 M NaCl pH 7.6 containing 0.1% Tween 20) and incubated with HRP-labeled streptavidin (Amersham) diluted 1/1500 in Tris-HCl buffer pH 7.6. After a second wash, ECL reagents were used for detection of biotinylated collagen fragments by chemiluminescence. Autoradiographs were scanned and full length and ¼ α-chains were quantified by computer analysis using the IP Lab Gel Scientific Image Processing program (Signal Analytics- Vienna, Virginia, USA). The estimation of collagenase activity was based on the densitometric data in terms of percentage biotinylated collagen degradation into ¼ α-chains.

\[
\% \text{ collagen degraded} = \frac{\text{density of } \frac{1}{4} \alpha_1 \text{ (I) chains}}{\text{densities of } \frac{1}{4} \alpha_1 \text{ (I) chains} + \alpha_1 \text{ (I) chains}} \times 100
\]

H. Preparation of samples for SBA

The total protein yield of γ-fractions and β-fractions from the discontinuous Percoll gradients was dependent on the donor and the initial amount of neutrophils isolated. From 1.5×10⁸ cells, the total protein obtained was 96 μg and 287 μg for γ-fractions and β-fractions respectively. In preparation for SBA analysis, various combination of these fractions were mixed and incubated for 4 hours rotating at 37°C. The samples included 150 μl of γ, 150 μl γ + 50 μl β, 150 μl of β with a total protein concentration of 0.130
μg/μl for the γ-fraction and 0.379 μg/μl for the β-fraction. An aliquot (100 μl) of the β fraction was activated with the addition of 50 μl 1 mM APMA and incubated at room temperature for 45 minutes. Then, 50 μl from samples were removed and combined with 6 μl 10X CAB and 2 μl biotinylated collagen for SBA analysis.
RESULTS

A. MT1-MMP mRNA expression in PMN

Expression of MT1-MMP mRNA was examined in whole cell lysates of peripheral blood neutrophils and human gingival fibroblasts by RT-PCR. PCR primers for a 236 bp GAPDH product were used as a positive control to ensure the presence of cellular mRNA (Fig. 1). The MT1-MMP primers identified a 580 bp MT1-MMP product in both the neutrophil and HGF samples. No gene product was identified in a separate reaction without reverse transcriptase to ensure the absence of genomic DNA.

B. Immunolocalization of MT1-MMP in HGF and PMN

Immunolocalization studies were done to determine the presence of MT1-MMP. As a positive control, human gingival fibroblasts (HGF) exhibited strong staining for MT1-MMP (Fig. 2A) and within the same field, the DAPI stained nuclei were evident (Fig. 2B). No significant immunoreactivity was observed in HGF preparations stained with an irrelevant antibody, anti-nebulin, (Fig. 2C) or with no primary antibody (Fig. 2E). Peripheral blood neutrophils stained with anti-MT1-MMP also showed bright staining (Fig. 3A). The neutrophils were identified based on morphology under light microscopy and DAPI staining demonstrated the multilobular appearance of the neutrophil nuclei. Neutrophil preparations stained with an irrelevant antibody, anti-nebulin (Fig. 3C) or with no primary antibody (Fig. 3E) showed no significant immunoreactivity. I used a nebulin antibody as a control because nebulin is a high molecular weight protein that is specifically localized in skeletal muscle myofibrils. This immunolocalization study was
Figure 1: MT1-MMP mRNA expression in HGF and PMN. MT1-MMP mRNA expression was tested by RT-PCR on samples of whole cell lysates of human gingival fibroblasts (HGF) and peripheral blood neutrophils (PMN). Primers 142 and 90 identified a 580 bp of MT1-MMP in PMN and HGF. The primer for GAPDH positive control also appears in both samples as 236 bp. —RT lanes were reactions with no reverse transcriptase produced no gene products.
Figure 2: Immunolocalization of MT1-MMP. A) Preparation of human gingival fibroblasts (HGF) stained with anti-MT1-MMP primary antibody and TRITC-conjugated secondary antibody. B) same field shown in panel A stained with DAPI. C) PMN stained with anti-nebulin primary antibody and TRITC-conjugated secondary antibody. D) same field shown in panel C stained with DAPI. E) PMN stained with no primary antibody and TRITC-conjugated secondary antibody. F) same field shown in panel E stained with DAPI.
Figure 3: Immunolocalization of MT1-MMP. A) Preparation of peripheral blood neutrophils (PMN) stained with anti-MT1-MMP primary antibody and TRITC-conjugated secondary antibody. B) same field shown in panel A stained with DAPI. C) PMN stained with anti-nebulin primary antibody and TRITC-conjugated secondary antibody. D) same field shown in panel C stained with DAPI. E) PMN stained with no primary antibody and TRITC-conjugated secondary antibody. F) same field shown in panel E stained with DAPI.
completed on neutrophil samples from three different donors with similar results. In all samples, the neutrophils incubated with anti-MT1-MMP antibody as the primary antibody exhibited strong fluorescence with minimal background signals.

C. Identification of MT1-MMP by Western Blot Analysis

Samples of human gingival fibroblasts, peripheral blood neutrophils and fractions thereof were separated by SDS-PAGE and transferred onto nitrocellulose membranes. After incubation with anti-MT1-MMP, three bands were identified at 86 kDa, 80 kDa, and 62 kDa for the human gingival fibroblast sample (HGF) (Fig. 4). The same banding pattern appeared for the plasma membrane fraction (γ) from neutrophils. For the whole cell neutrophil lysate sample (PMN), the 80 kDa and 62 kDa bands were present with additional bands at 69 kDa and 66 kDa. No bands were detected in the α and β fractions (Fig. 4). This Western Blot analysis was repeated with neutrophil samples from three other human donors and each blot produced the same banding pattern. The 62 kDa band is the latent form of MT1-MMP and was present in the human gingival fibroblast (HGF), whole cell neutrophil lysates (PMN) and the plasma membrane fraction (γ). MT1-MMP was not present in the specific (β-fraction) or in azurophilic (α-fraction) granules.

D. Verification of specific granules (β-fraction) as a source of latent MMP-8

Samples of purified human MMP-8 (Calbiochem), neutrophil plasma membrane (γ-fraction) and specific granules (β-fraction) were separated by SDS-PAGE and transferred onto nitrocellulose membrane. After incubation with anti-MMP-8, the purified human MMP-8 sample exhibited two bands at 65 kDa and 55 kDa which represented the latent
Figure 4: Identification of MT1-MMP. Samples of whole cell lysates of peripheral blood neutrophils (PMN) and postnuclear cavitate fractions of γ (plasma membrane), β (specific granules), α (azurophil granules) were tested by Western blot for immunoreactive MT1-MMP. The proform of MT1-MMP of 62 kDa is predominant in PMN and γ (plasma membrane) fractions. The positive control of human gingival fibroblast (HGF) also demonstrates the presence of MT1-MMP. The samples incubated with no primary antibody demonstrate minimal cross-reactivity.
and active form of MMP-8 respectively. The specific granules sample (β-fraction) exhibited only the 65 kDa latent form of MMP-8 whereas in the neutrophil plasma membrane sample (γ-fraction), MMP-8 was not detected (Fig. 5).

Collagenase activity of the specific granules sample (β-fraction) was tested by SBA (Fig. 6). Analysis of an aliquot (56.9 μg total protein) representing 6.5% of the β-fraction revealed negligible (1%) digestion of the biotinylated collagen. An equivalent aliquot (6.5% or 58.1 μg total protein) of specific granules from another donor digested 3% of the collagen. However, when an aliquot representing 4.3% or 37.3 μg total protein of β-fraction was treated with APMA, the collagenase activity of the specific granules sample generated 86% collagen digestion. Thus, the specific granules (β-fraction) contain latent MMP-8 that could be activated. Both specific granule samples (β-fraction) that were obtained from two different human donors produced similar results from the anti-MMP-8 Western Blots analysis and SBA.

E. Collagenase activity in neutrophil plasma membrane (γ-fraction)

The neutrophil plasma membrane sample (γ-fraction) contained MT1-MMP as demonstrated by the anti-MT1-MMP immunofluorescence (Fig. 3A) and Western blot analysis (Fig. 4), but the sample did not contain any forms of MMP-8 as demonstrated by the anti-MMP-8 Western Blot analysis (Fig. 5).

However, the neutrophil plasma membrane sample (γ-fraction) did exhibit some collagenase activity. Analysis by SBA showed that an aliquot (6.7% or 19.5 μg total
Figure 5: Identification of MMP-8 Western blot analysis with anti-MMP-8 monoclonal antibody demonstrates the presence of latent 65kDa MMP-8 in the β fractions and absence of MMP-8 in the γ fractions. Pure human MMP-8 serves as a positive control demonstrating the presence of both the latent and active form of MMP-8.
Figure 6: Collagenase activity in plasma membrane (γ-fraction) and specific granules (β-fraction). γ, β, APMA activated β, and γ+β samples were tested by SBA for collagenase activity. γ alone had some intrinsic collagenase activity while β alone had no activity. β can be activated by the addition of APMA. γ + β demonstrated an increase in digestion when combined.
protein) of plasma membrane fraction generated 28% collagen digestion (Fig. 6). Another equivalent aliquot (6.7% or 20.4 μg total protein) of plasma membrane fraction from another donor digested 24% of the collagen.

F. Increased collagenase activity of specific granules (β-fraction) latent MMP-8 by neutrophil plasma membrane

The incubation of an aliquot (19 μg total protein) representing 5% of neutrophil plasma membrane fraction with an aliquot (1.6% or 19 μg total protein) of specific granules sample (β-fraction) digested 54% of the collagen (Fig. 6). Aliquot mixtures of similar proportions from another donor generated 40% collagen digestion.
DISCUSSION

The main findings of this study are that MT1-MMP is expressed in peripheral blood neutrophils and is enriched in the plasma membrane fraction of these cells. In vitro experiments showed that components of the plasma membrane fraction can activate the latent collagenase from the specific granules of neutrophils.

A. Expression of MT1-MMP in Peripheral Blood Neutrophils

RT-PCR demonstrated that peripheral blood neutrophils expressed MT1-MMP mRNA while immunofluorescence and Western blots showed the presence of the MT1-MMP protein. I used human gingival fibroblasts as positive controls for MT1-MMP and, as expected, these cells showed constitutive expression of MT1-MMP mRNA and protein as previously described (Atkinson et al. 1995, Gilles et al. 1997). Many other cell types such as smooth muscle cells, endothelial cells, ameloblasts, odontoblasts, chondrocytes, and osteoclasts express MT1-MMP (Imai et al. 1997, Sato & Seiki 1996, Sato et al. 1997) however to the best of my knowledge, this is the first report of MT1-MMP expression by neutrophils.

In experiments using nitrogen-cavitated cells separated by density centrifugation in Percoll gradients, I localized MT1-MMP to the plasma membrane fractions as demonstrated by Western blots of the various neutrophil fractions. This finding is consistent with previous studies showing that MT1-MMP is found in the plasma membrane preparation of transfected COS-1 cells, human skin fibroblasts and osteoclasts (Sato et al. 1994, Atkinson et al. 1995, Sato et al. 1997). In view of the structure of MT1-
MMP which contains a 24 amino acid hydrophobic trans-membrane domain, it would be expected that MT1-MMP would be tethered to the neutrophil membrane by the trans-membrane region.

B. Forms of MT1-MMP

By Western blotting I only found the 62 kDa (latent) form of MT1-MMP in neutrophils. MT1-MMP exists in three forms. The latent form that contains a propeptide domain is 60 kDa form. Cleavage of this propeptide domain results in a 57 kDa form which can activate progelatinase A. However, in the absence of TIMP-2, this 57 kDa form undergoes autocatalytic conversion to a functionally inactive form (44 kDa) that lacks the entire catalytic domain but maintains the hemopexin-like domain and hinge region (Hernandez-Barrantes et al. 2000). The absence of the active and the functionally inactive MT1-MMP forms in the Western blots may be attributed in part to the neutrophil preparation method since isolation procedures can exert profound effects on the activation systems that regulate the proteolytic machinery of these cells (Pabst 1994). Cognizant of the tendency of neutrophils to be readily activated by inappropriate isolation procedures, special precautions were taken to prevent cell activation following venipuncture in healthy adult human volunteers. For example, as neutrophils can be activated by trace amounts of lipopolysaccharides (LPS), I used LPS-free solutions. disposable plastics and glassware baked at 180°C for several hours. It is thought that if these special precautions are taken, neutrophils isolated from circulating blood are largely in a “resting state” (Haslett et al. 1985). Thus in resting neutrophils, the armamentarium used for bacterial defence is inactive. Notably, the components of the NADPH oxidase
system are stored in granules and therefore in resting neutrophils the granule contents are not assembled and are not prepared for destruction of phagocytosed organisms (Weiss 1989). Similarly, the neutrophil collagenase (MMP-8) is stored in the latent, pro-enzyme form in the specific granules prior to release and extracellular activation (Doherty et al. 1994). By analogy I suggest that in resting neutrophils the MT1-MMP is in a latent form because it has not been activated to engage in proteolysis. Thus the 57 kDa active form and the 44 kDa functionally inactive form of MT1-MMP were not detected in the Western blot analysis. Currently, it is not known what mechanism may activate MT1-MMP on the surface of neutrophils. However, I speculate that the 57 kDa active form is present in activated neutrophils. It is likely that this activation mechanism would exert an important effect on the ability of MT1-MMP to activate MMP-8 and thereby initiate degradation of matrix proteins. Thus, if MT1-MMP can cleave the N-terminal propeptide of MMP-8, it may be able to regulate the collagenolytic activity of MMP-8.

In vitro, a number of activation mechanisms have been studied which produce different forms of MMP-8 and with varying levels of collagenolytic activity. For example, stromelysin-2 processes MMP-8 by a single-step activation mechanism by cleavage of the Gly$^{78}$-Phe$^{79}$ peptide bond in the N-terminal propeptide domain. This active MMP-8 displays very high specific collagenolytic activity (Knauper et al. 1996b). On the other hand, trypsin requires a two-step activation mechanism in which the first cleavage occurs at Arg$^{48}$-Phe$^{49}$ to generate an intermediate latent form and then a second cleavage at Arg$^{70}$-Cys$^{71}$ to produce an active MMP-8 (Knauper et al. 1990). MMP-8 activation by HgCl$_2$ follows a three-step mechanism where the first cleavage is at Asn$^{53}$-Val$^{54}$. Then,
autoproteolytic cleavage of Asp$^{64}$-Met$^{65}$ produces an intermediate form (Met$^{65}$ N-terminus) which displays only about 40\% of the maximum collagenolytic activity. Final activation occurs after autoproteolytic cleavage of either Phe$^{79}$-Met$^{80}$ or Met$^{80}$-Leu$^{81}$ (Blaser et al. 1991).

C. MMP-8 activation by MT1-MMP

Currently, the system(s) by which MMP-8 is activated in vivo is not known although, as discussed above, a large number of in vitro studies have implicated stromelysin (Knauper et al. 1996b), oxidants (Nagase 1997), as well as other proteases such as trypsin and chymotrypsin (Knauper et al. 1990).

Initial pilot experiments to determine if MT1-MMP can activate MMP-8 involved the incubation of recombinant MT1-MMP with latent rat MMP-8. Based on the SBA, this combination resulted in complete digestion of the biotinylated collagen. However, the latent rat MMP-8 which was supposed to be latent produced > 50\% digestion. Unfortunately, this experiment was not reproduced due to the lack of more recombinant MT1-MMP. Analysis of MT1-MMP mediated activation of MMP-8 was also complicated by the difficulty in obtaining a source of pure latent MMP-8. The latent rat MMP-8 most likely was activated during shipment or during storage, so I decided to use the specific granules from neutrophils as a source of latent MMP-8. As demonstrated by the Western blots (Fig. 5) and SBA (Fig. 6), the specific granules were a viable source of latent MMP-8 than can be activated by APMA.
Since only a limited amount of recombinant MT1-MMP was available, I tried to generate recombinant MT1-MMP by transfections of CHO cells with MT1-MMP plasmids. While the transfection was successful, the predominant form of MT1-MMP generated was the inactive form that lacked the catalytic domain. Consequently, the neutrophil plasma membrane was used as a source of MT1-MMP, a source that required large number of cells and arduous procedures.

As I was able to show the presence of MT1-MMP in association with the cell surface of neutrophils, I conducted experiments in which I combined neutrophil plasma membrane fractions with the contents of specific granules that contained latent MMP-8. In these experiments, the membrane preparation exhibited abundant MT1-MMP and moderate collagenase activity while the specific granules contained abundant latent MMP-8 but minimal collagenase activity. After combining the two fractions, collagenase activity was doubled. This finding indicates that components of the neutrophil membrane including MT1-MMP have the potential to activate latent MMP-8. While MT1-MMP exhibits collagenase activity, it is 5-7.1 fold less efficient than MMP-8 (Ohuchi et al. 1997). However, if MT1-MMP can process the latent form of MMP-8 to an active form, then this would provide an efficient and spatially segregated mechanism for activating MMP-8 on the surface of neutrophils. In this context, previous studies have demonstrated that MT1-MMP activates other MMPs such as latent gelatinase A and collagenase-3 (Sato et al. 1994, Knauper et al. 1996). Sato (1994) transfected MT1-MMP plasmid into human fibrosarcoma HT1080. These cell lines also secreted progelatinase A (66 kDa) into the culture supernatant. Plasma membrane fractions of the transfected HT1080 (20 µg
protein) were incubated with the conditioned medium from HT1080 that contained progelatinase A for two hours at 37°C. Gelatin zymography demonstrated that the plasma membrane generated the processed 64 kDa and 62 kDa gelatinase A. Similarly, Knauper (1996) incubated fibroblast-derived plasma membranes that contained MT1-MMP (15µl; 1mg/ml protein) with 50 ng of procollagenase-3. The plasma membranes processed the procollagenase-3 to a 48 kDa active enzyme as demonstrated by Western blot analysis. Taken together, these studies indicate that MT1-MMP activation of MMP-8 is feasible. However, that MT1-MMP can directly activate MMP-8 still needs to be established.

As mentioned above, in gingivitis, MMP-8 is predominantly latent, whereas, in periodontitis, it is conceivable that the combination of a susceptible host and the presence of periodontal pathogens provides a situation in which an activation cascade can lead to active MMP-8. I suggest that virulence factors and inflammatory cytokines may trigger specific enzymes which lead to activation of enzymes such as furin or plasmin. These enzymes in turn can activate latent MT1-MMP. The active MT1-MMP will then activate MMP-8 which leads to destruction of the periodontium. As many cells types express MT1-MMP, it is possible that within the periodontium or gingival crevice, other cell types (e.g. fibroblasts) that express MT1-MMP may be responsible for activation of MMP-8. However, because neutrophils are so abundant in acute inflammation and are the primary producers of MMP-8, it seems unlikely that other cells such as fibroblasts play a significant role in MMP-8 activation. That extensive but reversible destruction of gingival tissues occur in gingivitis suggests that there may be fundamental differences in the mechanism of tissue degradation between periodontitis and gingivitis.
D. Summary and Suggestions for Future Studies

In summary, my results demonstrate that neutrophils express MT1-MMP and the protein is located on the plasma membrane. The combination of plasma membrane fractions with latent MMP-8 indicates that components of the neutrophil membrane may be able to activate MMP-8. I suggest that one of these activating molecules may be MT1-MMP. However, the activation of MMP-8 by MT1-MMP still needs to be confirmed by more definitive experiments. For example, a potential future study could involve the use of an antibody that blocks the enzymatic activity of MT1-MMP or an assay that immunodepletes MT1-MMP from the plasma membrane fraction prior to incubation with latent MMP-8. Under these conditions, selective removal of the MT1-MMP by antibody should reduce the activation of MMP-8 and lead to lower collagenase activity as detected by the SBA assay.

In considering activation systems, I should also point out the involvement of TIMP-2 in the hypothetical activation of MMP-8 by MT1-MMP as there is increasing evidence that TIMP-2 plays an important role in regulating the activity of MT1-MMP. Strongin et al. (1995) suggested that TIMP-2 is required for progelatinase A (MMP-2) activation and Hernandez-Barrantes (2000) demonstrated that TIMP-2 regulated the effective cell-surface "concentration" of active MT1-MMP by adjusting the autocatalysis of the enzyme and consequently its availability for interacting with gelatinase A. Notably, in the absence of TIMP-2, there is uncontrolled autocatalysis of MT1-MMP that leads to production of the inactive 44 kDa form. However, when there is an excess of TIMP-2, the
enzymatic action of MT1-MMP is also inhibited. In effect, a future model of MT1-MMP activation of MMP-8 may involve controlling the level of TIMP-2 at the cell surface for appropriate regulation of MT1-MMP and its subsequent activation of MMP-8.

The ability to regulate the activity of MMP-8 at the cell surface confines collagenase activity close to the cell. This illustrates the concept of pericellular proteolysis (Werb 1997). Even though MMP-8 is released extracellularly, ECM degradation \textit{in vivo} is confined to the immediate pericellular environment of the cell (Andreasen \textit{et al.} 1997, Nakahara \textit{et al.} 1997). Because MT1-MMP is bound on the neutrophil plasma membrane surface, it provides the opportunity to concentrate the various components involved with ECM degradation close to the neutrophil. Thus, if MT1-MMP can activate MMP-8, then MT1-MMP may provide a mechanism for controlled localized degradation of the collagen matrix in periodontitis and other diseases in which there is a marked neutrophil inflammatory infiltration.
CONCLUSIONS

1. MT1-MMP is expressed in peripheral blood neutrophils.

2. MT1-MMP is found in the plasma membrane and not in the specific and azurophilic granules.

3. Specific granules from the β-fraction of neutrophils are a source of latent MMP-8 that can be activated by APMA.

4. The plasma membrane fraction has collagenase activity.

5. The collagenase activity of a plasma membrane fraction combined with specific granules of neutrophils is higher than the collagenase activity of the individual fractions.
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