Matrix Metalloproteinase Processing of Monocyte Chemokines

by

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
in
THE FACULTY OF GRADUATE STUDIES

Department of Biochemistry
and Molecular Biology

THE UNIVERSITY OF BRITISH COLUMBIA

April 17th, 2001

Abstract

Extracellular matrix degradation occurs in chronic inflammatory diseases such as arthritis and disturbances in connective tissue homeostasis contribute to various lung, neurological and cardiovascular diseases and is pivotal in tumor metastasis. The matrix metalloproteinase (MMP) family of endoproteinases is implicated in these processes by a general ability to degrade the structural components of the extracellular matrix. To understand the biological role of MMP proteolysis in physiological and pathological processes, it is necessary to identify biologically relevant substrates.

I initiated yeast two-hybrid screens to identify novel substrates of gelatinase A using the hemopexin C domain of the enzyme as bait. Initial screens of a cDNA library constructed from Concanavalin A-treated human gingival fibroblasts identified the chemokine monocyte chemoattractant protein (MCP)-3 as a hemopexin C domain binding protein. Incubation of MCP-3 with gelatinase A resulted in cleavage of MCP-3 at Gly^4-Ile^5 a preferred scissile bond sequence for the enzyme. The turnover rate, k_{cat}/K_m was determined to be 8,000 $M^{-1}s^{-1}$, more efficient than gelatinase A cleavage of gelatin. Indeed, cleaved MCP-3 was identified in human rheumatoid arthritis synovial fluids. Gelatinase A (and other MMP)-mediated cleavage of MCP-3 resulted in conversion of chemokine receptor agonist activity to a general acting antagonist, impairing the activity of several related chemokines.

The mechanistic importance of the hemopexin C domain in gelatinase A cleavage of MCP-3 was determined. The turnover rate was reduced to 500 $M^{-1}s^{-1}$

upon removal of the hemopexin C domain from the enzyme. Exogenous hemopexin C domain competed for cleavage whereas the collagen binding domain of gelatinase A did not. Specificity of MCP cleavage could be attributed to unique binding of both the gelatinase A and membrane-type (MT)-1 hemopexin C domains to only MCP-3 and not MCP-1, -2, or -4. Chemokine chimeras further demonstrated the importance of hemopexin C domain exosites in catalysis. My results provide evidence that MMP activity can contribute toward the reparative process in inflammation and that interactions of MMPs with chemokines provide a self-attenuating network to dissipate pro-inflammatory activities. I propose that MMP processing of chemokines is a new paradigm in chemokine and MMP biology in the regulation of inflammation.

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Thesis Format

The layout of this thesis represents an emerging method of presenting the scientific work that has been conducted during the doctoral training. Quoting from the most recent *Standards for the Ph.D. Degree in the Molecular Biosciences: Recommendations of the Committee on Education of the International Union of Biochemistry and Molecular Biology*: "it [the thesis] may consist of one or more published papers with a general introduction and a thorough discussion of the research project." Paraphrasing other recommendations, scientific endeavors are highly collaborative. Other individuals often help with the work to expedite publication. It is essential that before each Chapter/paper, the author state explicitly his/her contribution to the work. It is also unavoidable that there will be some duplication of methodology and introductory ideas. The author must work to avoid this as much as possible. This thesis has followed these guidelines and represents a body of work that was initiated, driven, and organized by the candidate, and includes manuscripts written by the candidate with editorial revisions by the project supervisor.

Acknowledgements

It's been a real pleasure the last 4-5 years—working in the Overall lab and living in Vancouver. I would like to acknowledge the support and training I received from Chris Overall. Without his support and encouragement many of the experimental work presented in this thesis would not have happened. Thanks for all the letters of support, bottles of champagne, giving me the opportunity to meet your friends and colleagues, and for being a good role model—particularly in the footwear department. I must also thank Tom Beatty and Ross MacGillivray for many thoughtful discussions, but especially Ross for the extra 'committee meetings'. Many past and present members of the Overall lab have also contributed to my enjoyment—past—Edwin, Bjorn, Angela, Jorge, and especially Maggie—present—George, Charlotte, Eric, Gayle, Todd, Heidi, Dennis but especially Andrea for accepting all of my spare change. Clive Roberts deserve extra mention as both a great wealth of science-related information and hilarious stories.

Importantly, there a great number of people that shared in many vices and helped the process take longer by most important distractions—Glen, John, Doug, Cam, Chris-there are many-so one mention is enough. Erin got special mention in my other thesis, yet she still deserves it—so here it is.

Chapter 1 Introduction

Perspective

Multi-cellular organisms are faced with the challenges of cell-cell communication during phases of development and environmental challenges. This requires a transmission of signals that alter cellular processes in response to various stimuli. Cells have numerous mechanisms for sending and responding to signals. A paradigm for cellular communication involves the activity of secreted molecules. These bioactive molecules can diffuse through the intercellular space, either through the extracellular matrix, the supportive structure between cells, or to distant regions through the vasculature and blood stream. The signaling molecules generally interact with cell-surface bound proteins on the receiving cell, which acts as a receptor for the signal. The interaction of ligand with its cognate receptor results in a number of actions that allow for the transduction of the signal (Pawson, 1995). One class of receptors is defined by a heptahelical transmembrane motif, and interacts with guanine nucleotide-binding proteins (Hall *et al.*, 1999). This causes a number of intracellular proteins to be recruited and activated, generally resulting in the alteration of transcription in the nucleus of the target cell (Edwards, 1994). There are many variations on this theme; however, the common result is that the receptor molecule changes the physiology of the cell upon interaction with the extracellular ligand.

The cell has evolved multiple mechanisms that modulate the signaling events during phases of development. It is apparent that signals between cells must be fine tuned, and not just binary responses. Many similar signaling pathways may converge on the same intracellular target, which can result in a gradient response fine tuning the cellular changes. Separate from intracellular biochemical effects that can determine the outcome of receptor binding, a number of extracellular events can alter the fate of cell signals. This thesis will deal with one very powerful and recently emerging method of modulating cell signals. Extracellular proteolysis has recently been put into the spotlight as a fundamental and critical aspect of modulating the communication and migration of cells (Murphy and Gavrilovic, 1999). There are many families of extracellular proteases that have been implicated in regulating the physiology of the cell, by either processing the ligands or the receptors. Two important families of enzymes include the serine proteases and the metalloproteinases. Recognizing that metalloproteinases are not just degrading enzymes, but can specifically process extracellular molecules to modulate activity, I sought to discover new substrates to gain a better understanding of MMPs in biological processes. In this introductory Chapter I will review the activity and characteristics of the matrix metalloproteinase (MMP) family of extracellular proteases. In addition, I will present a brief perspective on the process of inflammation and discuss the biology of a class of signaling molecules called chemokines. I have recently

discovered that chemokines form a novel class of MMP substrates. The specific proteolysis of chemokines modulates subsequent cellular responses in inflammation. This thesis will describe the interactions between these two classes of extracellular molecules, and the biological implications on cellular activity during immune responses to external environmental challenges.

Matrix Metalloproteinases

General Characteristics

In humans the matrix metalloproteinase family of extracellular proteinases is comprised of 21 members characterized to date. All members of this family have a very conserved domain structure by amino acid sequence (see Figure 1.1). With some exceptions, as detailed below, the regions typically shared amongst the MMPs can be characterized from N-terminus to C-terminus as a signal sequence required for secretion to the extracellular space, a pro-peptide domain required for enzyme latency, a catalytic domain, a linker region, and a hemopexin COOH-terminal (C) domain. The MMP proteins can be organized into four families, as defined by both their substrate repertoire and domain structure.

Collagenases

Vertebrate collagenase activity was first discovered in 1962 (Gross & Lapiere,

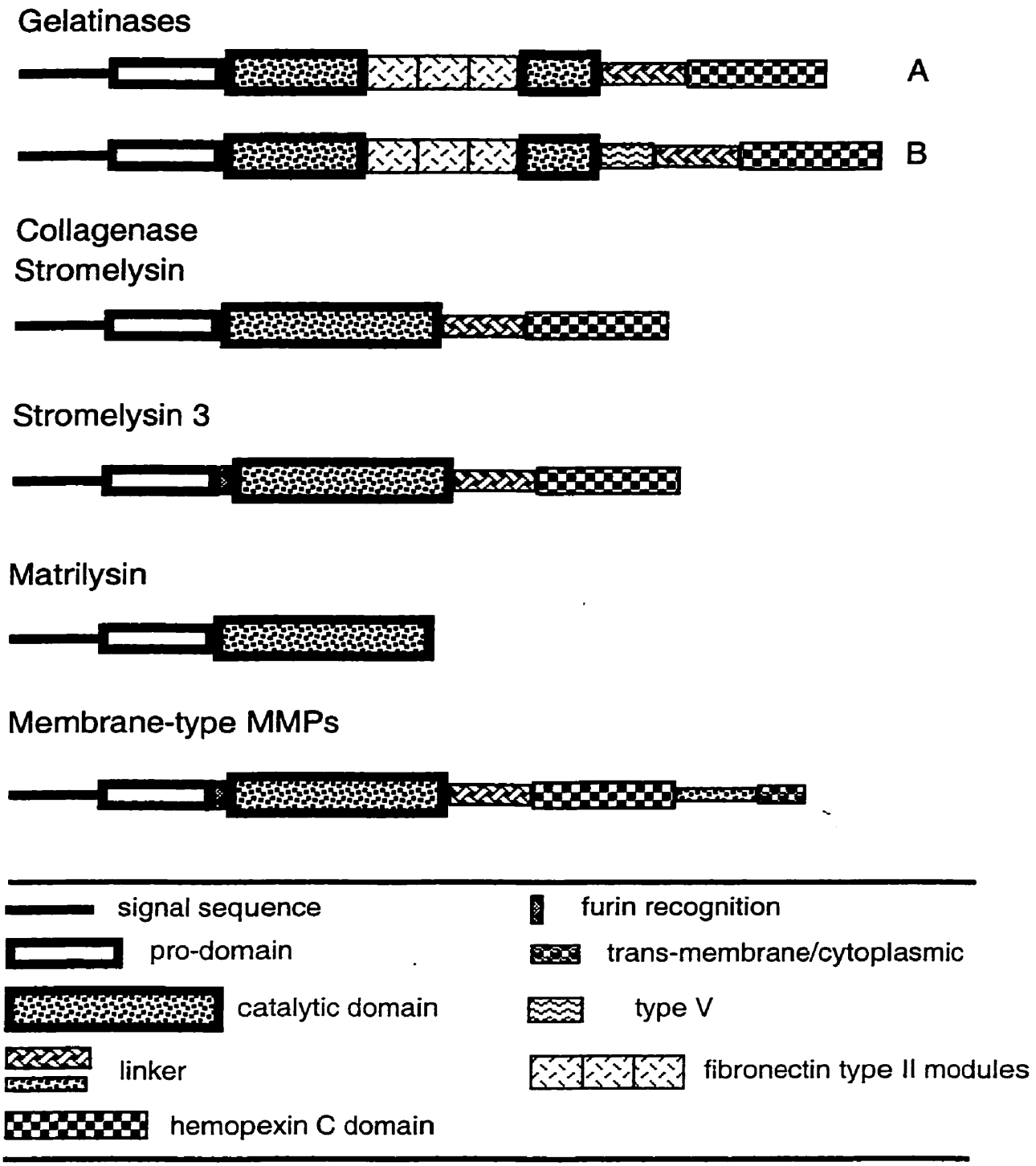


Figure 1.1 The matrix metalloproteinase family.

The MMP family members are shown according to their substrate specificity and domain organization. Each conserved domain contributes to the specific properties of the proteinase (see text for details).

1962) in the frog when it was recognized that tadpole tail resorption during development would require massive degradation of the structural collagen. The sequence of human interstitial collagenase (MMP-1) (Goldberg *et al.*, 1986) showed that it had the typical MMP domain structure as described above and as shown in Figure 1.1. MMP-1 degrades various interstitial collagens as well as α1-antitrypsin (Mast *et al.*, 1991). Collagenase 2 (MMP-8) was the second MMP to be identified, and was characterized from human neutrophils (Lazarus *et al.*, 1968). Accordingly, this enzyme is also known as neutrophil collagenase. This enzyme is unique among the MMPs as it is stored in specific granules, and is released during phagocytosis. Collagenase 3 (MMP-13) was characterized from breast carcinomas (Freije *et al.*, 1994) and was shown to be specifically overexpressed in malignant samples. MMP-13 displays the typical domain structure and substrate repertoire of the other collagenases with a strong preference for type II collagen, implicating this enzyme in cartilage pathologies (Otterness *et al.*, 2000). A potentially novel collagenase has been identified in Xenopus (collagenase 4, MMP-18; Stolow *et al.*, 1996) that shows very specific developmental expression patterns related to organogenesis.

Gelatinases

Gelatinase activity was first described in rheumatoid synovial samples (Harris and Krane, 1972), but it was not until 1988 that the complete sequence for

human gelatinase A was reported (Collier *et al.*, 1988) and was found to be identical to the type IV collagenase previously reported (Liotta *et al.*, 1981). This enzyme is 72-kDa in its zymogen form and after activation can cleave native type IV collagen. Accordingly, gelatinase A (MMP-2) also has the names type IV collagenase, and 72-kDa gelatinase. It shares the standard domain organization but has inserted within its catalytic domain a triple repeat of fibronectin type II modules which directly contribute to this enzymes ability to bind and degrade gelatin substrates (Steffenson *et al.*, 1995). The other gelatinase, gelatinase B (MMP-9) was first detected from human polymorphonuclear leukocytes (Sopata and Dancewicz, 1974). In 1989, the cDNA sequence was published (Wilhelm *et al.*, 1989) revealing that gelatinase B is the largest of the MMPs. In addition to the typical domain structure, it has the three fibronectin type II modules (like gelatinase A) but it also has a collagen type V-like domain inserted after the active site. Recently it has been shown that this sequence contributes to the high level of glycosylation of the enzyme (Mattu *et al.*, 2000). The biology of the gelatinases has been studied by targeted gene disruption studies in mice. Two separate reports have demonstrated that MMP-2 is important in tumor growth and metastasis (Itoh *et al.*, 1998), but not as a protease for the beta-amyloid precursor protein (Itoh *et al.*, 1997). The MMP-9 knock-out mice have bone and matrix abnormalities in addition to impaired metastasis (Vu *et al.*, 1998; Liu *et al.*, 2000; Coussens, *et al.*, 2000). However, on the whole, the gelatinase A/B-

deficient mice develop normally demonstrating the functional redundancy in the MMPs.

Stromelysins

A proteinase activity that was distinct from collagenase was identified in the early '70s and was purified in the early '80s from rabbit bone (Galloway *et al.*, 1983) and called proteoglycanase. Stromelysin 1 (MMP-3) was also named transin for 'transiently expressed', since it was isolated from rat fibroblasts that had undergone a malignant transformation (Matrisian *et al.*, 1988) and collagenase activating protein (Vater *et al.*, 1983) as it proteolytically removes the pro-peptide of collagenase. Stromelysin 2 (MMP-10) was discovered from cloning (Breathnach *et al.*, 1987) and shown to have over 70% homology in protein sequence. Both stromelysin 1 and 2 have the typical MMP domain organization. Stromelysin 3 (MMP-11) was identified in 1990 (Basset *et al.*, 1990). As deduced from its sequence, in addition to the typical MMP domains, stromelysin-3 contains at the end of its pro-peptide domain the sequence RKRR, which is the recognition sequence for the intracellular family of furin-like proteases. Indeed, stromelysin-3 is activated intracellularly and secreted as an active enzyme, not as a zymogen (Pei and Weiss, 1995). Stromelysin 1 has been shown to be involved in aspects mammary gland development (Sympson *et al.*, 1994; Lochter *et al.*, 1997) including embryonic implantation and post-weaning involution.

Phenotypes of stromelysin 1 deficient mice range from impaired wound contraction (Bullard *et al.*, 1999), impaired contact hypersensitivity response (Wang *et al.*, 1999), and compensatory increases in gelatinase expression (Lijnen *et al.*, 1998).

Membrane-types

As the name suggests, this family of MMPs has at the C terminus of its domain organization a trans-membrane domain and short cytoplasmic region that anchors the protein to the cell surface (Cao *et al.*, 1995). MT1-MMP was first described in 1994 (Sato *et al.*, 1994) from tumor cells of invasive lung carcinomas. As discussed briefly below, MT1-MMP is important as the physiological activator of progelatinase A. Six MT-MMPs have now been identified (Sato *et al.*, 1994; Takino *et al.*, 1995; Will *et al.*, 1997; Lopez-Otin *et al.*, 1999; Pei, 1999; Velasco *et al.*, 2000). MT4- and MT6-MMP differ from the other MT-MMPs in that they are anchored to the cell-surface by a glycosyl-phosphatidylinositol (GPI)-anchor (Itoh *et al.*, 1999; Kojima *et al.*, 2000), not via trans-membrane and cytoplasmic regions. Like stromelysin 3, the MT-MMPs have furin-recognition inserts that result in the intracellular activation of these enzymes. Interestingly, the MT-MMPs are resistant to inhibition by the tissue inhibitor of metalloproteinases (TIMP)-1 revealing an important distinction of this family from the other MMPs. Little is known regarding the in vivo activity of this

family beyond the important activity of MT1-MMP to activate the gelatinase A zymogen. However, various human tumors and cell lines have been shown to have high levels of MT-MMPs (Sato *et al.*, 1997) and unlike the gelatinase knock-out mice, MT1-MMP disruption results in profound problems in bone morphogenesis (Holmbeck *et al.*, 1999) and reduced angiogenesis (Zhou *et al.*, 2000).

Tissue inhibitors of metalloproteinases (TIMPs)

The activity of both secreted and membrane-bound MMPs is modulated by the endogenous soluble protein inhibitors named tissue inhibitors of metalloproteinases—TIMPs. As matrix degradation is the hallmark of many pathological situations, the levels of TIMPs will also be crucial in determining disease processes (Brew *et al.*, 2000). To date, four TIMP molecules have been characterized in humans. They share the domain organization of an N-terminal inhibitory domain, and a C-terminal protein interaction domain. The TIMPs are also characterized by 6 disulphide bonds, each domain having a three loop structure comprised of the 3 disulphide cross-links. Many bio-functions have been ascribed to the TIMPs; some appear to be linked to metalloproteinase inhibition, such as the apoptotic activity of TIMP-3 (Bond *et al.*, 2000), whereas others are not, for example the suppression of mitogenesis by TIMP-2 (Hoegy *et al.*, 2001).

TIMP-1

The production of a protein that inhibited collagenase activity was first reported in 1975 (Bauer *et al.*, 1975) from human fibroblasts and later from rabbit bone cultures (Cawston *et al.*, 1981). This inhibitor was very stable, but extremely sensitive to reductive alkylation, demonstrating the importance of the disulphide cross-links in activity. TIMP-1 was also identified independently as erythroid potentiating factor (Gasson *et al.*, 1985). In addition to its inhibitory activity against all MMPs with the exception of MT-MMPs, TIMP-1 binds to the hemopexin C domain of gelatinase B, the implications of which are unclear in regard to its ability to regulate MMP-9 activation and activity. Early studies indicated the anti-tumorigenic activity of this metalloproteinase inhibitor by down-regulating its expression (Khokha *et al.*, 1989) as well as inducing expression (Khokha *et al.*, 1992). TIMP-1 deficient mice have altered cyclicity during reproduction, with a decrease in length of the estrus period (Nothnick, 2000) and a concomitant decrease in TIMP-2 and TIMP-3 expression (Nothnick *et al.*, 1997).

TIMP-2

The discovery of other proteinaceous MMP inhibitors with a different molecular mass from TIMP-1 was facilitated by the technique of reverse zymography.

Reverse zymography involves the impregnation of both gelatin and gelatinase in the SDS-PAGE gel. Potential inhibitory activities are then loaded, renatured and protein-staining zones of non-clearance (where the gelatinase was not able to degrade the gelatin) indicate the presence and molecular mass of a gelatinase-inhibitory protein. TIMP-2 was identified from a human melanoma cell line (Stetler-Stevenson *et al.*, 1989) and SV-40-transformed lung fibroblasts (Goldberg *et al.*, 1989). Like TIMP-1, TIMP-2 also has various growth factor-like activities (Corcoran and Stetler-Stevenson, 1995) and analogous to TIMP-1 can bind to the hemopexin C domain of progelatinase A (Overall *et al.*, 1999). That TIMP-2 potentiates the activation of gelatinase A at the cell surface by its dual association with the hemopexin C domain of gelatinase A and the catalytic domain of MT1-MMP has been the focus of much work, and is described below. Indeed, the only noticeable defect in TIMP-2 knockout mice was a defect in pro-gelatinase A activation (Wang *et al.*, 2000; Caterina *et al.*, 2000) further demonstrating the redundancy of this protease-inhibitor system in the normal development of the organism.

TIMP-3

TIMP-3 is unique among the TIMPs in that it is not a soluble extracellular protein. TIMP-3 binds with high affinity to the extracellular matrix (Yu *et al.*, 2000). In addition, TIMP-3 has the unique distinction in the MMP/TIMP family of being

specifically associated with a human disease, Sorsby's fundus dystrophy (Fariss *et al.*, 1998). Interestingly, disease progression is associated with point mutations in TIMP-3 which typically result in the replacement of serines with cysteines. This results in TIMP-3 aggregates (predominately dimers) in the matrix of the eye, presumably via disulphide linkages. TIMP-3 is also unique among the MMPs in that it binds to the pro-forms of both gelatinase A and B (Butler *et al.*, 1999), and beyond its MMP inhibitory activity, TIMP-3 also inhibits the TNF-α converting enzyme (Amour *et al.*, 1998) which is a metalloproteinase, but of the ADAMs (a disintegrin and metalloproteinase domain) family.

TIMP-4

Human TIMP-4 is the most recently characterized TIMP and was the first MMP-TIMP cloned by analysis of expressed sequence tags from a human heart cDNA library (Greene *et al.*, 1996). Little is known regarding the in vivo activity of TIMP-4, however TIMP-4 over-expression in a human breast cancer cell line inhibited tumor growth and metastasis when injected into nude mice (Wang *et al.*, 1997). Interestingly, it has an analogous gelatinase A-binding activity as compared to TIMP-2 (Bigg *et al.*, 1997). However, where it has been established that TIMP-2 potentiates progelatinase A activation, TIMP-4 lacks this critical activity (Toth *et al.*, 2000; Bigg *et al.*, 2001).

MMP Domains

An important aspect of MMPs in biological processes is both the regulation and specificity of MMP proteolysis. The conserved domain structure (as shown in Figure 1.1) indicates the significance of these different domains as critical molecular determinants contributing to the various functions of the MMP family.

Pro-peptide domain

As noted above, the MMP proteins are generally secreted as inactive zymogens that require proteolytic processing to yield a proteolytically active protein. This 80 residue domain contains what is referred to as the cysteine switch sequence (PRCG[N/V]D) that is responsible for maintaining the enzyme in the latent form. The cysteine within this sequence has recently been shown to directly co-ordinate with the catalytic zinc ion in the active site of the enzyme in the crystal structure (Becker, *et al.*, 1995; Morgunova *et al.*, 1999). This occupancy prevents the substrates of the enzyme from interacting with the active site groove. The cysteine switch 'opens' upon proteolytic cleavage within the pro-peptide region. This results in a destabilization of the cysteine-zinc co-ordination, and the pro-peptide region no longer occupies the active site. The recent crystal structure of full-length gelatinase A revealed additional contacts between the pro-domain and the collagen binding domain which likely increases the stability of the zymogen (Morgunova *et al.*, 1999).

Catalytic domain

Functionally, this is the most important domain of the MMPs. The catalytic domain contains the active site cleft where substrate scissile bonds are presented for cleavage. Structural studies have shown the active site architecture at high resolution, and demonstrate the role of the co-ordinated zinc ion with the active site. The zinc is held in place by the classic zincin proteinase motif of sequence HExxHxxGxxH. Mechanistically, it is known that the glutamic acid residue is present to polarize a bound water molecule. Together with this polarized water molecule, the glutamate residue likely acts as a general acid/base that is essential for cleavage of the substrate (Becker *et al.*, 1995). Indeed, changing the catalytic glutamate to alanine abolishes MMP activity. This has been a successful strategy used to crystallize MMPs by avoiding autolysis.

Collagen binding domain

The two gelatinases (A & B) have inserted just N-terminal to the active site region in the catalytic domain, a domain which is comprised of three tandem repeats of fibronectin type II repeats. This region has been shown to bind many different types of collagen, both native and denatured (Steffensen *et al.*, 1995). It has been demonstrated that this domain contributes toward the type IV collagenolytic activity of these MMPs as demonstrated by deletion mutants of both gelatinase A

(Murphy *et al.*, 1994) and gelatinase B (Shipley *et al.*, 1996).

Linker region

A flexible spacer, termed the proline-rich linker or flexible hinge, connects the catalytic domain to the hemopexin C domain (see below). The function of this domain in MMPs is not well characterized. Hypotheses regarding the action of this region range from providing a molecular flex between the catalytic and hemopexin C domains to playing a direct role in the proposed intercollation triple helicase action required for collagen degradation (De Souza *et al.*, 1996). Indeed, mutation of proline residues in this domain results in significant reductions in collagenolytic activity (Knäuper *et al.*, 1997); however, mutation of proline residues always has the potential to significantly disrupt the pre-existing structure of peptide chains.

Hemopexin C domain

This domain derives its name from the fact that it has homology to the serum protein hemopexin, and also vitronectin. Notably, studies from one group have reported the binding of the gelatinase A hemopexin C domain to the $\alpha_v\beta_3$ integrin, which is know to bind vitronectin (Brooks *et al.*, 1996, 1998). The biological implications of this is not clear; however, a recent report by the same group has characterized a small molecule inhibitor of this interaction, and demonstrated its

anti-angiogenic activity (Silletti *et al.*, 2001). A striking feature of the hemopexin C domain is its 4-bladed beta-propeller structure (Bode, 1995; Murphy and Knäuper, 1997). The overall shape is that of a squat cylinder comprised of four beta sheets (comprising one hemopexin module) each forming a blade of the beta-propeller structure (Li *et al.*, 1995). Extensive packing faces on the sides of the blades arranged next to one another to form a very stable configuration, stabilized by a disulphide crosslink between cysteines which begin and end the domain. A central pore running through the middle of the hemopexin C domain contains one (Li *et al.*, 1995) or two (Gomis-Rüth *et al.*, 1996) Ca^{2+} ions that are structurally important, as chelation abrogates heparin and fibronectin binding by the isolated gelatinase A hemopexin C domain (Wallon and Overall, 1997).

The hemopexin C domain of collagenase 1 (Clark and Cawston, 1989), neutrophil collagenase (Hirose *et al.*, 1993), collagenase 3 (Knäuper *et al.*, 1997) and MT-1MMP (Tam and Overall, unpublished data) bind native collagens. That this association is important in the catalysis of these substrates is demonstrated by deletion mutants (Clark and Cawston, 1989; Murphy *et al.*, 1992) and disruption of hemopexin C domain structure by removal of the disulphide cross-link (Windsor *et al.*, 1991). These alterations result in proteins that have lost collagenolytic activity, but are still competent on small peptide or denatured substrates. Interestingly, deletion of the hemopexin C domain of collagenase-3 does not affect cleavage of type I and II collagens and fibronectin (Knäuper *et al.*,

1997). Thus, exosites on the hemopexin C domain of collagenases play an important role in the triple helicase activity required for some fibrillar collagens to potentiate catalysis.

In contrast to the collagenases, the gelatinases are less dependent on the hemopexin C domain for catalysis of both native and denatured collagen (gelatin) as demonstrated by deletion studies of both gelatinase A (Murphy *et al.*, 1992) and gelatinase B (O'Connell *et al.*, 1994). This difference is likely attributable to the presence of exosites on the collagen binding domain which are known to bind collagens (Steffenson *et al.*, 1997). The gelatinase hemopexin C domain does however have exosites for other molecules including heparin, fibronectin (Wallon and Overall, 1995), and binds TIMPs (O'Connell *et al.*, 1994; Overall *et al.*, 1999, 2000). The fact that the gelatinases seem less dependent on the hemopexin C domain for catalysis has prompted some authors to suggest the primary role of the gelatinase hemopexin C domain is to stabilize and direct the interaction of these MMPs with the TIMPs. Indeed, the gelatinase hemopexin C domains are critical in binding the TIMPs, which influence the proteolytic activation of the zymogens; however, the data in this thesis details the importance of the gelatinase A hemopexin C domain in directing catalysis of chemokine substrates.

Role of MMP domains in zymogen activation

Although somewhat removed from the focus of this thesis, one must consider the

activation of these enzymes and the molecular determinants involved, before discussing the roles of MMPs in physiological and pathological processes. In particular, much work has focused on and delineated the cell-surface activation of gelatinase A (Murphy *et al.*, 1999). This MMP is unique in that the proteolytic activation occurs via a four protein assembly at the plasma membrane. Gelatinase A undergoes two proteolytic events before achieving complete activation. The first cleavage occurs at residue Asn^{37}-Leu^{38} (Will *et al.*, 1996), by the cell-surface activator of gelatinase A, MT1-MMP (Sato *et al.*, 1994). The second proteolytic event is an autocatalytic intermolecular processing at residue Asn^{80}-Tyr^{81}, resulting in the fully active proteinase.

How the first proteolytic event occurs has been the focus of much work, and is now generally accepted to be the result of the following process. Gelatinase A is secreted in the zymogen form to the extracellular space. It is then bound in complex with TIMP-2 via the C-domain of TIMP-2 and the hemopexin C domain of gelatinase A (Murphy *et al.*, 1999). This stable complex is then localized to the cell-surface by means of the TIMP-2 receptor, the active form of MT1-MMP (Strongin *et al.*, 1995). This interaction occurs via the free N-domain of TIMP-2 and the active site domain of MT1-MMP. It has been shown by x-ray crystallography that the N-terminal cysteine of TIMP-2 co-ordinates with the catalytic zinc in the active site (Fernandez-Catalan *et al.*, 1998). This tri-molecular complex is then poised to activate the latent enzyme; however, this

requires another active molecule of MT1-MMP since the 'receptor' MT1-MMP has been inactivated by interaction with the TIMP-2. A fourth molecule must then assemble in this multi-protein complex to initiate the first cleavage in the pro-peptide region of the gelatinase A zymogen.

Although the four-protein complex is now accepted as the physiological mechanism of gelatinase A activation, important questions are still unresolved. How is the gelatinase released from the complex? Does the auto-proteolytic event also occur at the cell surface? Does fully active gelatinase A remain in complex with TIMP-2? These are very important considerations in the biology of gelatinase A, but are tangential to the work of this thesis and as such will only be considered briefly in the action of gelatinase A on substrates within the extracellular matrix. It is important to note the key role of the protein/protein interactions between discrete domains of these molecules in the activation cascade.

Role of MMP catalytic and exosite domains in proteolytic activity

The catalytic activity of MMPs is controlled by two very important structural elements: 1] the architecture of the active site cleft that dictates both the binding efficiency of the main chain of the substrate within the groove and the specificity of the scissile bond by subsite pockets that surround the catalytic zinc ion; and 2] substrate binding sites outside the active site groove termed exosites (Overall,

2000).

Active site

As noted above, the catalytic zinc ion is co-ordinated by three histidines in the metzincin signature motif HExxHxxGxxH. Proteolysis is initiated by nucleophilic attack by a polarized water molecule on the carbonyl of the substrate scissile bond. An important molecular determinant of substrate specificity is the different amino acid residues which make up the active site subsites (Kiyama *et al.*, 1999). Perhaps the most critical residue in the substrate is that of the P_1' immediately after the cleavage site. The active site subsite S_1' is responsible for accommodating this residue. Hence, the size and chemical properties of the MMP S_1' subsite residue are crucial in determining peptide bond preference for cleavage.

Exosites

Also contributing to substrate cleavage are additional substrate binding sites on discrete domains or smaller functional modules located away from the active site of the enzyme named exosites (Overall, 2000). In this first instance, exosites can increase the affinity of the binding interaction between the substrate and enzyme. Additional binding sites would increase the chance of a productive interaction. In its simplest description exosites favor substrate binding, which will increase the

local concentration, decreasing the association constant K_m, thereby improving the kinetics of the reaction.

Exosites have the ability to not only modulate but also broaden the substrate specificity profile of the enzyme by providing additional contact surfaces not influenced by the active site subsites. Molecules bound by exosites will have the potential of being cleaved by being brought in proximity to the catalytic site. This will serve to extend the spectrum of molecules that an MMP will degrade. In this way the function of the proteinase is refined and can be made more specific and efficient (Overall, 2000).

In addition to tethering substrates to the enzyme that potentiates cleavage, exosites may be involved in the preparation of the substrate prior to cleavage. Many components of the extracellular matrix, most notably collagen, are known to be substrates for the MMP family, and are resistant to proteolysis due to their rigid 3-dimensional quaternary structure. It is unavoidable that a substrate that is too large to fit into the active site groove must be somehow 'prepared' for cleavage, as a separate yet important aspect of proteolysis. A perfect example of this is the degradation of native collagen. Fibrillar collagen adapts a rigid triple helical coil that is approximately 15 angstroms in diameter. The width of the active site of the enzyme is approximately 5 angstroms. It is apparent that the collagen coil must be opened or 'melted' so the substrate can enter the active site for cleavage. This triple helicase activity of collagenases has

been shown to be dependent on exosites on the hemopexin C domain (Windsor *et al.*, 1991; Murphy *et al.*, 1992) and a loop outside the active site groove, as deletion-mutant enzymes cannot cleave native collagen. These mutant enzymes are catalytically competent on small peptide analogue substrates that do not require this type of substrate presentation into the active site (Chung *et al.*, 2000). Thus, exosites outside the active site provide additional important properties to the enzyme and greatly influence catalytic outcomes.

MMPs in biology

MMPs are a family of extracellular endoproteinases that together can degrade most components of the extracellular matrix. Accordingly, they are implicated in most processes that involve matrix degradation. The action of the MMP family has always been considered a "double-edged sword". During normal phases of development the extracellular matrix must be remodeled in a tightly regulated manner; however, during certain disease processes matrix destruction can be both a cause and an effect. Importantly, the activity of the MMP family is implicated in both the beneficial and detrimental aspects of cellular biology. To understand the cellular and tissue functions of MMPs, especially where aberrant or uncontrolled protease activity leads to disease, the substrate repertoire must be elucidated. Once the biological substrates are identified rational drug therapeutics can be designed for the intervention of disease progression.

MMPs in physiology

During many normal developmental processes the action of MMPs is required. These can be roughly categorized into the areas of reproduction, development, and maintenance. A well-studied example of this is the process of human reproduction (Fata *et al.*, 2000). Specifically, during postpartum uterine involution co-ordinated MMP expression, notably matrilysin, appears important for regulated remodeling to occur (Rudolph-Owen *et al.*, 1997). MMP expression and activity patterns are also linked to alterations of the mammary gland structure during normal development (Rudolph-Owen and Matrisian, 1998; Benaud *et al.*, 1998 Schedin *et al.*, 2000). Indeed, overexpression of stromelysin-1 can result in an epithelial to mesenchymal transition leading to tumor formation (Sternlicht *et al.*, 1999). Bone remodeling events which involve both degradation and remodeling of the matrices require regulated proteolysis including the cathepsins and MMPs (Uusitalo *et al.*, 2000).

MMPs in pathology

In addition to the important role MMPs play in the natural development of the organism, MMPs are also implicated in many pathological conditions. These include arthritis (Vincenti *et al.*, 1994; Malemud *et al.*, 1999), periodontitis (Ryan *et al.*, 1996), various lung (Corbe *et al.*, 2000; Parks and Shapiro, 2001),

neurological (Rosenberg, 1995) and cardiovascular diseases (Li *et al.*, 2000). Disturbances in connective tissue homeostasis that can determine many disease outcomes can be attributed to de-regulation of MMP activity resulting in loss of normal tissue architecture. This can involve either dissolution of the supportive matrix or excessive matrix production leading to fibrosis.

Notably, the degree of tumor metastasis is a very important role attributed to the MMP family. The process of tumorigenesis and the important role of MMP activity is somewhat tangential to this thesis but merits brief consideration as it is this connection which has driven much research into MMP activity and the design of general MMP inhibitors as therapeutic agents.

Most human cancers are correlated with excessively high levels of MMP expression. In particular, MMP over-expression is associated with aggressive tumor growth and an increase in the likelihood of metastasis (McCawley and Matrisian, 2000) which is the most deadly aspect of this disease. In order for a tumor to grow beyond approximately 2 cm in size nutrients must be delivered (Werb *et al.*, 1999). This requires the establishment of a circulation, a process referred to as angiogenesis. Unlike normal cells, many tumor cells constitutively express MMPs or have reduced TIMP expression (Stetler-Stevenson *et al.*, 1993). Indeed, gelatinase A knock-out mice show a significant reduction in experimental metastasis (Itoh *et al.*, 1998). The gelatinase A activator MT1-MMP is overexpressed in HT-1080 fibrosarcomas (Sato and Seiki, 1996) and is

associated with highly invasive breast cancer cell lines (Gilles *et al.*, 1997; Pulyaeva *et al.*, 1997). Accordingly, the activity of the MMP family has focused interest in the biopharmaceutical industry as important therapeutic drug targets in the treatment of various cancers. Indeed, in the United States alone there are over 200 patented synthetic MMP inhibitors (Woessner and Nagase, 2000).

Another key role for MMPs in the survival of an organism is during wound healing (Pilcher *et al.*, 1999). Following injury a complex series of events are initiated that generally results in an inflammatory reaction. During the inflammatory response many different proteases are involved in the healing process (Parks, 1999). Particularly required are the matrix metalloproteinases, as the remodeling and regeneration of the extracellular matrix is fundamental in the resolution of the host response and repair of the wound.

Inflammation

General consideration

The inflammatory response is a very general response to any non-host substance or tissue trauma. To inflame is defined as to "set afire" and is an appropriate term to describe the physiological response of the body when defending against the multitude of challenges from the external environment. The cardinal signs of inflammation are redness, heat, swelling, and pain (rubor, calor, tumor, dolor). A distinction is generally made between acute and chronic

inflammation, although many of the mechanisms are shared.

Acute inflammation

Acute refers to an abrupt and severe inflammatory reaction that is generally short in duration. It is associated with a response involving an exudative reaction that allows for the accumulation of serum proteins due to vascular permeability. The contents of the exudate include clotting factors, complement, and antibodies. Following the initial inflammatory exudate is an active directed transmigration of inflammatory cells to the site of challenge—first polymorphonuclear neutrophils (PMNs), then mononuclear cells (predominately macrophages). In addition to bacterial cell wall products, degraded extracellular matrix proteins, and some activated complement proteins, chemokines are critical chemoattractants in establishing a productive infiltration of effector cells. Additionally, to migrate through the extracellular matrix to the site of injury the inflammatory cells secrete MMPs, notably either neutrophil collagenase (MMP-8 by PMNs), or interstitial collagenase (MMP-1 by macrophages) to aid in the dissolution of the supportive matrix. The main role of the PMNs and macrophages in inflammation is for the phagocytosis of the foreign material. To aid in phagocytosis, opsonins in the exudate coat the foreign agent that initiated the inflammatory response. Opsonization is also the recognition signal for the PMNs and macrophages for engulfment and phagocytosis.

The duration of the vascular permeability and time taken to resolve the inflammation is dependent on a number of factors. They include histamines, various mediators derived from arachidonic acid, and the complement system. Clearance of the remaining fluid is accomplished through the lymphatic system that can deliver the foreign antigens to the lymph nodes to initiate and strengthen immunity.

Chronic inflammation

Diseases such as rheumatoid arthritis, tuberculosis, periodontitis, and leprosy are the result of chronic inflammatory reactions. While there is no clear dividing line between acute and chronic reactions a fundamental mechanistic difference can be defined. Acute inflammation involves an exudative process and chronic inflammation can be characterized as a proliferative process, where there is a proliferation of fibroblasts and vascular endothelium, in addition to an unrelenting influx or infiltration of inflammatory cells. Specifically, an accumulation of neutrophils that release many proteolytic enzymes, most importantly MMPs and serine proteases (neutrophil elastase, cathepsin G), are involved in the tissue disruption and degradation associated with many chronic inflammatory diseases (Yager and Nwomeh, 1999). Acute responses can transform into a chronic state, if the causal agent is not removed in due time. Indeed, chronic inflammation is often caused by ineffective or inappropriate repair mechanisms and has been

termed "frustrated repair". This results in a complete loss of proper tissue architecture due to imbalances in matrix deposition and processing, which often leads to fibrosis.

Chronic inflammation can be blamed on a prolonged infiltration and accumulation of effector cells at the site of injury, likely also the result of inappropriate chemotactic signals. Based on the experimental work in this thesis, it is our hypothesis that MMPs play an important regulatory role during inflammation with a functional interplay with the chemokine family.

Chemokines

General characteristics

Before considering the details of the chemokine families, it is important to distinguish between inflammatory and homing chemokines. Homing chemokines are constitutively expressed and are responsible for a number of homeostatic activities including maturation and homing of lymphocytes. For example, stromal derived factor (SDF)-1 is a homing chemokine implicated in hematopoietic cell regulation (Maekawa and Ishii, 2000). Indeed, mice lacking the SDF-1 gene are impaired in lymphopoiesis and have low numbers of myeloid bone-marrow precursor cells (Nagasawa *et al.*, 1996). This indicates that SDF-1 attracts progenitor B-cells into the microenvironment of stromal cells and may be required for morphogenesis as SDF-1 is constitutively expressed by many tissues.

Inflammatory chemokines are produced by most tissues and leukocytes in response to the presence of inflammatory cytokines that are induced during pathological conditions. These chemokines are important participants in the inflammatory reaction (Feng, 2000). The accumulation and infiltration of effector cells from the blood stream is a hallmark of inflammation. This movement of cells through the blood vessel walls and through the extracellular matrix towards the site of injury involves adherence and directed migration of the inflammatory cells. The small molecule proteins that contribute towards this process were originally referred to as chemotactic cytokines. Since the discovery of the prototypical chemoattractant, interleukin (IL)-8 this family of molecules has been termed the chemokines. Even though the chemokine family in humans has grown to include upwards of 50 proteins the biological activities that defined IL-8 are more-or-less applicable to the entire family (Baggiolini *et al.*, 1995). In a very general sense chemokines have three important activities. Through activation of leukocyte integrins chemokines initiate adhesion of leukocytes to the vascular endothelium (Luscinskas *et al.*, 2000). Chemokines direct the active migration of leukocytes through the endothelial junctions and extracellular milieu to the site of injury (Howard *et al.*, 1996). Lastly, chemokines are activators of the effector cells initiating the release of oxygen intermediate free radicals and degradative enzymes that contribute towards the host response to injury (Furie and Randolph, 1995).

Chemokine families

Chemokines can be divided into different families based on the positioning and number of conserved cysteine amino acid residues that form essential disulphide bonds. Starting from the N-terminus, the first occurring cysteine residue crosslinks with the third and the second with the fourth. They are named accordingly, C, C-C, C-X-C, and C-X_3-C chemokines depending on the spacing of the first and second cysteine residues. The chemokines are all thought to share a similar structural motif (Clark-Lewis, 1995, see Figure 1.2) based on the structures of at least 15 proteins. A flexible N-terminal segment before the first cysteine is disulphide-bridged to a central backbone of 3 β-strands and loops, crosslinked by cysteines two and four. At the C-terminus is a α-helix terminated by a flexible C-terminal strand. All chemokines exert their cellular effects through binding and activation of heptahelical receptors that are coupled to GTP-binding proteins (Rossi, 2000). Fourteen receptors have been identified for the 50 or so ligands (see Table 1.1). The receptors are named according to their ligand specificity; eight C-C receptors (CCR-1-8), five C-X-C receptors (CXCR-1-5) and one C-X3-C receptor (CX3CR-1) have been characterized. Receptor binding and activation requires two important regions of the chemokine. An exposed loop of the structured backbone is thought to be responsible for initial receptor recognition and interaction. From the initial contact, this allows for

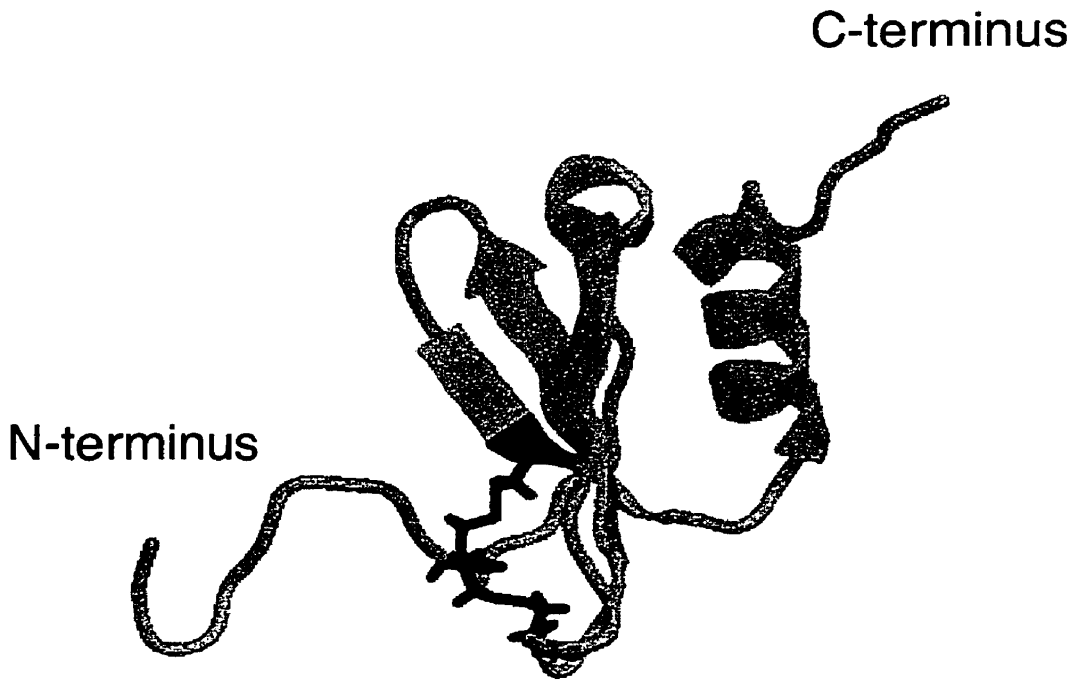


Figure 1.2 Solution structure of MCP-3.

The structure of MCP-3 as determined by nuclear magnetic resonance (NMR) spectroscopy is shown (Kim *et al.*, 1996). MCP-3 adopts the typical alpha-beta fold as determined for the structures of 15 other chemokines. The 20 residues of the flexible N-terminus are critical for both chemokine receptor binding and activation (Clark-Lewis *et al.*, 1995). The structured core is thought to provide a scaffold for these sites of interaction. The disulphide bonds are shown in black.

Chemokine Receptor	Chemokine Ligand	Cell target
CCR-1	MCP-3, RANTES, MIP-1α	Mø, T, NK, PMN
CCR-2A/B	MCP-1, -2, -3, -4	Mø, T, NK
CCR-3	Eotaxin-1, -2, -3, MCP-3, RANTES	Eo, Ba, T
CCR-4	TARC, MDC	T, NK, DC
CCR-5	MIP-1α/β, RANTES	Mø, T, DC
CCR-6	MIP-3a/LARC	T, DC
CCR-7	ELC/MIP-3β	Mø, T, DC
CCR-8	I309/TCA, TARC	Mø, T
CXCR-1	IL-8, GCP-2	PMN
CXCR-2	IL-8, GRO, NAP-2	PMN
CXCR-3	IP-10, MIG, ITAC	T
CXCR-4	SDF-1α/β	almost all
CXCR-5	BCA-1	B
CX3CR-1	Fractalkine	Mø, NK, T

Table1.1 Robustness of the chemokine system.

The redundancy in both cell receptor usage of the chemokines, and cell receptor expression on the various effector cells is shown. MCP-monocyte chemoattractant protein; RANTES-regulated upon activation, normal T cell expressed and secreted; MIP-macrophage inflammatory protein; TARC-thymus and activation-regulated chemokine; MDC-macrophage-derived chemokine; LARC-liver and acitvation-regulated chemokine; ELC-EBV ligand chemokine; TCA-T cell activation gene; GCP-granulocyte chemotactic protein; IL-interleukin; GRO-growth-related oncogene; NAP-neutrophil-activating peptide; IP-interferon-inducible protein; MIG-monokine induced by interferon; ITAC-interferon-inducible T cell alpha chemoattractant; SDF-stromal cell-derived factor; BCA-B cell-attracting chemokine. Mø-macrophage; T-T cell; NK-natural killer; PMN-polymorphonuclear; Eo-eosinophil; Ba-basophil; DC-dendritic cell; B-B cell.

the proper presentation of the flexible N-terminus to activate or trigger the receptor. Many mutational analyses have studies characterized synthetic and natural analogues of chemokines that are still able to bind the receptor but are lacking this triggering capacity (Wells *et al.*, 1996).

Chemokine receptor signaling

The different intracellular signaling events initiated upon chemokine-ligand binding are dependent on *Bordetalla pertussis* toxin-sensitive heterotrimeric guanosine triphosphate (GTP)-binding proteins. Following chemokine binding the G-proteins associate with the receptor resulting in the exchange of guanosine triphosphate (GDP) for GTP. This activation initiates a classical signaling pathway of G-protein-linked cell-surface receptors (Alberts *et al.*, 1994). The G-proteins dissociate and activate membrane-associated phospholipase C (PLC). The action of PLC creates the second messengers phosphatidylinositol 1, 4, 5-bisphosphate (IP_3) and diacyl-glycerol (DAG). This leads to mobilization of internal calcium stores and activation of protein phosphorylation cascades. In addition, chemokine receptors are known to activate tyrosine kinases (Mellado *et al.*, 1998) and mitogen-activated protein kinase pathways (Kampten *et al.*, 1998). Following ligand-induced activation the receptor becomes desensitized to repeated stimulation by agonists by phosphorylation of the cytoplasmic region and rapid internalization. This desensitization is considered crucial in allowing

the cell the continuing ability to respond to and sense the chemoattractant gradient (Murphy, 1996).

Redundancy

An interesting feature of the chemokine and chemokine receptor system is the redundancy in both number and action (see Table 1.1). Most chemokines bind and activate more than one cognate chemokine receptor. Adding to the redundancy, most leukocytes express a variety of chemokine receptors. The result of this versatility is that cellular responses to injury will be robust, regardless of the precise expression patterns and levels of chemokines produced (Mantovani, 1999). This is best exemplified by the production of various chemokine and chemokine receptor gene disruptions in mice. Disruptions in CCR-1, -2, -5, and CXCR-1, and -2 have minimal effects on the growth and development of the organism. Certain alterations in cell-specific recruitment are observable (Bush *et al.*, 2000; Blease *et al.*, 2000; Dawson *et al.*, 2000), however the redundancy of the chemokine system guarantees a sufficient fidelity for the normal development of the organism.

Chemokine antagonists

The discovery in 1996 that certain chemokine receptors, in conjunction with the T-cell clusters of differentiation (CD)-4 antigen, act as recognition and entry

points for the human immunodeficiency virus (HIV) sparked incredible interest in the chemokine field (Bleul *et al.*, 1996; Oberlin *et al.*, 1996). It was observed that chemokines, notably stromal-derived factor (SDF)-1α, and chemokine antagonists could prevent HIV infection. The interconnection between HIV and chemokines has resulted in much focus on chemokines as potential therapeutic targets for HIV. That chemokine agonists and antagonists are important during inflammation has been the other major focus in the chemokine field (Baggiolini, 1998). As discussed above, the N-terminal region of chemokines is essential in receptor activation. This led to studies characterizing the activity of various synthetic chemokine antagonists. It has been established that for the most part minimal modification of the N-terminus of RANTES, MCP-1, MCP-3, and others (Proost *et al.*, 1998; Gong *et al.*, 1996) results in the creation of effective receptor antagonists.

Interestingly, chemokine analogues have also been isolated from cell cultures following treatment with various cytokines that models an inflammatory reaction. Monocyte chemoattractant protein -1 and -2, and RANTES have been isolated with truncations at both the N-terminus and C-terminus (Proost *et al.*, 1998). The majority of natural chemokine truncations are anywhere from 1-7 residues removed from the flexible termini. It is established that the serine protease CD 26 (dipeptidyl peptidase IV) can remove the first two residues of various proteins when proline is the penultimate residue. The biological

implications of this processing is not clear; however, CD 26 cleavage does alter the bioactivity of its substrates and could be an important mechanism in HIV infection (Ohtsuki *et al.*, 2000).

Relevance

The data in this thesis details our identification of the MCP chemokine family as novel and biologically relevant substrates of the MMP family. MMP proteolysis results in N-terminal truncations (generally a tetra-peptide) of the MCPs. In addition, we detail the importance of gelatinase A hemopexin C domain exosites in the processing of the chemokine substrates.

Novel targets of MMP activity

Historically, the MMP family has been considered responsible for the degradation of the structural components of the extracellular matrix. While it is clear that matrix components do comprise a major class of substrates many recent reports have characterized novel cell bound and soluble extracellular substrates. Within this new repertoire of substrates are the membrane-bound precursor form of tumor necrosis factor (TNF)-α (Gearing *et al.*, 1994), the ectodomain of the fibroblast growth factor receptor-1 (Levi *et al.*, 1996), heparin-binding epidermal growth factor-like growth factor (Suzuki *et al.*, 1997), and various soluble factors including IL-1β (Ito *et al.*, 1996), insulin-like growth factor binding protein (IGFBP)

-3 (Fowlkes *et al.*, 1994), -5 (Thrailkill *et al.*, 1995), and the blood clotting factors fibrinogen and factor XII (Hiller *et al.*, 2000). Interestingly, the action of MMPs on the cell-surface bound substrates serves to enhance or activate the activity upon release from the membrane whereas the cleavage/degradation of the soluble factors results predominantly in the inactivation of action. This new substrate repertoire of MMPs places their activity in processes where modulation of the biological effects of these molecules is key in both physiological and pathological events. The concept of MMP proteolysis as a processing activity, as opposed to degradation, is an important emerging concept of MMP biology and will likely open new avenues of both basic research and therapeutic intervention of the MMP family of proteinases. However, to identify new substrates, particularly those present in low amounts, novel strategies need to be adopted beyond the serial approaches of routine degradation assays.

Thesis hypothesis and experimental design

It was our hypothesis that the gelatinase A hemopexin C domain contains protein-binding exosites that contribute to the substrate profile of the enzyme. These potential binding interactions can bring proteins in close proximity to the catalytic center of the enzyme. To test this hypothesis we set out to identify proteins that bind to the isolated hemopexin C domain. There are several screening technologies available which attempt to identify putative protein-protein

interactions. We used the yeast two-hybrid system to identify protein partners for the gelatinase A hemopexin C domain. While the yeast two-hybrid system has been a successful methodology for the identification of intracellular protein-protein interactions, it has been used far less frequently for the identification of extracellular protein interactions. Presumably this is due to the presence of disulphide crosslinks in extracellular proteins which would likely not form in the reducing environment of the yeast nucleus. In addition, there were no examples in the literature of identifying protease substrates using a protein domain outside of the active site. We saw this as an excellent opportunity to use the well-established methodology in a completely novel way. We were able to demonstrate the utility of this system with domains of extracellular proteins. Specifically, Chapter 3 details the initial characterization of a novel gelatinase A substrate, monocyte chemoattractant protein (MCP)-3, and the important implications of this in inflammation. Chapter 4 presents data describing the importance of the gelatinase A hemopexin C domain in the catalysis of MCP-3 and Chapter 5 presents initial evidence that MMP processing of chemokines is a new paradigm in chemokine and MMP biology in the regulation of inflammatory responses.

Chapter 2 General Methods

RNA isolation

Human gingival fibroblasts were used to generate a cDNA library for the yeast two-hybrid system. Cell cultures were grown to 80% confluence, followed by treatment with 25 µg/ml Concanavalin (Con) A for 24 hours. Cell layers were harvested and RNA was isolated by lithium chloride extraction (Overall and Sodek, 1990). Briefly, cells were collected by centrifugation and washed in diethyl pyrocarbonate (DEPC)-treated sterile water. Cells were lysed in extraction buffer (6 M urea, 3 M LiCl, 10 mM sodium acetate, 10 mM Tris, pH 8.0) and collected by centrifugation. The precipitant (RNA fraction) was collected and resuspended in 0.1 M sodium acetate, 0.5% SDS. The RNA pellet was washed and separated from protein fraction by phenol extraction and isolated by ethanol precipitation. The resultant RNA was dissolved in distilled water and quantified by ultraviolet absorbance spectroscopy (where 40 µg/ml RNA has an optical density of 1 at 260 nm) and stored at –70 °C until use.

cDNA synthesis

Total RNA was applied to a poly-T Sepharose resin, the column was washed with wash buffer 1 (0.5 M LiCl, 10 mM Tris, 1 mM EDTA, 0.1% SDS, pH 7.5) then wash buffer 2 (0.15 M LiCl, 10 mM Tris, 1 mM EDTA, 0.1% SDS, pH 7.5). The mRNA was eluted with elution buffer (2 mM EDTA, 0.1% SDS). The mRNA was

quantified by ultraviolet absorbance spectroscopy as above. cDNA synthesis was performed using a commercially available kit, Timesaver (Pharmacia Biotech, U.S.A) following the manufacturer's instructions. Briefly, an RNA:cDNA duplex was synthesized from the mRNA template. Double-stranded cDNA fragments were then synthesized from the hybrid template. In order to clone these fragments into the yeast two-hybrid vector, short Eco R1/Not 1 DNA adapters were ligated onto the ends of the pool of cDNA fragments. This was done by incubation with 1 U of T4 DNA ligase (New England Biolabs) in a buffer containing 30 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, 10 mM 1, 4-dithiothreitol (DTT), 1 mM adenosine 5'-triphosphate (ATP) according to manufacturer's instructions.

Library construction, isolation and purification

The cDNA fragments with EcoR1 adapters (from above) were ligated using T4 DNA ligase into the yeast two-hybrid Gal4-DNA binding domain vector pGAD424 (Clontech). The ligation products were introduced into library-efficiency DH5-α *E. coli*. cells (Gibco-BRL) by electroporation (Bio-Rad, Gene-Pulser). Following electroporation, cells were plated onto 100-150 cm^2 petri dishes containing Luria-Bertani (LB)-agarose + ampicillin (100 μg/ml) medium and incubated at 37 °C for 24 h. The colonies from plates were scraped into LB + ampicillin liquid cultures and incubated with agitation at 37 °C for an additional 24 h. The cells were

collected by centrifugation, and washed with phosphate-buffered saline (PBS). The library plasmid DNA was isolated using a commercially available "maxi-prep" kit (Qiagen, U.S.A.) according to manufacturer's instructions. Briefly, the cell pellets were suspended in lysis buffer, then a differential precipitation buffer, and the cellular debris was removed by centrifugation. The supernatant was applied to a DNA binding column, washed, and eluted into TE (10 mM Tris-HCl, 1 mM ethylenediamine tetraacetate) buffer and stored at -70 °C until use.

Gelatinase A hemopexin C domain—Gal4 DNA-binding domain plasmid construction

Human cDNA, corresponding to the gelatinase A hemopexin C domain was amplified by the polymerase chain reaction (PCR). PCR reactions were assembled in a 100 µl volume containing 1 mM in each of the four deoxyribonucleotide triphosphates, 0.1 µg cDNA template, 100 pmoles each of 5' primer—5'-CCGAATTCGGGGCCTCCCCCTGACATT-3' and 3' primer—5'-GCACGTCGACTCAGCAGCCTAGCCAGTC-3'. The PCR reaction was done in a Perkin Elmer Gene Amp 2400 thermocycler for 30 cycles consisting of: (1) denaturation at 94 °C for 30 s, (2) annealing at 55 °C for 10 s, (3) extension at 72 °C for 60 s. The PCR amplification product was purified by agarose gel electrophoresis purification (Qiagen) and was treated with the restriction endo-nucleases EcoR1 and Sal1. The fragment was ligated into the Gal4-DNA binding

domain vector pGBT9, and sequenced to confirm the DNA fidelity. DNA was amplified and purified by a "maxi-prep" kit (Qiagen) as above.

Yeast transformation

Large-scale yeast transformations were performed according to manufacturer's instructions (Clontech, U.S.A.). Briefly, yeast cells (strain HF7c) were grown to mid-log phase in liquid culture (yeast peptone dextrose, YPD-20 g/l peptone, 10 g/l yeast extract, 2% dextrose). The cells were collected by centrifugation and washed with sterile water. The cells were then resuspended into a PEG/lithium acetate buffer (40 % polyethylene glycol, 100 mM lithium acetate). Library plasmid DNA, 'bait' plasmid DNA, and salmon sperm DNA (used as a carrier) were added to the yeast suspension. The solution was placed into a shaker at 37 °C for 30 min. The yeast suspension was then adjusted to 10% DMSO. The transformation mixture was then incubated at 42 °C for 15 min. Yeast cells were collected by centrifugation, resuspended in TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5) and plated onto synthetic deficient (SD, 6.7 g/l yeast nitrogen base without amino acids) medium lacking the amino acids tryptophan (trp) and leucine (leu) (SD-trp$^-$leu$^-$, to determine efficiency). The yeast cells were also plated onto and SD-trp$^-$leu$^-$his$^-$ (also lacking the amino acid histidine, to isolate yeast cells expressing interacting proteins) and incubated at 30 °C for 5-7 days.

Colony selection and characterization

Following incubation, robust individual yeast colonies were picked off the primary transformation plates and re-streaked onto SD-trp$^-$leu$^-$his$^-$ plates and incubated at 30 °C for 3 days. Individual colonies were tested for activation of the LacZ reporter gene by a colony liquid β-galactosidase assay. Yeast cells were grown to mid-log phase in liquid SD-trp$^-$leu$^-$his$^-$ medium, cells were harvested, and protein extracts were released from the yeast cell by freezing under liquid nitrogen. These crude protein preparations were quantified for β-galactosidase activity by incubating in Z-buffer (50 mM sodium phosphate, 10 mM KCl, 1 mM $MgSO_4$, pH 7.0) with colour-producing reagent (O-nitrophenyl β-D-galactopyranoside) as per manufacturer's instructions (Clontech).

Plasmid isolation

The yeast colonies that exhibited robust growth on selective medium, and displayed significant β-galactosidase activity (relative to manufacturer's positive controls, p53/SV40 T antigen, Clontech, U.S.A.) were selected for isolation of plasmid DNA encoding the library cDNA. Briefly, yeast cells were grown for 16 h at 30 °C and harvested by centrifugation. The cell pellets were resuspended in yeast lysis solution (2% TX-100, 1% SDS, 100 mM NaCl, 10 mM Tris, 1.0 mM EDTA, pH 8.0), phenol:chloroform:isoamylalcohol (25:24:1 solution) and acid-washed glass beads, and the mixture was vortexed vigorously for 5 min.

Samples were centrifuged to separate phases, and the aqueous phase was collected. Nucleic acid was precipitated by the addition of 0.8 volumes of isopropyl alcohol and centrifugation. The DNA pellets were dried under a vacuum, and resuspended into TE buffer. Isolated DNA was introduced into the *E. coli* strain DH5-α by electroporation, bacteria were plated onto LB-agar plates containing 100 µg/ml ampicillin, and plates were incubated for 12 h at 37 °C. Individual colonies were selected for growth in liquid culture and plasmid DNA isolation using a commercially available kit (mini-prep, Qiagen). The isolated plasmid DNA was characterized as either 'bait' plasmid or library plasmid by restriction endonuclease digestion.

Confirmation of interacting cDNA

Isolated library plasmids were then re-transfected into the yeast strain HF7c alone, or in combination with the gelatinase A hemopexin C domain bait plasmid. Library plasmids that only supported growth on SD-his$^-$ plates in conjunction with the bait plasmid were selected for further characterization by sequence analysis of the cDNA encoding the interacting protein.

DNA sequence analysis and identification of cDNA encoding potential interactions

Sequence analysis of isolated plasmids was done by PCR using the Perkin

Elmer Applied Biosystems Big Dye Terminator reaction mix. Briefly, 8.0 µl terminator ready reaction mix was added to 100 ng DNA template, 3.2 pmol each 5' and 3' library plasmid-specific primers, in a total volume of 20 µl. The PCR cycles (25) consisted of the following three steps: (1) denaturation at 96 °C for 10 s, (2) annealing at 50 °C for 5 s, (3) extension at 60 °C for 4 minutes. The reaction products were purified according to manufacturer's instructions and were analyzed on an automated sequencer (NAPS sequencing unit, University of British Columbia). Derived sequences were identified by world wide web-based BLAST searches.

Protein expression and purification

The human cDNAs corresponding to the gelatinase A hemopexin C domain (done by Eric Tam), collagen binding domain (done by Bjorn Steffensen) and the MT1-MMP hemopexin C domain (done by Eric Tam) were amplified by PCR from full-length cDNA clones. The amplified DNA was sub-cloned in the expression vector pGYMX (which encodes for an N-terminal His_6-tag) and DNA sequences were verified by PCR as detailed above. The expression plasmids were transformed *E. coli* strain DH5-α and proteins expressed by growing bacteria in liquid culture for 24 h at 37 °C. The cell pellets were collected by centrifugation, and bacterial inclusion bodies were isolated by sonication. The precipitated proteins were recovered from inclusion bodies by extraction with 6 M GuHCl in

PBS. The solubilized proteins were re-folded by step-wise dialysis into PBS with 150 mM NaCl. The re-folded proteins were applied to a Ni-Sepharose column (Pharmacia) and eluted by a salt gradient elution (Overall *et al.*, 2000).

General Antibodies

A mouse monoclonal antibody that recognizes human MCP-3 was purchased from Peprotech Laboratories (California, U.S.A.). An antibody that recognizes the Histidine-tag on the expressed proteins was generated in rabbits (done by Yili Wang). The peptide His-His-His-His-His-His-Gly-Gly-Cys was synthesized (NAPS) and conjugated to the immunogenic protein Keyhole Limpet Hemoglutinan (KLH) by disulphide linkages (Wallon and Overall, 1997). This chimera was mixed with Freunds complete adjuvant and injected intramuscularly into white New Zealand rabbits to initiate an immune response. Rabbits were then injected intradermally every 3 weeks with chimera protein mixed with Freunds incomplete adjuvant. When antibody production was judged to be optimum [by enzyme-linked immunosorbent assay (ELISA)-assay of serum against peptide], rabbits were sacrificed for blood harvest. Antibodies were purified from serum by affinity-chromatography. The peptide His-His-His-His-His-His-Gly-Gly-Cys was immobilized onto agarose beads (BioRad). The serum was applied to the resin to allow binding of antibodies. Following extensive washes with PBS, antibodies were eluted with 1.0 M triethanolamine, pH 11.5, and

neutralized by addition of Tris buffer to 100 mM, pH 8.0.

Epitope Antibodies

In order to identify and distinguish the very low *in vivo* quantities of full-length MCP-3 (1-76) and the MMP-cleaved form of MCP-3 (5-76), we designed antibodies that would only recognize either full-length (α-1-76) or the MMP-cleaved form of MCP-3 (α-5-76) using a neo-epitope strategy. To do this, peptides corresponding to the first 5 residues of MCP-3 (NH_2-Gln-Pro-Val-Gly-Ile-Gly-Gly-Cys) and the residues corresponding to the new N-terminus following MMP cleavage (NH_2-Ile-Asn-Thr-Ser-Thr-Gly-Gly-Cys) were synthesized (NAPS) and conjugated to KLH. The antisera were raised in rabbits as detailed above and affinity purification of α-1-76 and α-5-76 was performed against native protein (not the peptides), MCP-3 (1-76) and MCP-3 (5-76) respectively. Since the antibodies will react with the free amino-group at the new terminus, it was necessary to immobilize the proteins to a support resin via the C-terminal carboxyl moiety. This was done using amino-alkyl Affi-gel-102 according to manufacturer's instructions (BioRad, USA). Following immobilization, resin was washed extensively with PBS. In order to increase the overall specificity and reduce cross-reactivity, α-1-76 serum was first applied to the MCP-3 (5-76) resin, and α-5-76 was first applied to the MCP-3 (1-76) resin. Following this pre-adsorption, each serum was then applied to its respective resin. The antibodies

were isolated as described above. This procedure resulted in no cross-reactivity of these affinity-purified antibodies.

ELISA-based binding assay

The chemokines (as described in Figure legends) that were tested for interaction with the gelatinase A hemopexin C domain, MT1-MMP hemopexin C domain, or collagen binding domain were immobilized onto 96-well plates (protein concentration 5 μg/ml) for 16 h at 4 °C. Solutions were removed from wells, and blocking buffer (2.5% BSA) was added and incubated for 1 h at 25 °C, to reduce non-specific binding to the plastic. Following removal of blocking buffer, serial dilution solutions of the hemopexin C domain, MT1-MMP hemopexin C domain or collagen binding domain were added and incubated for 1 h at 25 °C. Following extensive washing (PBS-tween 0.05%), affinity-purified anti-His_6 anti-sera was added at a dilution of 1/500 in PBS-tween for 1 h at 25 °C. Alkaline phosphatase-conjugated secondary antibody (BioRad) was then added at a dilution of 1/1000 in PBS-tween. Alkaline phosphatase levels were quantified by addition of a commercially available p-nitrophenyl substrate (Sigma) on an ELISA plate reader (Molecular Devices) at 405 nm.

Chemical Cross-linking

Purified MCP-3 or gelatinase A hemopexin C domain (in quantities indicated in

Figure legends) were cross-linked in isolation or as mixtures by the addition of 0.5% glutaraldehyde for 20 min at 22 °C. The cross-linking reaction was stopped by addition of an equivalent volume of 2X SDS sample buffer (120 mM Tris-HCl, pH 6.8, 10% glycerol, 3% sodium dodecyl sulfate (SDS) and 0.1% bromophenol blue). The samples were electrophoresed through 15% polyacrylamide gels containing 0.1% SDS in Laemmli's running buffer (25 mM Tris, 192 mM glycine, and 0.1% SDS) (Laemmli, 1970). Following electrophoresis, the gels were stained with silver nitrate as per the manufacturer's protocol (BioRad).

Gelatinase A overlay assay

In order to identify overall binding associations between the chemokine substrate and gelatinase A, we used a modification of the ELISA-binding assay. Briefly, proteins to be tested for gelatinase A binding were immobilized onto 96-well plates as described above. Following a blocking step with 2.5% BSA, a solution of gelatinase A enzyme (either full-length or hemopexin C domain-truncated) was applied to the wells. After 1 h incubation at 25 °C and extensive washing, SDS-PAGE sample buffer was applied to the wells to extract the bound enzyme. The samples were assayed for gelatinase A activity (which correlates with protein binding activity) by gelatin zymography.

Calcium and chemotaxis assays

In order to the determine bioactivity of various chemokines, or derivative chemokines following MMP cleavage, a monocyte-like cell line (THP-1, ATCC) was used in two *in vitro* assays. The first assay monitored the release and increase of free intracellular calcium in response to chemokine receptor binding. To do this, THP-1 cells were loaded with a calcium sensitive fluorescent dye, Fluo-3AM (Molecular Probes, U.S.A.) in modified Guy's buffer (10 mM HEPES, 150 NaCl, 1 mM $MgCl_2$, 5 mM KCl, pH 7.4) with the addition of 0.38 mg/ml pluronic F127 to aid in uptake. Following 3 washes in modified Guy's buffer (without dye and detergent), cells were resuspended in 25 mM HEPES, 150 mM NaCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 3 mM KCl, pH 7.4. Additions of chemokines were as indicated in Figure legends, and fluorescence was monitored with a spectrofluorimeter (Perkin-Elmer 650-10B) using an excitation wavelength of 506 nm and an emission wavelength of 526 nm.

In the second assay (done by Neidy Gong) THP-1 cell trans-migration was assessed in disposable transwell trays (Costar, U.S.A.) with 6.5 mm diameter chambers and a membrane pore size of 3 µm. The chemokines were added to the lower well as indicated in the Figure legends in 600 µl HEPES-buffered RPMI-1640 medium supplemented with 10 mg/ml BSA. The THP-1 cells ($1x10^6$ cells/100 µl) were added to upper well in medium minus chemokine. After 1.5 h, the number of cells that had migrated to the lower well was determined by light

microscopy.

Mouse models of inflammation

In order to assess the *in vivo* bioactivity of the full-length and MMP-cleaved forms of the chemokines, we used a modification of a skin inflammation model procedure described previously (Van Damme *et al.,* 1992; done by Dr. C.A. McCulloch). Briefly, CD-4 mice were injected subcutaneously (as detailed in Figure legends) and killed 18 h after injection. Paraffin sections transverse to the skin were stained with hematoxylin and eosin and examined by light microscopy to enumerate mononuclear infiltration.

To monitor chemokine activity in a separate model of inflammation, we used the irritant zymosan to induce peritonitis (Ajuebor *et al.*, 1999: done by Dr. C.A. McCulloch). Briefly, mice were injected in the intra-peritoneal space with 500 µl PBS +/- 1 mg zymosan. To confirm zymosan-induced peritonitis after 24 h, cells were counted from intra-peritoneal washouts. The control mice had 100,000 cells/ml whereas zymosan-injected mice had 3,900,000 cells/ml, indicating efficient induction of peritonitis. To test for antagonist activity saline or MMP-cleaved MCP-3 (5-76) was injected into the intra-peritoneal space, following 24 h treatment with zymosan, as indicated in Figure legends. Following an additional 24 h incubation, mouse macrophage cell counts were obtained from intra-peritoneal washouts using a monoclonal macrophage specific F4/80

antibody.

The following Chapter represents a manuscript that was published in the journal Science (*Science*, **289**, 1202-1206, 2000). The data incorporated into this paper represents the major discoveries and advances made by the candidate during his Ph.D. studies. Due to space restraints and formatting of the journal, it is written in a very concise style. Much time and effort was made by the candidate to edit versions of the manuscript to the satisfaction of the editor of the journal. As such, the paper has not been re-written for this thesis, but methods that were used in the studies but not described in the manuscript can be found in Chapter 2.

In addition, there are other authors on the manuscript, making it difficult to assess the contribution of the candidate. The candidate, who is first author on the paper, did the following work that makes up the majority of discoveries detailed in the paper:

cDNA library synthesis

Yeast two-hybrid plasmid construction and screening

Plasmid rescue and identification of monocyte chemoattractant protein 3

In vitro analysis of MCP-3 binding and cleavage by gelatinase A

Cell culture experiments

Calcium flux assays (with the assistance of Dr. J.H. Gong)

The candidate wishes to acknowledge the important contributions and

collaborations by the following:

J.H. Gong, who did the chemotaxis and cell binding assays.

E. Tam, for providing purified gelatinase A hemopexin C domain.

C.A.G. McCulloch for *in vivo* analysis of MCP-3 in mouse models of inflammation.

I. Clark-Lewis for providing chemically synthesized chemokines.

C.M. Overall for help in experimental design and important editorial suggestions.

Chapter 3 Inflammation dampened by gelatinase A cleavage of monocyte chemoattractant protein 3.

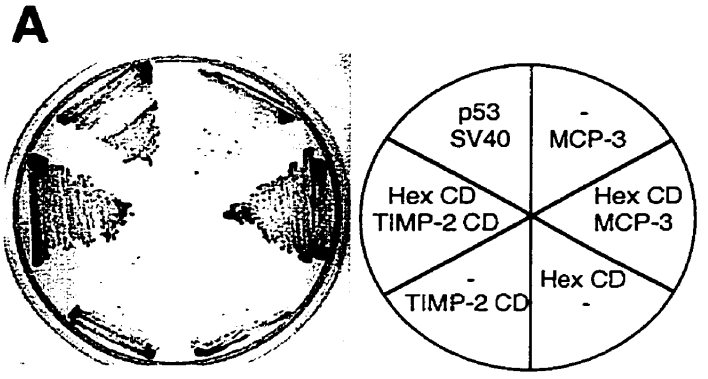
Summary

Tissue degradation by matrix metalloproteinase (MMP)-2 (gelatinase A) contributes to the pathogenesis of inflammation and tumor metastasis. Recognizing the catalytic importance of substrate-binding exosites outside the MMP catalytic domain, we initiated a search for novel MMP substrates using the gelatinase A hemopexin carboxy (C) domain as 'bait' in the yeast two-hybrid system. We report the first use of the two-hybrid system to screen for extracellular proteinase substrates with the discovery that chemokines are a novel class of MMP substrate. Here we show that gelatinase A specifically binds to monocyte chemoattractant protein (MCP)-3 through hemopexin C domain interactions leading to MCP-3 cleavage at Gly^4-Ile^5. By calcium mobilization, chemotaxis, and *in vivo* models of inflammation, we demonstrate that gelatinase A-cleaved MCP-3 is inactive as a chemoattractant. Moreover, the gelatinase A-cleaved MCP-3 is converted to a CC receptor antagonist that regulates the action of related chemokines and mononuclear cell responses. Our results reveal that in addition to MMPs being effectors of the inflammatory response, they can also dampen the course of inflammation by destroying chemotactic gradients and functionally inactivating others. This suggests that MMPs are both effectors and regulators of the inflammatory response.

Degradomics, the identification of biologically relevant substrates for the increasing number of recognized proteinases, is a challenge of proteomics. Although yeast two-hybrid screening (Fields and Song, 1988) has identified intracellular protein-protein interactions, a screening rationale using a proteinase catalytic domain for bait is tenuous because cleavage of library-encoded substrate would preclude detection. Therefore, to identify potential matrix metalloproteinase (MMP) substrates, a substrate-binding exosite domain, the hemopexin carboxyl (C) terminal domain (Gomis-Rüth *et al.*, 1996; Wallon and Overall, 1997) that is structurally and functionally distinct from the catalytic domain, was used in a two-hybrid screen. Collagen binding by this domain of collagenolytic MMPs is a prerequisite for cleavage. Therefore, we hypothesized that other proteins that bind to this domain may also be proteinase substrates.

To determine the suitability of the two-hybrid system for extracellular proteins, interaction between the single disulphide bonded gelatinase A (MMP-2) hemopexin C domain and the C domain of the tissue inhibitor of metalloproteinase (TIMP)-2 which contains 3 disulphide bonds was assessed (Fig. 3.1A). Deletion (Ward *et al.*, 1994) and domain swapping (Nguyen *et al.*, 1994) studies indicate that these domains interact in the cellular activation and localization of gelatinase A to cell surface membrane type (MT)-MMPs (Sato *et al.*, 1994). Association was detected (Fig 3.1A, B), indicating that protein

Figure 3.1 MCP-3 interactions with the gelatinase A hemopexin C domain. **(A)** In the yeast two-hybrid assay only the yeast transformants Hex CD/TIMP-2 C domain, Hex CD/MCP-3, and p53/SV40 (positive control) showed growth on medium lacking histidine. **(B)** β-Galactosidase levels in yeast expressing the indicated fusion proteins. **(C)** Cross-linking of MCP-3 and recombinant hemopexin C domain. MCP-3 alone, or in the presence of 0.5 molar equivalent (+), 1.0 molar equivalent (++), or 2.0 molar equivalents (+++) of hemopexin C domain **(D)** ELISA binding assay of MCP-3 against recombinant gelatinase A hemopexin C domain (Hex CD) or collagen binding domain (CBD).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TIMP-2 CD
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MCP-3
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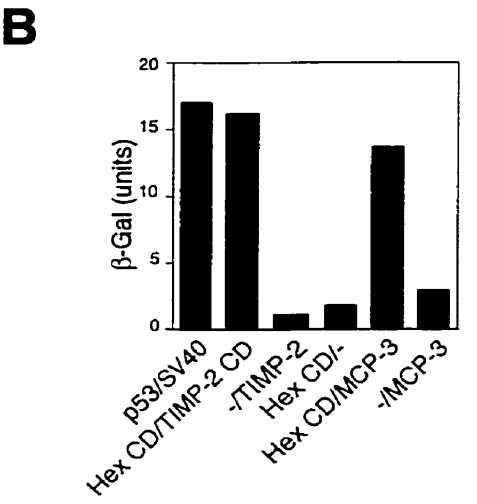
TIMP-2 CD
Hex CD
-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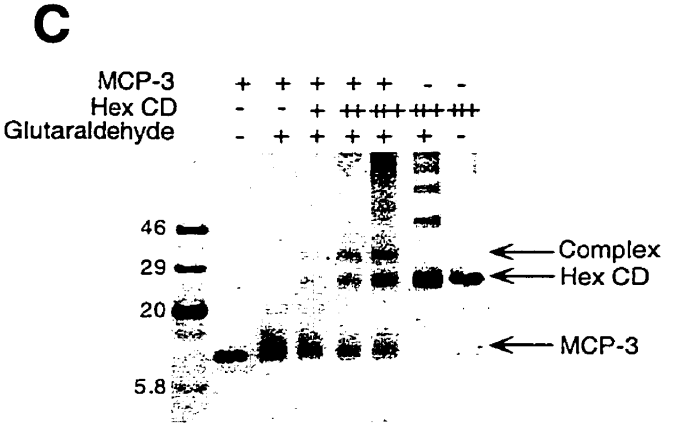
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20
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Complex
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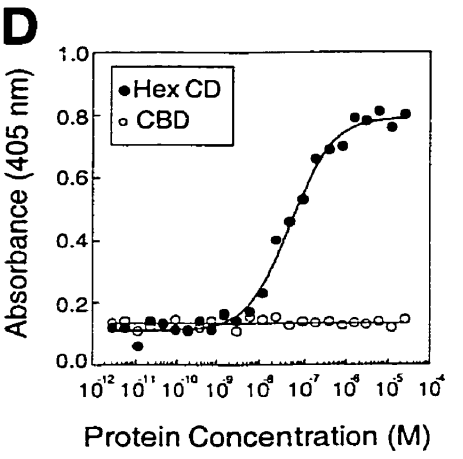
MCP-3


D
Hex CD
CBD
Absorbance (405 nm)
1.0
0.8
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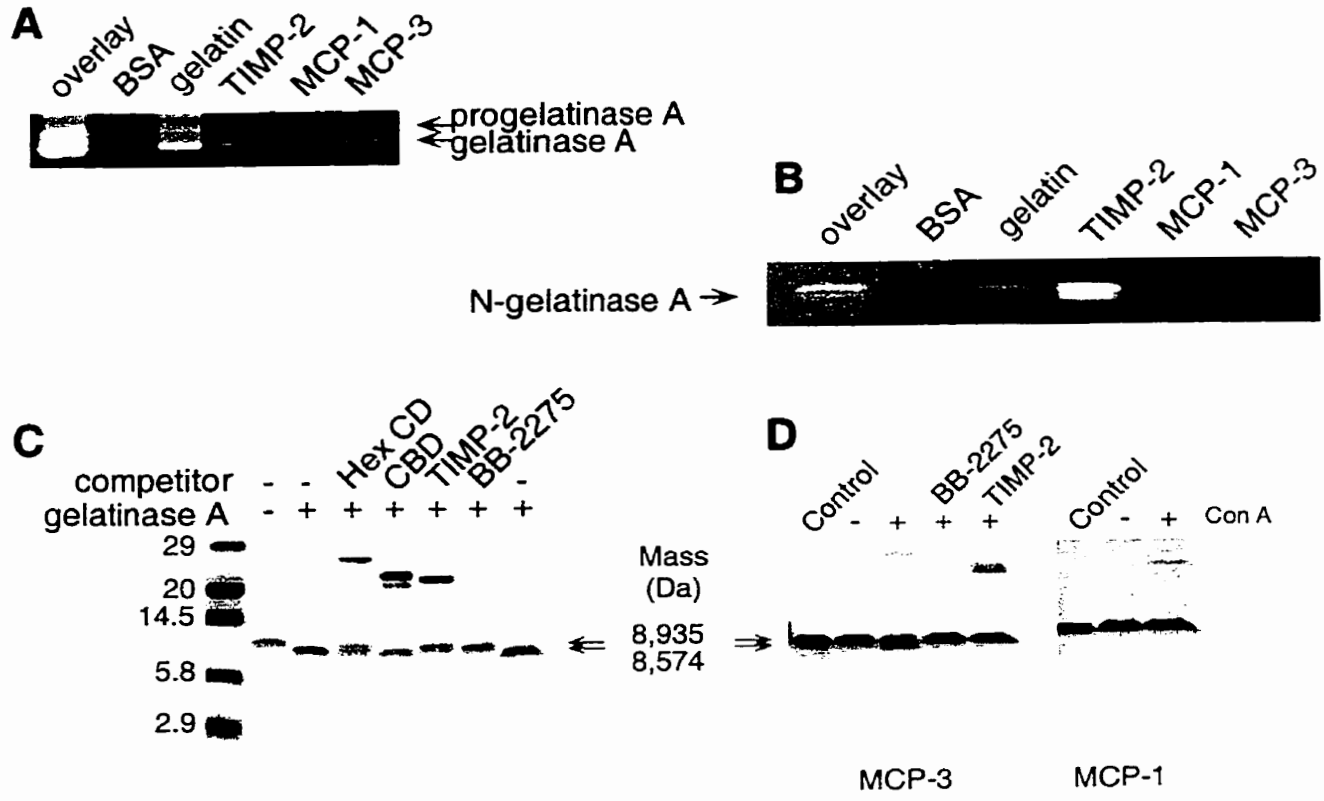
0.4
0.2
0.0
Protein Concentration (M)

expression and folding in yeast at 30 °C generated a stable, functional protein fold in domains that normally contain disulphide bonds.

A cDNA library was constructed from Concanavalin A-treated human gingival fibroblasts, which stimulates extracellular matrix degradation by activation of gelatinase A (Overall and Sodek, 1990). Using the human gelatinase A hemopexin C domain in yeast two-hybrid screens monocyte chemoattractant protein (MCP)-3 was identified as a potential binding protein (Fig. 3.1A, B). The 76-amino acid residue MCP-3 is one of several tissue derived CC-chemokines that recruits monocytes and other leukocytes in inflammation and osteosarcoma (Opdenakker *et al.*, 1993). The hemopexin C domain had a comparable interaction with both MCP-3 and the TIMP-2 C domain (Fig. 3.1B). Chemical cross-linking of synthetic MCP-3 to recombinant hemopexin C domain verified this interaction (Fig. 3.1C). The cross-linked MCP-3-hemopexin C domain had the mass of a 1:1 bimolecular complex, whereas MCP-3 alone was not cross-linked. Furthermore, the hemopexin C domain showed saturable binding to MCP-3 by an ELISA-based binding assay (Fig. 3.1D). Absence of binding by recombinant gelatinase A collagen binding domain protein (Steffensen *et al.*, 1995) comprised of three fibronectin type II modules confirmed specificity. Full-length gelatinase A also bound MCP-3 (Fig. 3.2A), whereas truncated gelatinase A lacking the hemopexin C domain (N-gelatinase A) did not (Fig. 3.2B). No interaction was observed between gelatinase A and the related CC

Figure 3.2 Gelatinase A binding and cleavage of MCP-3. **(A)** Gelatin zymography of enzyme capture film assay of pro and active gelatinase A against bovine serum albumin (BSA), gelatin, TIMP-2, MCP-1, and MCP-3. *Overlay*, recombinant enzyme used. **(B)** Gelatin zymography as in A, but with hemopexin-truncated gelatinase A (N-gelatinase A) used as overlay. **(C)** Tricine gel analysis of MCP-3 cleavage by gelatinase A in the presence of equimolar amounts (relative to MCP-3) of recombinant hemopexin C domain, collagen binding domain, TIMP-2, or 10 μM BB-2275 (British Biotech Pharmaceuticals, U.K.). **(D)** Tricine gel analysis (Schagger and Von Jagow, 1987) of human fibroblast-mediated MCP-3 cleavage. Fibroblast cultures were treated with Concanavalin A for 24 h at 37 °C. After 16-h incubation with MCPs in the presence of the MMP inhibitors indicated (concentrations as in **C**). **(E)** Electrospray mass spectrometry, NH_2-terminal Edman sequencing, and tricine gel analysis of MCP-3 cleavage products produced by recombinant gelatinase A activity. MCP-3 (5 μg) was incubated with 100 ng, 10 ng, 1 ng, 100 pg, 10 pg, 1 pg, and 100 fg recombinant gelatinase. **(F)** Electrospray mass spectrometry and tricine gel analysis of MCP-1, -2, -3, and -4 after incubation with recombinant gelatinase A. The NH_2-terminal sequence of the MCPs is shown with the Gly-Ile scissile bond in MCP-3 in bold.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B
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MCP-1
MCP-3
N-gelatinase A
C
competitor
gelatinase A
Hex CD
CBD
TIMP-2
BB-2275
29
20
14.5
5.8
2.9
Mass
(Da)
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8,574
D
Control
BB-2275
TIMP-2
Control
Con A
MCP-3
MCP-1
E
Mass
(Da)
N-terminal sequencing
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8,574
I-N-T-S
MCP-3
gelatinase A
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F
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MCP-2
MCP-3
MCP-4
gelatinase A
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5.8
Mass
(Da)
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8,580
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MCP-1
Q-P-A-S-V-S-I-P-I-T-
MCP-2
Q-P-V-G-I-N-T-S-T-T-
MCP-3
Q-P-D-A-L-N-V-P-S-T-
MCP-4
1
5
10

chemokine MCP-1. As controls, full-length and N-gelatinase A did bind to gelatin and TIMP-2 by the collagen binding domain and active site (Howard *et al.*, 1991) respectively. Hence, these data demonstrate a specific requirement for the hemopexin C domain of gelatinase A for binding MCP-3.

Incubation of MCP-3 with recombinant gelatinase A resulted in a small but distinct increase in electrophoretic mobility of MCP-3 on tricine gels (Fig. 3.2C) that the MMP specific inhibitors TIMP-2 and the hydroxamate inhibitor, BB-2275, blocked. Exogenous recombinant gelatinase A hemopexin C domain decreased MCP-3 cleavage whereas the collagen binding domain had no effect (Fig. 3.2C). In addition, the k_{cat}/K_m decreased from 8,000 $M^{-1}s^{-1}$ for full-length gelatinase A cleavage of MCP-3 to 500 $M^{-1}s^{-1}$ for N-gelatinase A confirming the mechanistic importance of the hemopexin C domain in MCP-3 degradation. These kinetic parameters for full-length gelatinase A cleavage of MCP-3 were higher than for denatured type I collagen (gelatin), 7,580 $M^{-1}s^{-1}$ (Young *et al.*, 1995) indicating that MCP-3 should be efficiently cleaved *in vivo*. Indeed, MCP-3 but not MCP-1 was cleaved by cultured human fibroblasts following Concanavalin A-induced gelatinase A activation (Fig. 3.2D). Cleavage of MCP-3 by other MMPs was also examined (McQuibban and Overall, Chapter 5). Although gelatinase A was the most efficient of these proteinases, collagenase-3 (MMP-13) and MT1-MMP also processed MCP-3, further favoring the cleavage of MCP-3 *in vivo*. However, MCP-3 was not a general MMP substrate since matrilysin (MMP-7), which lacks

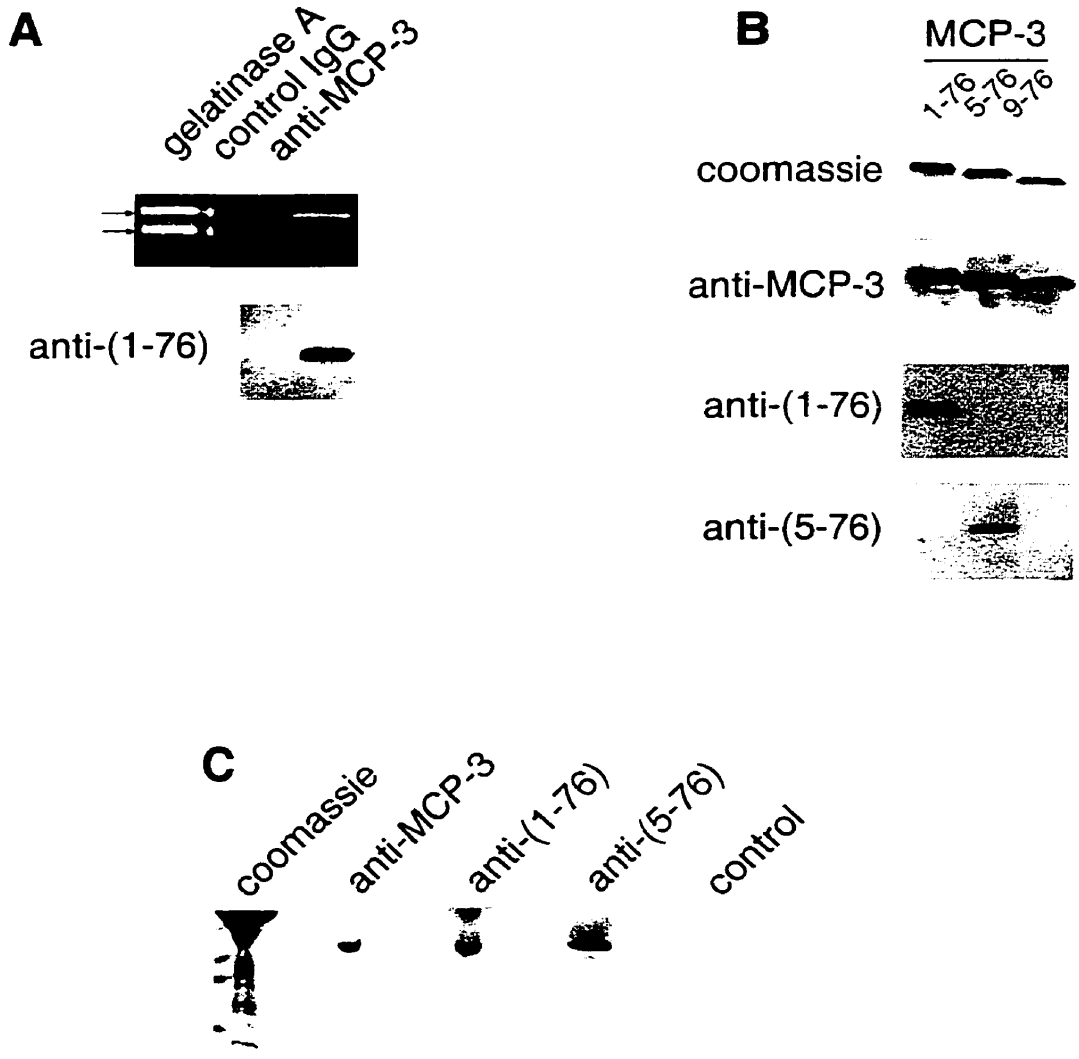
a hemopexin C domain, and the leukocyte MMPs, collagenase-2 (MMP-8), and gelatinase B (MMP-9) did not cleave MCP-3.

To identify the cleavage site in MCP-3, electrospray mass spectroscopy was performed. The mass of the gelatinase A-cleaved MCP-3 was 8,574 Da both *in vivo* (Fig. 3.2D) or *in vitro* (Fig. 3.2E) a reduction from the mass of the full-length molecule (8,935 Da) by the exact mass of the NH_2-terminal four residues. NH_2-terminal Edman sequencing confirmed the scissile bond to be Gly^4-Ile^5 (Fig. 3.2E), a preferred sequence for gelatinase A cleavage (Netzel-Arnett *et al.*, 1993) that is absent in other MCPs not cleaved by gelatinase A (Fig. 3.2F), including mouse MCP-3 (McQuibban and Overall, unpublished data).

A monoclonal antibody to human MCP-3 co-immunoprecipitated pro-gelatinase A, but not the active enzyme, from the synovial fluid of an arthritis patient (Fig. 3.3A). Full-length MCP-3 was identified in these immunocomplexes with an affinity-purified antibody raised against the NH_2-terminal 5 residues of MCP-3 (Fig. 3.3B). Gelatinase A-cleaved MCP-3 was also detected in human rheumatoid synovial fluids with affinity-purified anti-neoepitope antibody that recognizes the free amino group of the cleaved MCP-3 at Ile^5, but not the full-length MCP-3 nor another truncated MCP-3 (residues 9 to 76) (Fig. 3.3B, C). Hence, these data demonstrate that MCP-3 interacts with and is cleaved by gelatinase A *in vivo*.

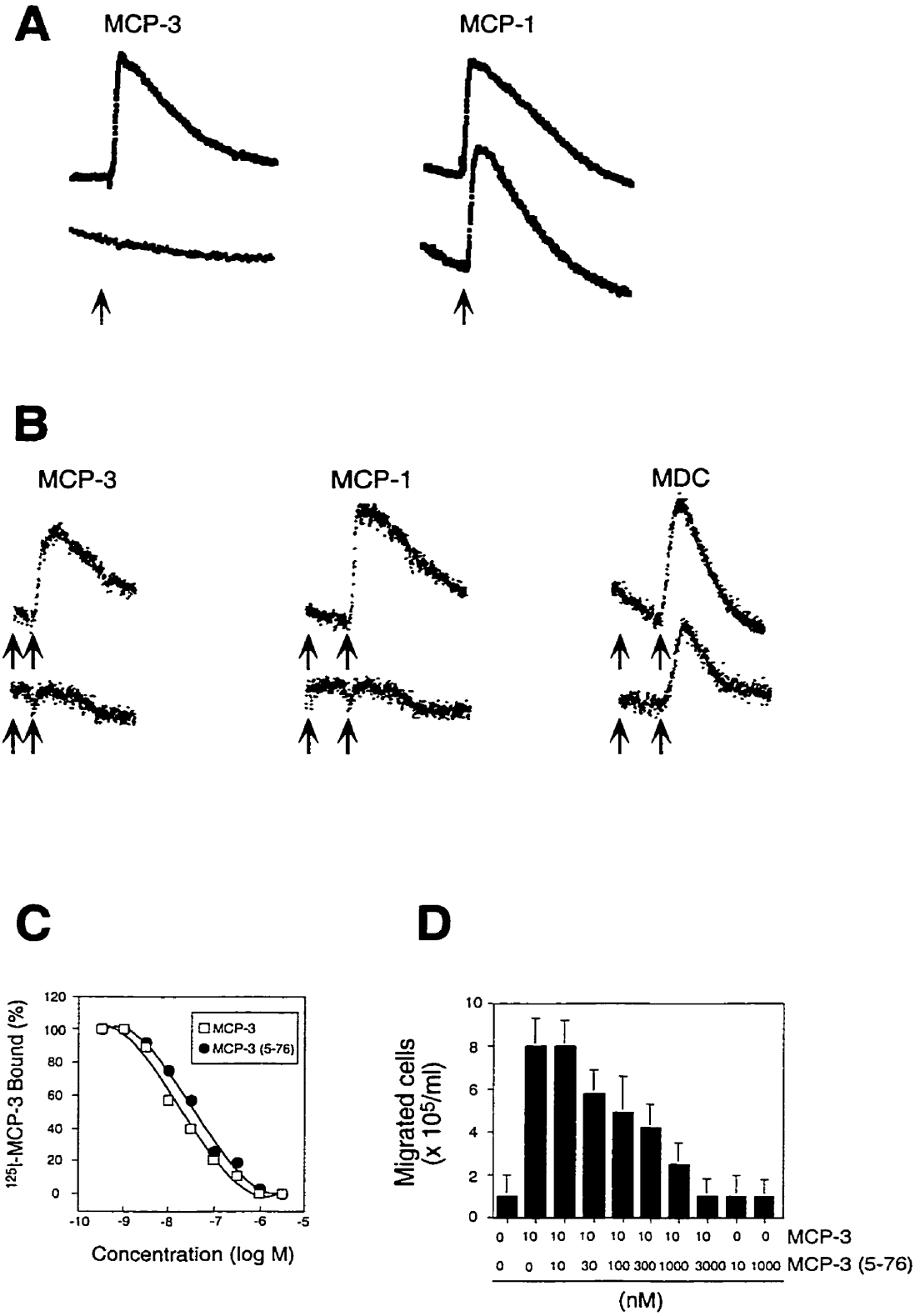
MCP-3 binds leukocyte CC-receptors-1, -2, and -3 mobilizing intracellular

Figure 3.3 Identification of *in vivo* human MCP-3/progelatinase A complexes and MCP-3 (5-76) in human synovial fluid. (A) MCP-3 was immunoprecipitated with an anti-MCP-3 monoclonal antibody from 200 µl human synovial fluid. Gelatin zymography (top panel) and western blotting with rabbit anti-MCP-3 (1-76) antibody (bottom panel) of the complexes. **(B)** Characterization of affinity purified antibody raised against residues 1 to 5 of MCP-3 (anti-1-76) and the neoepitope at residues 5 to 9 of MCP-3 (anti-5-76). **(C)** Identification of MCP-3 (5-76) in human rheumatoid synovial fluid. Total protein is shown following SDS-PAGE and Coomassie staining. Detection of full-length and gelatinase A-cleaved forms of MCP-3 in human synovial fluid by western blotting with antibodies as indicated. Control blot indicates anti-(5-76) pre-adsorbed with synthetic MCP-3 (5-76) before use.


A
gelatinase A
control IgG
anti-MCP-3
anti-(1-76)
B
MCP-3
1-76
5-76
9-76
coomassie
anti-MCP-3
anti-(1-76)
anti-(5-76)
C
coomassie
anti-MCP-3
anti-(1-76)
anti-(5-76)
control

calcium stores leading to directed cell migration that recruits and activates a variety of leukocytes. NH_2-terminal truncation of synthetic MCP-1 and MCP-3 at different sites has variable effects on their agonist activity (Gong and Clark-Lewis, 1995; Gong *et al.*, 1996). Gelatinase A-mediated removal of the first four residues of MCP-3 resulted in the loss of CC-receptor-1 and -2 activation in THP-1 cells, a monocyte cell line which expresses these two receptors. Neither gelatinase A-cleaved MCP-3 in the presence of 1/1000 gelatinase A (Fig. 3.4A) nor synthetic MCP-3 (5-76) [MCP-3 residues 5 to 76, corresponding to the gelatinase A-cleaved form] (Fig. 3.4B) elicited a calcium response. In addition to loss of CCR agonist activity, MCP-3 (5-76) antagonized the subsequent response not only to MCP-3, but also to MCP-1 (Fig. 3.4B) and macrophage inflammatory protein (MIP)1-α (see Chapter 5). MCP-1 only binds CCR-2 and MIP-1α binds to CCR-1 and CCR-5, confirming the CCR-1 and CCR-2 antagonist activity of MCP-3 (5-76). MCP-3 (5-76) did not block the calcium response to macrophage-derived chemokine (MDC), which binds CCR-4, a receptor not bound by MCP-3 (Fig. 3.4B). The physiological relevance of MCP-3 antagonism was confirmed by cell binding assays. Scatchard analysis showed that synthetic MCP-3 (5-76) bound THP-1 cells with high affinity (K_d of 18.3 nM) similar to that of full-length MCP-3 (K_d of 5.7 nM) (Fig. 3.4C). In transwell cell migration assays MCP-3 (5-76) was not chemotactic, even in amounts 100-fold higher than full-length MCP-3 (Fig. 3.4D). MCP-3 (5-76) also functioned as an

Figure 3.4 Cellular responses to gelatinase A-cleaved MCP-3. **(A)** Loss of intracellular calcium mobilization by MCP-3 following gelatinase A-cleavage. Fluo-3AM loaded THP-1 monocytes were treated (arrows) with 5 nM MCP-3 or MCP-1 (top scans) or respective chemokine incubated first with gelatinase A for 18 h (bottom scans). The data are presented as relative fluorescence emitted at 526 nm. **(B)** Intracellular calcium mobilization by MCP-3, MCP-1, and MDC. Fluo-3AM loaded THP-1 monocytes or a B-cell line transfected with CCR-4 (for MDC) were first exposed to either 0 nM (left arrow, top scans) or 500 nM MCP-3 (5-76) (left arrow, bottom scans), followed by MCP-3 (30 nM), MCP-1 (5 nM), and MDC (5 nM) as indicated (right arrow, top and bottom scans). **(C)** THP-1 cell receptor binding of MCP-3, and MCP-3 (5-76). **(D)** Transwell assay of monocytes treated with MCP-3 and MCP-3 (5-76) at the indicated amounts.


A
MCP-3
MCP-1
B
MCP-3
MCP-1
MDC
C
125I-MCP-3 Bound (%)
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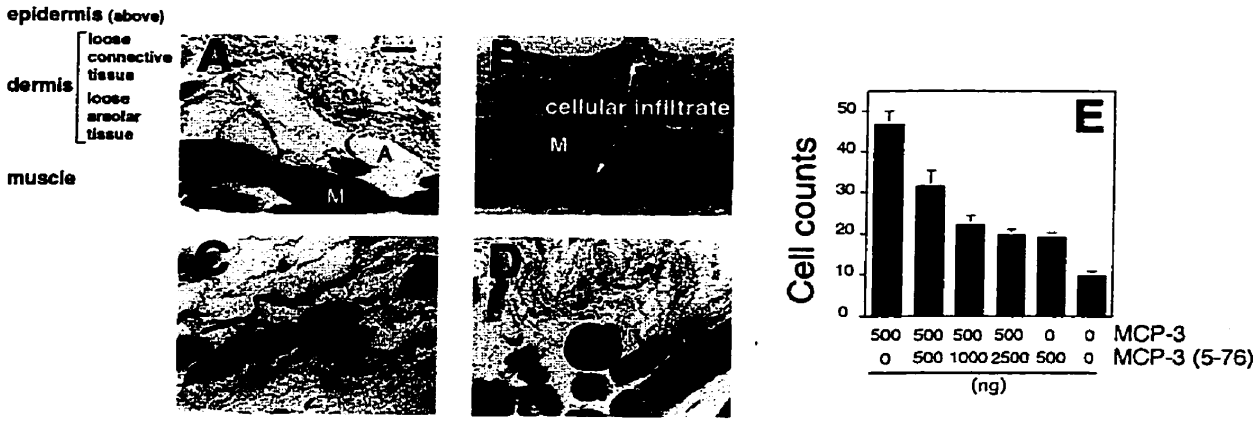
100
80
60
40
20
0
-10
-9
-8
-7
-6
-5
Concentration (log M)
MCP-3
MCP-3 (5-76)
D
Migrated cells
(x 10⁵/ml)
10
8
6
4
2
0
0 10 10 10 10 10 10 10 0 0 MCP-3
0 0 10 30 100 300 1000 3000 10 1000 MCP-3 (5-76)
(nM)

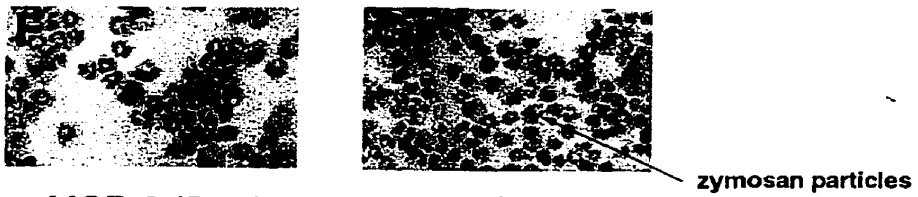
antagonist in a dose dependent manner to inhibit the chemotaxis directed by full-length chemokine (Fig. 3.4D). Thus, MMP inactivation of MCP-3 also generates a broad-spectrum antagonist for CC-receptors that retains strong cellular binding affinity and modulates the response to a number of related chemoattractants.

To examine the biological consequences of gelatinase A cleavage of MCP-3 in inflammation, various mole ratios of full-length MCP-3 and gelatinase A-cleaved or synthetic MCP-3 (5-76) were injected into mice subcutaneously. Full-length, but not cleaved MCP-3, induced infiltration of mononuclear inflammatory cells with associated matrix degradation at 18 h post-injection (Fig. 3.5A-D). A statistically significant reduction in the mononuclear cell infiltrate in response to as little as a 1:1 mixture of MCP-3 (5-76) with MCP-3 was observed (Fig. 3.5E). In a separate mouse model of inflammation, the cellular infiltrate in 24-h zymosan A-induced peritonitis was attenuated after intraperitoneal injection with MCP-3 (5-76). Consistent with morphometric examination of the cell content in the peritoneal cavity (Fig. 3.5F, G), FACS analysis of peritoneal washouts showed that macrophage (F4/80+) cell counts were reduced by ~40% at both 2 and 4 hours following MCP-3 (5-76) treatment (Fig. 3.5F, G).

Chemokine inactivation and clearance *in vivo* is not well understood (Van Damme *et al.*, 1999). While several examples are known in which metalloproteinase activity activates cytokines (Gearing *et al.*, 1994) and α-defensins (Wilson *et al.*, 1999), this is the first demonstration to our knowledge of

Figure 3.5 Animal responses to gelatinase A-cleaved MCP-3. Light micrographs of haematoxylin and eosin stained subcutaneous tissue sections (tissue region as shown) of mice injected with 100 µl of **(A)** saline/buffer control **(B)** MCP-3, 1 µM **(C)** gelatinase A-cleaved MCP-3, 1 µM and **(D)** 2:1 molar ratio of gelatinase A-cleaved MCP-3:full-length MCP-3, 1 µM:0.5 µM. Bar=20 µm; M, muscle; A, adipocyte; C, loose connective tissue. **(E)** After sub-cutaneous injections with MCP-3 and MCP-3 (5-76) mixtures the infiltrating mononuclear cells were enumerated and expressed as cells/75,000 μm^2 (n=5, error bar=S.D., $p<0.01$ compared with MCP-3). **(F)** and **(G)** Haematoxylin and eosin stained cytospins of intraperitoneal washouts of zymosan A-treated mice injected 24 h later with **(F)** MCP-3 (5-76) or **(G)** saline for 4 h. Macrophage cell counts by FACS analysis for each group of mice are presented below (n=5, +/- S.D., $p<0.05$ by ANOVA).





+MCP-3 (5-76) +saline

F4/80+ cells/ml (x 100,000)

2 h	7.95 +/- 1.52	12.8 +/- 0.5
4 h	9.01 +/- 1.18	13.9 +/- 4.4

the extracellular inactivation of a cytokine *in vivo* by MMP activity. The relative amounts of intact and cleaved MCP-3 that are present after pathophysiological cleavage will regulate chemotaxis and the extent of inflammation. Our identification of the importance of gelatinase A in the pathophysiological processing of MCP-3 reveals a new intersection of two distinct pathways that regulate the extracellular environment and the immune response. Notably, gelatinase A is largely derived from stromal cells and is not usually expressed by leukocytes (Opdenakker *et al.*, 1991); these cells express MMP-8 and gelatinase B, both of which do not cleave MCP-3. Therefore, leukocyte proteolytic activity is unlikely to disrupt cognate chemokine gradients. Overall, the activity of several tissue-derived MMPs, and gelatinase A in particular, can contribute to the cessation of the host response, an important aspect of healing and tissue resolution. Since TGF-β1, a growth factor that orchestrates wound repair (Letterio and Roberts, 1998), stimulates gelatinase A but represses other MMPs (Overall *et al.*, 1991), we suggest that the interactions of MMPs with chemokines provide a self-attenuating network to dissipate pro-inflammatory activities. Therefore, MMPs are both effectors and regulators of inflammation.

The following Chapter represents a manuscript that will be submitted to the Journal of Biological Chemistry. The manuscript represents many biochemical aspects of the gelatinase A cleavage of MCP-3. The format of this journal is traditional, and includes the relatively full descriptions of the work presented. As such, there may be some duplication in methodology and in introductory comments. The data presented in this Chapter represents work done solely by the candidate.

Chapter 4 Exosites on the matrix metalloproteinase hemopexin C domain direct efficient proteolysis of monocyte chemoattractant protein 3.

Summary

Several recent reports suggest that matrix metalloproteinases (MMPs) are important processing enzymes, expanding the substrate repertoire of this enzyme family beyond that of extracellular matrix components. MMPs are multi-domain proteins with each domain contributing unique characteristics to the enzyme. In a comparison of substrate-binding properties of the gelatinase A collagen binding domain and the hemopexin C domain, we previously reported that all substrates examined bound the collagen binding domain except fibronectin. Here we demonstrate the importance of hemopexin C domain exosites in the proteolysis of a chemokine substrate, monocyte chemoattractant protein (MCP)-3. Full-length gelatinase A cleaved MCP-3 more efficiently than N-gelatinase A, which lacks the hemopexin C domain. Gelatinase A cleavage of MCP-3 is inhibited by exogenous addition of the hemopexin C domain, but not the collagen binding domain. The interaction between MCP-3 and the hemopexin C domain (K_d of 0.4 μM) increased the k_{cat}/K_m from 500 $M^{-1}s^{-1}$ to 8000 $M^{-1}s^{-1}$ but was not sufficient to retard substrate exchange and cleavage by retention of the cleaved form, MCP-3 (5-76), which had a similar K_d (1.0 μM). Only MCP-3 and not MCP-1, -2, and -4, bound to the hemopexin C domain, and it alone contains the preferred gelatinase A scissile bond sequence Gly-Ile.

MCP-3 mutants and chimeras with MCP-1 revealed the pivotal role of substrate-binding exosites for cleavage, compared to the flanking sequences of the scissile bond. The importance of hemopexin C domain substrate-binding exosites was further demonstrated with MT1-MMP suggesting that this may be a paradigm in MMP structure and function.

Introduction

Matrix metalloproteinases are members of a family of zinc-dependent endoproteinases that can collectively degrade the proteins of the extracellular matrix (Nagase and Woessner, 1999; Werb, 1997). Accordingly, MMPs have been implicated in human disorders involving the turnover and remodeling of the extracellular matrix (Curran and Murray, 1999), and traditionally have been considered important in the biology of extracellular matrix destruction. However, recent studies have demonstrated that the substrate repertoire of the MMP family is rapidly expanding beyond the structural components of the extracellular matrix and MMP gene disruption studies in mice have displayed a variety of effects (Itoh *et al.*, 1998; Holmbeck *et al.*, 1999; Coussens *et al.*, 2000; Haro *et al.*, 2000). Indeed, many biologically active molecules have been revealed as substrates. Our recent yeast two-hybrid identification of monocyte chemoattractant protein-3 as a MMP substrate (McQuibban *et al.*, 2000), as well as α-defensin (Wilson *et al.*, 1999), serpins (Mast *et al.*, 1991; Liu *et al.*, 2000), interleukin 1 beta (Ito *et al.*, 1996), and insulin-like growth factor binding proteins (Fowlkes *et al.*, 1994) suggest that MMPs should also be viewed as processing enzymes. Following proteolysis by MMPs, the function of these substrates is altered, changing both activity and specificity to influence cell behavior. Thus, it is important to identify substrates of MMPs in order to understand the role of these proteinases in biological processes and to identify new drug targets to treat disease.

One of the key issues of MMP proteolysis is how substrate specificity is determined, and by what molecular determinants. The prototype MMP enzyme is comprised of three well-conserved domains that have been implicated in substrate binding, catalysis, and tissue inhibitor of metalloproteinase (TIMP) binding: the prodomain is responsible for enzyme latency, the catalytic domain is important for fine peptide bond specificity and cleavage, and the hemopexin C domain is essential for collagen triple helicase activity in the collagenases (Lauer-Fields *et al.*, 2000; Chung *et al.*, 2000) and for binding TIMPs in the gelatinases (Murphy *et al.*, 1999). The two gelatinases A and B (MMP-2 and MMP-9) have inserted within the catalytic domain three fibronectin type II repeats, which we and others have shown to be important in gelatinolysis (Steffensen *et al.*, 1995; Banyai *et al.*, 1994, Murphy *et al.*, 1994). Substrate interactions outside the catalytic center with exosites are important for optimal alignment and presentation of scissile bonds to the active site, for increasing the local concentration of substrate, and for substrate preparation (Overall, 2000). The critical role of the hemopexin C domain in substrate cleavage was first shown for triple helicase activity of MMP-1 during collagenolysis (Clark and Cawston, 1989). However, the role of the hemopexin C domain in binding other substrates in the collagenases or other MMPs is not well characterized. Indeed, none of the native collagens cleaved by gelatinase A bind the hemopexin C domain (Wallon and Overall, 1997) which instead bind the collagen binding

domain. In view of the TIMP binding properties of the hemopexin C domain of gelatinase A and gelatinase B, many have considered that this was the major role of this domain in gelatinolysis. We hypothesized that the hemopexin C domain contains binding sites for other proteins that may prove to be novel substrates, and initiated a yeast two-hybrid screen to identify interacting proteins. One of the proteins cloned was monocyte chemoattractant protein (MCP)-3. We recently reported that this chemokine was an *in vivo* substrate for gelatinase A, and demonstrated the profound biological impacts of this cleavage in modulating inflammation (McQuibban *et al.*, 2000).

Here we have characterized in detail the role of the gelatinase A hemopexin C domain in chemokine recognition, binding, and catalysis. Our studies reveal the relative dominance of substrate-binding exosites on the hemopexin C domain compared with the active site in contributing to efficient catalysis of specific substrates. This has important implications in the design of MMP inhibitors that may lead to the development of substrate-specific inhibitors. This novel rationale for substrate-specific MMP inhibition in disease may reduce side effects.

Materials and Methods

Proteins

Gelatinase A hemopexin C domain, collagen binding domain, and MT1-MMP hemopexin C domain proteins were expressed and purified from bacteria as previously described (Overall *et al.*, 2000). Briefly, *E. coli* strain Le392 carrying the expression plasmid was grown in liquid culture for 24 h at 37 °C. Bacterial pellets were harvested by centrifugation and the inclusion bodies were isolated by sonication and lysozyme digestion. Proteins were extracted from the inclusion bodies using 6 M GuHCl, and re-folded by stepwise dialysis (Steffensen *et al.*, 1995). Refolded proteins were purified by Ni-Sepharose (Pharmacia Biotech) chromatography. Recombinant human full-length gelatinase A, hemopexin-truncated gelatinase A (N-gelatinase A), and TIMP-2 were the generous gift of Dr. R. Fridman (Wayne State, Detroit, U.S.A.). Recombinant human sMT1-MMP (corresponding to the catalytic and hemopexin C domain) was provided by British Biotech Pharmaceuticals (Oxford, U.K.). All chemokines were chemically synthesized, re-folded, and purified as previously described (Gong *et al.*, 1996). ^{125}I-labeled MCP-3 was purchased from Pharmacia Biotech (U.S.A.).

MMP assays

All enzyme reactions were performed at 37 °C in CAB buffer (20 mM Tris, 150

mM NaCl, 5 mM $CaCl_2$, pH 7.4) in a total volume of 20 µl, with enzyme and chemokine amounts given in Figure legends. Reactions were stopped by the addition of SDS-PAGE sample buffer. Cleavage of chemokines was monitored by Tris-tricine SDS-PAGE and by electrospray mass spectrometry. Turnover rates were calculated by monitoring product accumulation during time course reactions. The k_{cat}/K_m specificity constants were derived from k_{obs} derived from the slope of ln[S] plotted against time.

Anti-sera

Antibodies were generated against a peptide (anti-1-76: NH_2-Gln-Pro-Val-Gly-Ile-Gly-Gly-Cys, corresponding to the N-terminal 5 residues of MCP-3) conjugated to the immunogen protein keyhole limpet hemocyanin (KLH). Anti-sera, designated α-1-76, were isolated from New Zealand white rabbits as previously described (Steffenson *et al.*, 1995). Anti-His_6 antibodies were affinity-purified by column chromatography with immobilized His_6 peptide. Bound antibodies were eluted with 1.5 M triethanolamine, pH 11.5, and neutralized with 100 mM Tris, pH 8.0. Anti-1-76 antibodies were affinity-purified by column chromatography over immobilized full-length MCP-3 (1-76) protein bound at the C-terminal to Affi-gel 102 resin (BioRad). Bound antibodies were eluted as above.

Protein binding assays

Enzyme-linked immuno-adsorbent (ELISA) assays were performed as previously described (Steffenson *et al.*, 1995). Protein binding assays with ^{125}I-MCP-3 were performed by first immobilizing recombinant gelatinase A hemopexin C domain onto 96-well plates (Costar, U.S.A.). Following a blocking step with 2.5% bovine serum albumin (BSA) to prevent any non-specific binding to the plastic, ^{125}I-MCP-3 was added to the wells and incubated for 1 h at 25 °C in PBS-0.01% Triton X-100. Following extensive washes, increasing concentrations of either unlabelled MCP-3 (1-76) or MCP-3 (5-76) were added and incubated for 1 h at 25 °C. Supernatants were collected and ^{125}I-MCP-3 that was released was quantified using a gamma X-ray sensor cell (Beckman Scientific, U.S.A.) and K_d values were determined by Scatchard analysis.

Cell culture

Early passage human gingival fibroblasts were grown to 80% confluence in D-MEM (Gibco-BRL) supplemented with 10% newborn calf serum. Cells were washed with PBS, then treated with Concanavalin A (20 µg/ml) (Overall and Sodek, 1990) and MCP-3 was added at a concentration of 1 µM. After 24 h incubation at 37 °C, cell culture supernatants were removed and assayed for gelatinase A activation by gelatin zymography.

Results

The gelatinase A hemopexin C domain increases catalytic efficiency

To determine the importance of the hemopexin C domain in MCP-3 cleavage we utilized a hemopexin C domain-truncated form of gelatinase A. MCP-3 was cleaved only ~ 50% by N-gelatinase A after 16 h of incubation (Figure 4.1). In comparison, full-length enzyme efficiently cleaved all the MCP-3 at Gly^4-Ile^5 [MCP-3 (5-76)] as shown by electrospray mass spectrometry. Enzyme specificity was demonstrated by the absence of MCP-3 cleavage by the closely related gelatinase B (Figure 4.1). To confirm the importance of the hemopexin C domain in catalysis, we found that exogenous hemopexin C domain competed for gelatinase A cleavage of MCP-3 in a concentration dependent manner, whereas the collagen binding domain of gelatinase A did not (Figure 4.2).

The hemopexin C domain binds full-length and cleaved MCP-3

The MCP-3 cleavage assays revealed the importance of the hemopexin C domain in determining the rate of cleavage of this protein. The binding affinity (K_d) of MCP-3 for recombinant hemopexin C domain was determined to be 0.4 μM by use of ^{125}I-labeled MCP-3 (Figure 4.3). We also found that cleaved MCP-3 (5-76) bound to the hemopexin C domain with a similar affinity of 1.0 μM.

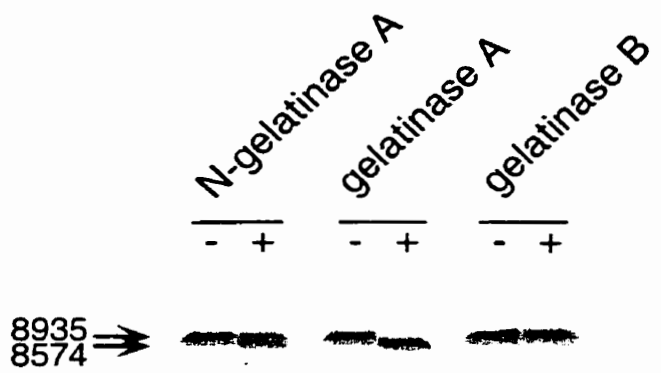


Figure 4.1 Cleavage of MCP-3 by gelatinases.

MCP-3 (1 µg) was incubated with 100 ng of enzyme at 37 °C for 16 hours. Reaction products were analyzed on Tris-tricine gels and by electrospray mass spectrometry. Despite these conditions of high enzyme/substrate ratio, gelatinase B was unable to cleave MCP-3.

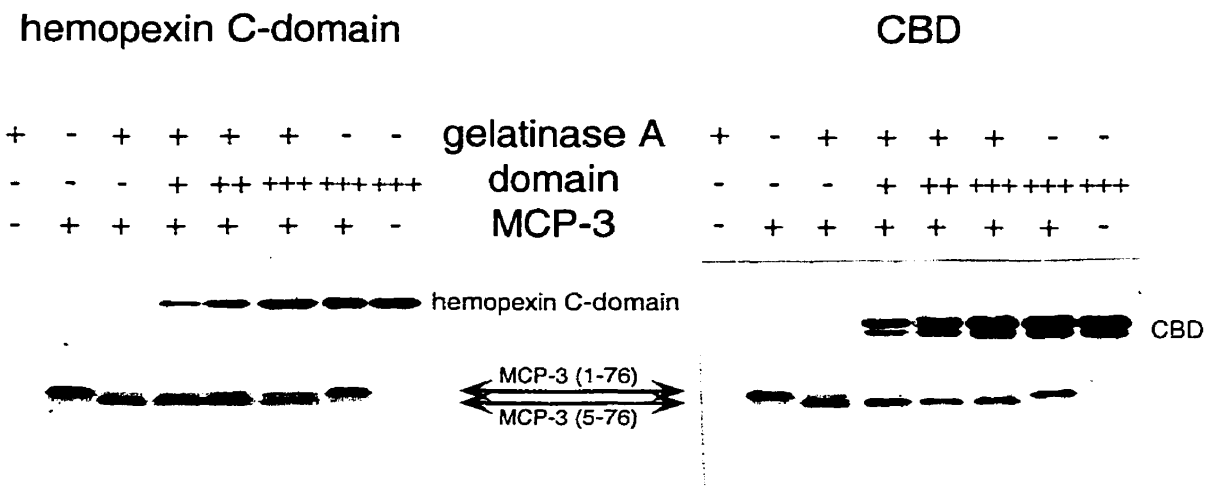


Figure 4.2 Inhibition of MCP-3 cleavage by the hemopexin C domain.

MCP-3 (1 µg) was incubated with 1 ng gelatinase A at 37 °C for 1 hour. Increasing amounts of hemopexin C domain (+, 1 molar equivalent; ++, 5 molar excess; +++, 10 molar excess/chemokine) but not collagen binding domain (CBD) (+, 1 molar equivalent; ++, 5 molar excess; +++, 10 molar excess/chemokine) resulted in inhibition of gelatinase A-mediated MCP-3 cleavage.

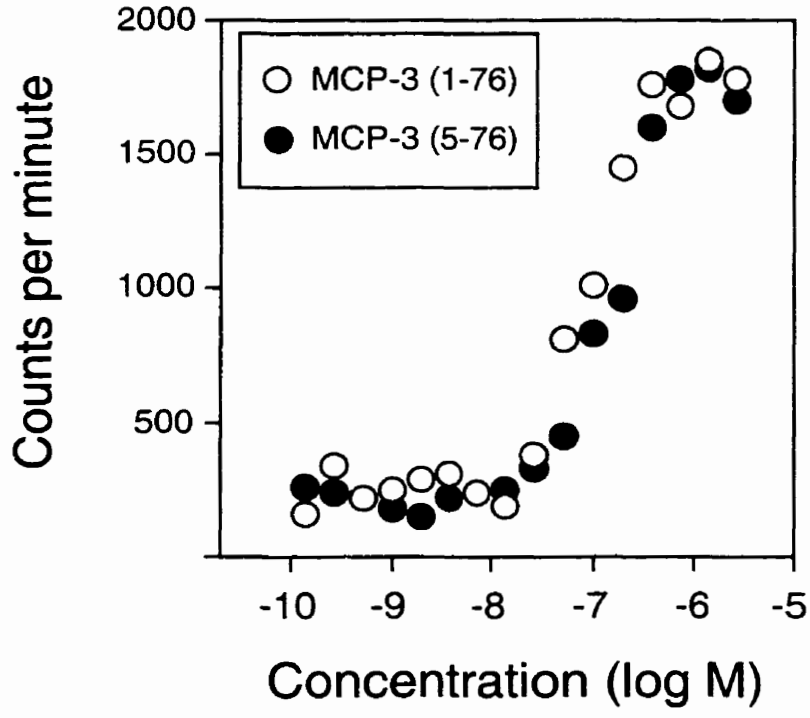


Figure 4.3 Both MCP-3 (1-76) and MCP-3 (5-76) bind to the hemopexin C domain.

[^{125}I]-labelled MCP-3 was bound to immobilized gelatinase A hemopexin C domain. Bound chemokine was competed with either unlabelled MCP-3 (1-76) (open circles) or unlabelled MCP-3 (5-76) (closed circles).

MCP-3 (5-76) does not inhibit gelatinase A cleavage of MCP-3

In view of the similar binding affinities of full-length and cleaved MCP-3 for the hemopexin C domain, we determined whether MCP-3 (5-76) product accumulation could inhibit cleavage of MCP-3 (1-76) by binding the hemopexin C domain. Cleavage assays of full-length MCP-3 were performed in the presence of increasing amounts of MCP-3 (5-76), hemopexin C domain, or MCP-1 (Figure 4.4). To monitor the degradation of full-length MCP-3 in the presence of increasing amounts of the added MCP-3 (5-76), which migrates very close on the gel, we designed and raised an antibody against the N-terminal 5 residues of MCP-3 such that upon gelatinase A cleavage of MCP-3, immunoreactivity is lost. Control blots confirmed no reactivity of the affinity-purified anti-sera to MCP-3 (5-76) (see Figure 3.3B), nor to MCP-1 (not shown) which differs in its N-terminal sequence. As shown in Figure 4, neither MCP-3 (5-76) nor MCP-1, which was added as a control, inhibited the cleavage reaction, even at 50-fold molar excess. To control for the utility of the assay and anti-sera, recovery of immunoreactivity was observed when increasing amounts, up to only 5-fold molar excess, of hemopexin C domain were added to the reaction, which we have already demonstrated is inhibitory (Figure 4.2).

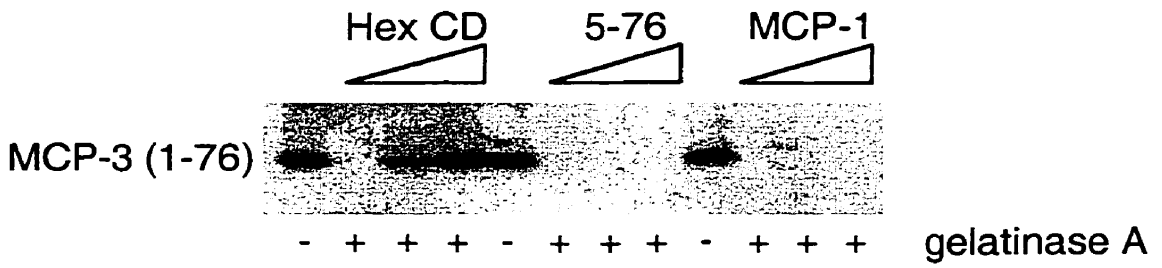


Figure 4.4 MCP-3 (5-76) does not inhibit gelatinase A cleavage of MCP-3 (1-76).

To determine whether the residual binding between MCP-3 (5-76) and the hemopexin C domain of gelatinase A would result in product inhibition, we performed competition assays. To monitor cleavage, affinity-purified anti-sera was used that only recognizes uncleaved MCP-3 (1-76). MCP-3 (0.1 µg) was incubated with 0.1 ng gelatinase A at 37 °C for 16 hours. To test for inhibition, up to 5- and 50-fold molar excess of either exogenous hemopexin C domain, MCP-3 (5-76), or MCP-1 were added to the reaction mixture. Immunoreactivity shows the presence of the full-length uncleaved molecule, and indicates efficient inhibition of the cleavage reaction. This was only seen with added hemopexin C domain.

The TIMP-2-hemopexin C domain interaction does not preclude MCP-3 binding and cleavage

TIMP-2 binds to both progelatinase A and the active form of the enzyme at two sites on the hemopexin C domain (Overall *et al.*, 1999) and active site (Murphy *et al.*, 1999), respectively. Since gelatinase A is normally found complexed with TIMP-2, we tested whether the TIMP-2/hemopexin C domain interaction would prevent or alter MCP-3 binding and therefore MCP-3 cleavage. In an ELISA-type binding assay, TIMP-2-hemopexin C domain complex bound MCP-3 with the same relative affinity as TIMP-2-free hemopexin C domain (Figure 4.5A) indicating different binding sites. Since the hemopexin C domain is essential for gelatinase A activation by MT1-MMP (Ward *et al.*, 1994), we determined whether MCP-3 binding would modulate progelatinase A activation. Human fibroblasts treated with the lectin Concanavalin A activate and release gelatinase A in complex with TIMP-2 (Overall and Sodek, 1990). We asked whether the presence of molar excess MCP-3 in the medium would alter this hemopexin C domain-dependent process. Gelatin zymography of the conditioned medium (Figure 4.5B) demonstrated that MCP-3 did not alter the TIMP-2/MT1-MMP dependent proteolytic processing of progelatinase A to its activation intermediate nor to its fully active form. In addition, incubation of MCP-3 in these Con A-treated cell cultures resulted in cleavage of MCP-3 (McQuibban *et al.*, 2000).

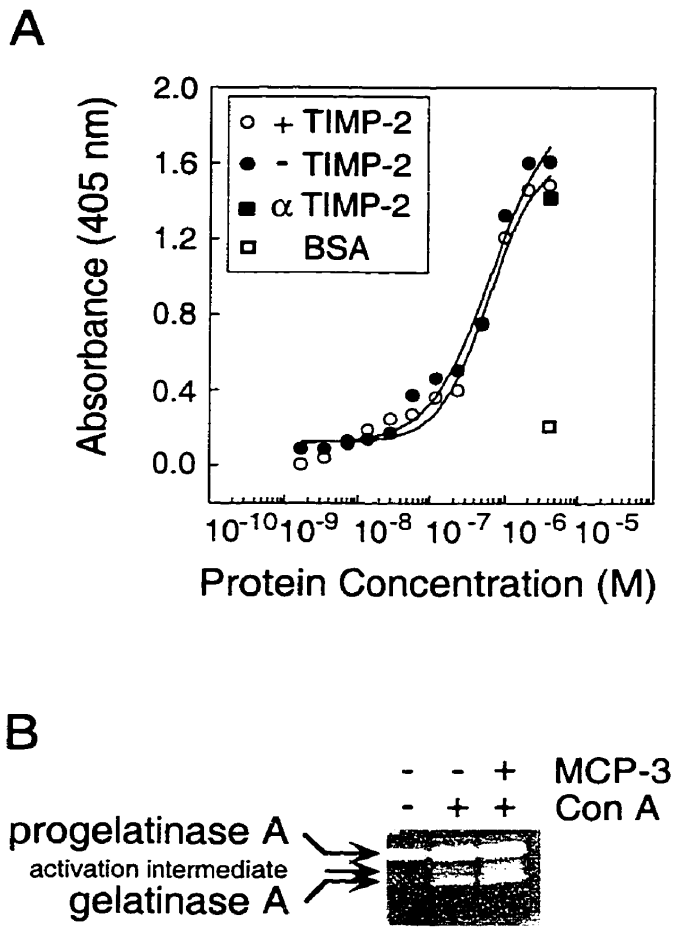


Figure 4.5 MCP-3 binding of the hemopexin C domain occurs independently of TIMP-2 association.

A; An ELISA-type binding assay was used to determine whether TIMP-2 binding to the hemopexin C domain would disrupt subsequent MCP-3 interaction. TIMP-2 was bound to immobilized hemopexin C domain, followed by addition of MCP-3. Immunodetection of bound MCP-3 reveals equivalent interaction, regardless of TIMP-2 association. α-TIMP-2 and BSA are shown as a controls.

B; Human fibroblast cells were treated with Concanavalin A (20 μg/ml) for 24 hours in the presence (10 μM) or absence of MCP-3. Gelatin zymography demonstrates that MCP-3 does not inhibit the Con A-mediated activation of gelatinase A.

MT1-MMP specifically cleaves MCP-3

The MT1-MMP-gelatinase A proteolytic axis is important for collagen and gelatin degradation in inflammation and cancer (Ellerbroek and Stack, 1999). Since MT1-MMP activity precedes gelatinase A activity, we assessed the MCP cleavage properties of MT1-MMP. Like gelatinase A, MT1-MMP cleaved only MCP-3 and not MCP-1, -2, or -4, in a reaction that was inhibited by the hydroxamate MMP inhibitor BB-2237 (Figure 4.6). Electrospray mass spectrometry identified the MCP-3 scissile bond to be Gly^4-Ile^5, the same as that for gelatinase A.

Only MCP-3 is bound by the gelatinase A and MT1-MMP hemopexin C domains

The MCP family of CC chemokines is composed of at least 4 closely related family members in man (Van Collie *et al.*, 1999). We have previously reported that only MCP-3 is cleaved by gelatinase A (McQuibban *et al.*, 2000). Here we have also shown that only MCP-3 is cleaved by MT1-MMP (Figure 4.6). A possible explanation for this specificity could be a pre-requisite for hemopexin C domain binding of substrate in order to facilitate cleavage. Therefore, we tested the relative binding affinities of the gelatinase A and MT1-MMP hemopexin C domains to MCP-1, -2, -3, and -4 using an ELISA-type binding assay. Only MCP-3 was bound by the hemopexin C domain of both gelatinase A (Figure

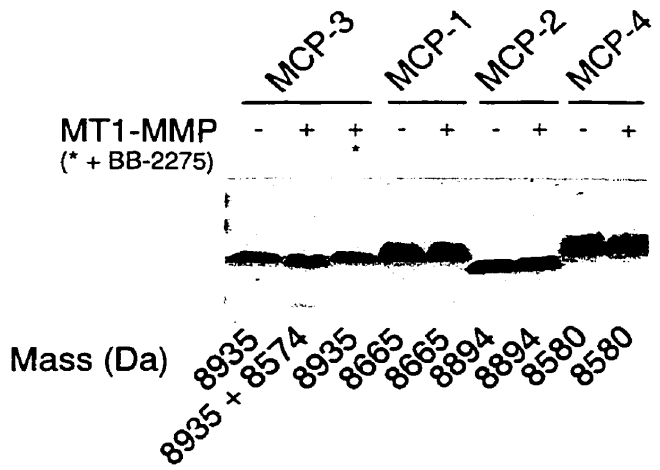


Figure 4.6 MT1-MMP proteolytic specificity towards MCP-3.

Chemokine (1 µg as indicated) was incubated with 1 ng sMT1-MMP for 4 hours at 37 °C. Reaction products were analyzed on Tris-tricine gels and by electrospray mass spectrometry. The general MMP hydroxamate inhibitor BB-2275 was used at 10 µM.

4.7A) and MT1-MMP (Figure 4.7B). Thus, the binding specificity coincides with the specificity of MCP chemokine cleavage by these two enzymes.

Hemopexin C domain binding is a requirement for efficient proteolysis

Since gelatinase A and MT1-MMP process only MCP-3 and only MCP-3 is bound by the hemopexin C domain of these enzymes, we determined the specific requirement of MCP-3-binding exosites on the hemopexin C domain for efficient proteolysis compared with sequence specificity of the scissile bond. Of note, MMP-7, which lacks a hemopexin C domain, does not cleave MCP-3, but gelatinase B also does not. Therefore, there is likely a specific site or sites on some MMP hemopexin C domains that can bind MCP-3 or a specific spatial alignment of bound substrate with the catalytic domain that facilitates cleavage. To test this hypothesis, two chemokine chimeras were generated: MCP-3 (1-10)/MCP-1 (11-76), in which the first 10 residues are MCP-3, and the remaining 66 residues are from MCP-1, which does not bind the hemopexin C domain (Figure 4.7A); MCP-3 (Gly^4->Ala^4), in which the scissile bond sequence in MCP-3 was mutated to that of the corresponding sequence in MCP-1 [which is not cleaved (McQuibban *et al.*, 2000)]. Cleavage of these chimeras was compared to that of wild type MCP-3 (1-76) (Figure 4.8). At an enzyme:substrate ratio of 1:1000, MCP-3 (Gly^4->Ala^4) was cleaved relatively efficiently by gelatinase A (turnover rate of 1,000 $M^{-1}s^{-1}$ as compared to 8000 $M^{-1}s^{-1}$ for MCP-3), whereas

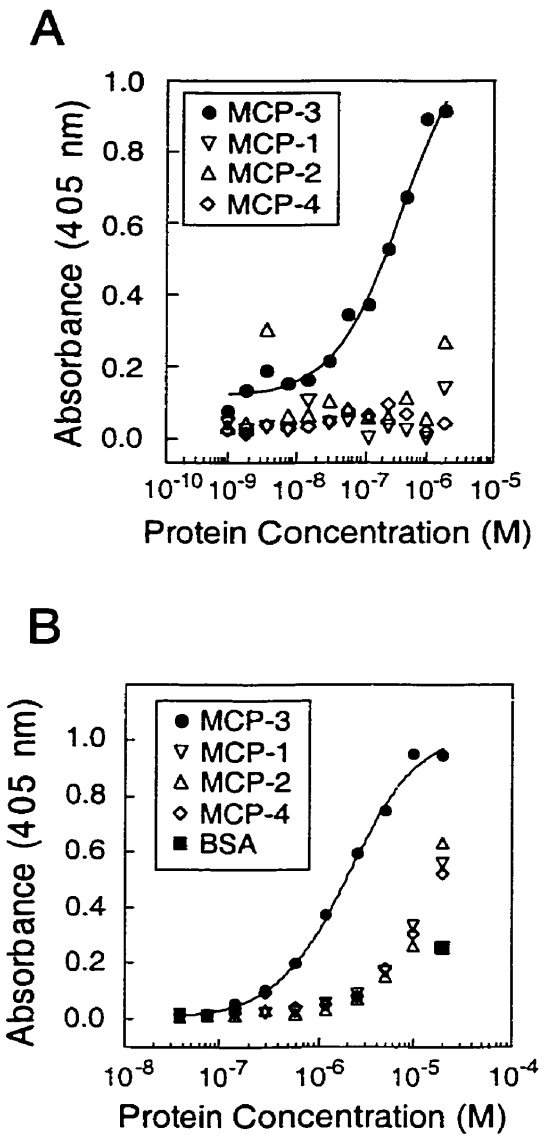


Figure 4.7 The gelatinase A and MT1-MMP hemopexin C domains only bind MCP-3.

In an ELISA-type binding assay, 0.5 µg of each chemokine was coated onto the microwell plate, and overlaid with a serial dilution of (**A**) gelatinase A hemopexin C domain, or (**B**) MT1-MMP hemopexin C domain. Immuno-detection of bound protein revealed that only MCP-3 showed significant interaction with both hemopexin C domains.

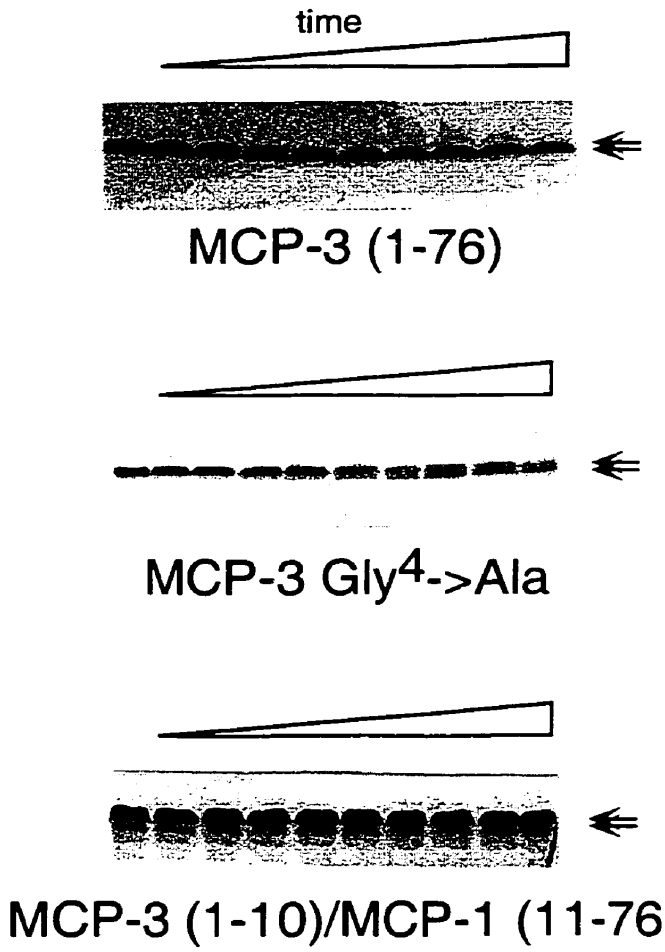


Figure 4.8 The MCP-3/hemopexin C domain interaction is required for efficient catalysis.

Wild-type, chimeric, or mutant chemokine (10 µg, as indicated) was incubated with 10 ng of gelatinase A and incubated for 0, 10 min, 30 min, 1 h, 2 h, 3 h, 4 h, 5 h, 6 h, and 7 h. Reaction products were analyzed on Tris-tricine gels followed by staining with Coomassie brilliant blue. The k_{cat}/K_m specificity constant was calculated from k_{obs}.

MCP-3 (1-10)/MCP-1 (11-76) was not cleaved under these conditions. However, following prolonged incubation with molar equivalents of enzyme and chemokine, partial cleavage of MCP-3 (1-10)/MCP-1 (11-76) did occur (not shown), from which we estimated the turnover rate to be $\leq 0.4\ M^{-1}s^{-1}$. Importantly, electrospray mass spectrometric analysis (Figure 4.6) showed the scissile bond of the cleaved chimeras to be at position 4-5 in the amino acid sequence, regardless of the chimera. These data suggest that binding by the hemopexin C domain efficiently promotes and directs substrate cleavage by optimally presenting the substrate to the active site cleft, even in the case of amino acid sequences not naturally cleaved by the enzyme.

Discussion

To discover new MMP substrates we recently described our novel strategy that utilized yeast two-hybrid screens to identify proteins that interacted with proteinase exosite domains. The rationale of the screen was based on our hypothesis that proteins that bind the hemopexin C domain may be MMP substrates. MCP-3 was identified as a gelatinase A hemopexin C domain interactor that was cleaved by the enzyme resulting in loss of agonist activity. Moreover, cleavage both *in vitro* and *in vivo* transformed this chemoattractant to a wide-acting chemokine receptor antagonist. Here, we have biochemically characterized the relative roles of the catalytic and hemopexin C domains in the catalysis of MCP-3 and shown the powerful effect of substrate binding exosites in promoting cleavage by MMPs—the first demonstration for any substrate other than collagen by collagenases.

The hemopexin C domain of gelatinase A binds MCP-3 leading to efficient catalysis (k_{cat}/K_m of 8,000 $M^{-1}s^{-1}$) as shown by a truncated form of gelatinase A that lacks the hemopexin C domain and which cleaves MCP-3 with a specificity constant of only 500 $M^{-1}s^{-1}$ (McQuibban *et al.*, 2000; Chapter 3). This data demonstrates the importance of an exosite(s) on the hemopexin C domain in contributing to efficient catalysis. The binding affinity of the hemopexin C domain for MCP-3 (1-76) and MCP-3 (5-76) is essentially the same indicating a relatively

small contribution in binding by the N-terminal sequence. Interestingly, the region of MCP-3 that is cleaved is disordered in solution (Kim *et al.*, 1996); however, the protein has a highly structured central domain comprised of a 3-stranded anti-parallel beta-sheet, covered on one face by a C-terminal alpha-helix. Since both the cleaved and full-length forms of MCP-3 bind the hemopexin C domain, we propose that on binding the central globular domain the flexible N-terminus of MCP-3 is presented to the active site groove of gelatinase A.

The importance of this exosite-binding interaction was also confirmed by competition experiments. Further, in experiments calibrated to produce 50% cleavage of MCP-3, addition of either exogenous hemopexin C domain or TIMP-2 resulted in complete loss of cleavage (not shown). As a control for these competition experiments the gelatinase A collagen binding domain, another distinct exosite domain, was unable to compete for gelatinase A cleavage of the chemokine. These data support the concept that the chemokine binding exosite(s) on the hemopexin C domain specifically contribute to and enhance the catalytic reaction. Indeed, only MCP-3 (of a four member family) is cleaved by gelatinase A and only MCP-3 is bound by the hemopexin C domain, strongly supporting this concept.

The catalytic specificity of MT1-MMP for MCP-3 is also likely the result of hemopexin C domain exosites as this domain showed preferential binding to MCP-3 compared to the other three MCPs tested. However, the presence of a

hemopexin C domain does not necessarily confer catalytic competence against MCP-3. Gelatinase B, the MMP that is most closely related to gelatinase A, does not cleave MCP-3. We predict that the gelatinase B hemopexin C domain lacks exosites for MCP-3 and does not bind the chemokine. However, it is also possible that the distant placement of the gelatinase B hemopexin C domain imparted by the O-linked glycosylated extended linker connecting the catalytic and hemopexin C domains positions the domain too far from the catalytic domain to potentiate cleavage (Mattu *et al.*, 2000). Nonetheless, it has recently been shown that gelatinase B can cleave the chemokine IL-8 enhancing its biological activity (Van Den Steen *et al.*, 2000), but not the related chemokines NAP-2 or RANTES (McQuibban and Overall, unpublished data) demonstrating that gelatinase B can cleave chemokines, but that the cleavage is specific.

Perhaps the most compelling data supporting the concept of the hemopexin C domain exosites being critical in chemokine proteolysis is the analysis of the hybrid and mutant MCP-3 chemokines. Ablation of the binding interaction between chemokine and enzyme abolished catalysis despite the presence of the scissile bond in the correct context from S_4 to S_6'. Moreover, cleavage of the chemokine still occurs even if the scissile bond sequence is mutated provided the exosite-binding interaction is maintained. These data strongly suggest that the binding interaction between chemokine and hemopexin C domain exosites favors the introduction of the flexible scissile sequence into the active site to form an anti-parallel β-sheet that is required for cleavage.

In vivo chemokine expression is often transient and the levels of

chemokine are very low, typically in the nM or pM range. Therefore, a binding domain that increases the likelihood of a productive interaction between a rare substrate and a proteinase is predicted to promote more efficient catalysis *in vivo*. However, it is a fine balance to optimize substrate-binding affinity to be sufficient to potentiate cleavage, but not too strong to inappropriately retain cleaved product that would inhibit subsequent binding and cleavage of new substrate. Indeed, we measured the K_d of the interaction between full-length and MMP-cleaved MCP-3 and found it to be only in the micromolar range, an affinity that is generally not regarded as particularly strong. This concept has important implications in the biology of multidomain proteinases such as MMPs and the serine proteinase networks involved in complement activation, clotting, and fibrinolysis. In a proteinase family variation in exosite binding domains would be a facile method to increase substrate diversity. Thus, it is likely that new substrates will be found which specifically bind to the hemopexin C domains of other MMPs.

The location of the MCP-3 binding exosite on the hemopexin C domain is at present unknown and is now under active study in our laboratories. Notably, TIMP-2 binding does not preclude the MCP-3 interaction and MCP-3 binding and cleavage does not inhibit gelatinase A activation by formation of the trimeric complex with TIMP-2 and MT1-MMP. Since TIMP-2 binds on the outer rim of the hemopexin C domain at the junction of beta-blades III and IV, a site orientated away from the catalytic domain (Overall *et al.,* 1999), our data excludes this region from MCP-3 binding. From the 3D structure of the spatial relationship

between the catalytic and the hemopexin C domains of gelatinase A (Morgunova *et al.,* 1999), the MCP-3 exosite is likely on the top face of the domain, possibly on its uppermost rim at the intersection of beta blades I and II, which forms an extended lip with the active site cleft.

An interesting consideration in gelatinase A function *in vivo* is the presence or absence of TIMP-2 bound to the hemopexin C domain of the active enzyme. Since we have demonstrated that MCP-3 can still bind the hemopexin C domain even if complexed with TIMP-2 for efficient cleavage *in vivo,* it matters not whether TIMP-2 is bound to the hemopexin C domain of active gelatinase A.

Gelatinase A has been implicated in both physiological and pathological processes with the pivotal role of extracellular matrix degradation during tumor metastasis (Lochter *et al.*, 1998) spawning interest in MMP inhibitors as therapeutic agents (Yu *et al.*, 1997). Initial studies with the broad-spectrum peptide-based active site MMP inhibitor Marimastat have demonstrated some clinical efficacy; however, side effects from inhibiting metalloproteinase action have been problematic (Wojtowicz-Praga *et al.*, 1998). Our data demonstrating the importance of exosite interactions outside the active site leads us to suggest that inhibitors could be designed that disrupt a specific substrate-binding interaction, but spare other enzyme activities, such as those directed by the collagen binding domain. Exosite-based inhibitors would be predicted to specifically inhibit enzymic activity against a limited subset of the substrate repertoire resulting in highly selective inhibitors with a consummate reduction in potentially harmful side effects. Since our data shows that MCP-3 binding and

cleavage does not disrupt the gelatinase A/TIMP-2 interaction, the design of an inhibitor of chemokine or other substrate-binding to the hemopexin C domain is even more appealing, as the cell surface biology and localization of the enzyme may not be disrupted.

This Chapter represents a manuscript that has been submitted to the Journal of Immunology. The manuscript details an initial enquiry into the possibility that multiple chemokines will be substrates for multiple MMPs. This manuscript deals exclusively with the monocyte chemoattractant protein family. The data presented in this Chapter represents work done by the candidate with technical assistance for chemotaxis experiments provided by Neidy Gong. Chemokines were provided by Dr. Ian Clark-Lewis.

Chapter 5 Chemokine gradient disruption by matrix metalloproteinase activity.

Summary

CC chemokines are potent chemoattractants for the directed migration of leukocytes to sites of host challenge. Chemokine receptor usage specifies cell type migration and receptor affinities dictate potency. We have recently identified monocyte chemoattractant protein (MCP)-3 as a substrate for gelatinase A of the matrix metalloproteinase (MMP) family of extracellular endoproteinases. Here, we have screened and shown that MCP-1, -2, -3, and -4 are cleaved by several of these important inflammatory proteinases resulting in altered bioactivity of the cleaved chemokine. Precise MMP trimming of the N-terminus of MCPs ablates activity and creates effective receptor antagonists. These findings lead us to suggest a new paradigm in chemokine biology. MCP production attracts leukocytes at the onset of challenge. Pro-inflammatory cytokine-induced MMP expression by the stroma then attenuates the chemoattractant gradient, antagonizing further infiltration and facilitating tissue resolution. Thus, the traditional view of MMPs being effectors of tissue demolition and facilitating leukocyte extravasation is broadened to include a homeostatic regulatory role in modulating inflammation in healing and disease.

Introduction

Chemokines are potent chemoattractants produced locally in tissues for the directed migration of leukocytes to sites of host challenge in infection, inflammation and cancer (Foxman *et al.*, 1997). Tissue gradients of inflammatory chemokines attract and maintain inflammatory cells at sites of irritation. Chemokines can be divided into families according to the position and spacing of N-terminal cysteine residues. Presently, the C, CC, CXC, CX_3C families are recognized (Zlotnik and Yoshie, 2000). The CC chemokine family includes 23 unique proteins with varying specificities and affinities for 10 different CC receptors. The monocyte chemoattractant protein (MCP) CC family targets multiple leukocyte subsets (monocytes, basophils, eosinophils, dendritic cells, and natural killer cells) whereas in the initial phases of inflammation, CXC chemokines are important for attracting polymorphonuclear leukocytes. Consequently, chemokines are important mediators of many pathologies, including chronic inflammatory and auto-immune diseases where the coordinated expression of MCPs and resultant leukocyte infiltration correlates with disease progression (Schluger and Rom, 1997; Baggiolini, 1998).

An important question in chemokine biology is how the chemoattractant signal is squelched to restrict prolonged transmigration that allows for clearance of the inflammatory infiltrate resulting in tissue resolution. Cell culture production of proteolytically processed chemokines has been reported (Proost *et al.*, 1998;

Wuyts *et al.*, 1999) and the effects of engineered truncated chemokines on cell behavior have been characterized (Gong and Clark-Lewis, 1995; Stuyf *et al.*, 1998). Considerable emphasis has been placed on the role of the serine exopeptidase dipeptidyl peptidase IV/CD 26 (Van Damme *et al.*, 1999), which can process many bioactive molecules with a penultimate N-terminal proline. However, the activity of CD26 in chemokine cleavage and inactivation *in vivo* has not been demonstrated.

The matrix metalloproteinase (MMP) family of zinc-dependent endoproteinases are either secreted or cell membrane bound enzymes with a broad substrate specificity that collectively can degrade most components of the extracellular matrix (Basset *et al.*, 1997). Matrix proteolysis is a hallmark of inflammatory reactions. Indeed, leukocyte extravasation and migration requires matrix cleavage. In particular, leukocyte-derived collagenase-2 (MMP-8) and gelatinase B (MMP-9) are the prominent early proteolytic mediators of matrix degradation allowing effector cell egress to the site of tissue damage (D'Haese, 2000; Coussens *et al.*, 2000). Following this initial leukocyte proteolytic phase, stromal cells, in response to pro-inflammatory cytokines secreted by the infiltrate, produce MMPs to amplify the tissue destructive phase. A provisional matrix is then deposited in association with decreased MMP expression and an increase in tissue inhibitor of metalloproteinase (TIMP) expression. In the final stages of healing, the provisional wound matrix is remodeled. General MMP production

ceases under the influence of transforming growth factor (TGF)-β1 which orchestrates wound healing by pleiotropic effects including the stimulation of extracellular matrix production (Overall *et al.*, 1991). Traditionally, structural matrix components are viewed as the major substrate class for MMPs (Nagase and Woessner, 1999). However, it is now becoming appreciated that MMPs have a wider substrate repertoire that includes bioactive molecules. Indeed, MMPs can cleave and release pro-tumor necrosis factor (TNF)-α (Gearing *et al.*, 1994), fibroblast growth factor receptor (Levi *et al.*, 1996), serpins (Mat *et al.*, 1991), and the MMP-7 dependent activation of α-defensin (Wilson *et al.*, 1999) which is an important step in regulating innate immunity.

We have recently reported the use of the yeast two-hybrid system to screen for potential MMP extracellular substrates (McQuibban *et al.*, 2000; Chapter 3). Screening resulted in the identification of monocyte chemoattractant protein (MCP)-3 as a binding protein and substrate for gelatinase A (MMP-2). This was a specific interaction since gelatinase A did not cleave MCP-1, -2, or -4. Since MMP substrate specificity overlaps to form a robust proteolytic system, we have now investigated whether other MMPs also process MCP-3 in a similar manner to gelatinase A, and whether other MMPs cleave the MCPs not cleaved by gelatinase A. Here we report that MCP-1, -2, -3, and -4 are also susceptible to efficient proteolysis by multiple MMPs to typically remove a N-terminal tetrapeptide. This results in loss of agonist activity to generate powerful receptor

antagonists. These data lead us to propose a new model whereby inflammatory induced MMP activity leads to the dissipation of inflammatory cell infiltrates, which in turn results in reduced MMP expression in a homeostatic feedback loop—aberrations of which may contribute to chronic inflammatory pathologies.

Materials and Methods

Production of MMPs and MCPs

Recombinant MMPs were expressed in CHO cells and purified from the conditioned CHO-SFM culture medium. Briefly, conditioned culture medium was collected and enzymatic activity was purified by gelatin-Sepharose chromatography for gelatinases. The other MMPs were purified by combinations of heparin-agarose and dye-ligand chromatography as appropriate. MT1-MMP was produced in its soluble, transmembrane deleted form. MCP-1, -2, -3, and -4 were chemically synthesized using solid phase methods, the polypeptides were purified by reverse phase HPLC and folded using air oxidation (Gong *et al.*, 1996).

Enzyme analysis

p-aminophenylmercuric acetate (APMA)-activated MMP (10 ng) and 10 µg MCP-3 were mixed together in CAB buffer (100 mM NaCl, 5 mM $CaCl_2$, 20 mM Tris, pH 8.0) and incubated at 37 °C. Aliquots were removed at 1 hr intervals and product accumulation was monitored by densitometric analysis of Coomassie-stained Tris-tricine SDS-PAGE gels. The k_{cat}/K_m specificity constant was calculated from graphical determination of the k_{obs}. Electrospray mass spectrometry of the reaction products was used to identify the scissile bond.

Calcium and chemotaxis assays

Monocytic THP-1 cells were loaded with Fluo-3AM for 30 min at 37 °C. The fluorescence was monitored with a Perkin-Elmer 650-10B spectrofluorimeter using an excitation wavelength of 506 nm and an emission wavelength of 526 nm. Desensitization assays were performed by sequential addition of MMP-cleaved MCP-3 or buffer control, followed by the full length chemokine. THP-1 cell migration was assessed in transwell trays (Costar). MCP-3 and MMP-cleaved MCP-3 were added to the lower well, and THP-1 cells (1×10^7 cells/ml) to the upper well. The percent migration was calculated by dividing the mean number of migrating cells in response to chemokine by the mean number of cells migrating in response to medium alone.

Results and Discussion

MMPs are implicated in many physiological processes involving matrix turnover (Werb and Chin, 1998), but direct evidence for this is limited. However, MMPs are clearly identified with pathologies, including arthritis and tumor metastasis (Curran and Murray, 1999) where it is assumed they have primarily a matrix degradative role. A broader substrate repertoire is now being revealed by recent studies that include signaling molecules like MCP-3. Since MMPs are differentially expressed in many tissues and cell types, we have now determined the specificity of MMP-mediated processing of MCP-3. Eight recombinant MMPs were tested for their catalytic competence against the chemokine. Incubation of MCP-3 with MMP-1, -2, -3, -13, and -14 resulted in a small but distinct increase in electrophoretic mobility on Tris-tricine gels whereas MMP-7, -8, and -9 were unable to cleave MCP-3 (Figure 5.1). Electrospray mass spectrometry was used to identify the MCP-3 scissile bond that was Gly^4-Ile^5 for each proteolytically competent MMP. The pattern of MMP cleavage suggests that this is a specific ability of the MMP family. Indeed, matrilysin (MMP-7), which is thought to be the MMP with the most promiscuous proteolytic activity, did not cleave MCP-3. Thus, in addition to the primary sequence, additional elements of substrate specificity are clearly important, including binding by the hemopexin C domain of these MMPs (McQuibban *et al.*, 2000; Chapter 4). Since previous reports (Proost *et al.*, 1998a, 1998b) have characterized N-terminal truncated

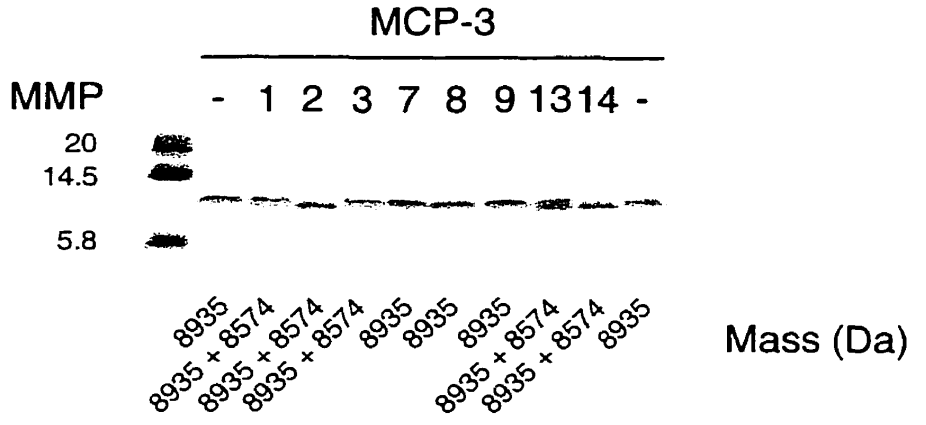


Figure 5.1 Specificity of MMP cleavage of MCP-3.

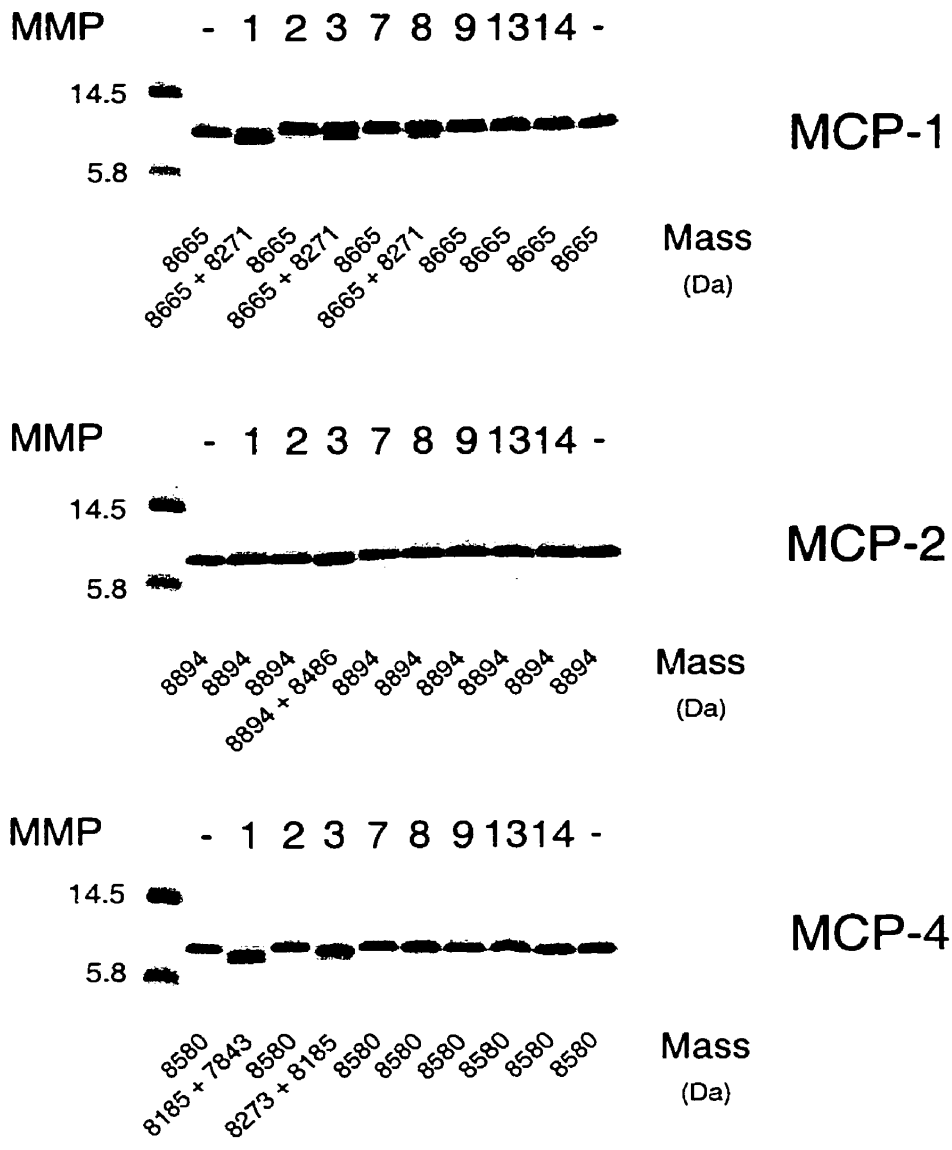
Electrospray mass spectrometry and Tris-tricine gel analysis of MCP-3 cleavage products produced by the recombinant MMP activity. APMA-activated MMP (1 ng) was incubated with 1 µg MCP-3 in MMP assay buffer at 37 °C for 8 hours. Reaction was stopped by the addition of SDS-PAGE sample buffer.

forms of MCP-1 and -2 isolated from cell cultures, we tested the ability of MMP-1, -2, -3, -7, -8, -9, -13, and -14 to cleave MCP-1, -2, and -4. Figure 5.2 shows that MCP-1 is efficiently cleaved at Ala^4-Ile^5 by collagenase 1 (MMP-1), to a lesser extent (20% of total by densitometric analysis) by stromelysin 1 (MMP-3), and to a very minor extent (5% of total) by neutrophil collagenase 2 (MMP-8). In contrast, MCP-2 was only susceptible to cleavage at Ser^4-Val^5 by stromelysin 1. Interestingly, MCP-4 was trimmed at Asp^3-Ala^4 and Ala^4-Leu^5, by both collagenase 1 and stromelysin 1 and also at Val^7-Pro^8 by collagenase 1, as summarized schematically in Figure 5.2. Hence, these data demonstrate the similar pattern of cleavage and general property of MMP cleavage across the MCP chemokine family.

The physiological likelihood of *in vivo* chemokine cleavage by MMPs was revealed by the high kinetic turnover rates (Table 5.1). Notably, gelatinase A and MT1-MMP are the most efficient at cleaving MCP-3 *in vitro*, but interestingly neither cleaves MCP-1, -2, or -4. This points to a unique role for MCP-3 cleavage by MT1-MMP and gelatinase A. MT1-MMP is the physiological activator of gelatinase A, and forms a cell-surface receptor for this enzyme (Strongin *et al.*, 1995). MT-MMPs are also critical for cell migration in collagen (Hotary *et al.*, 2000), an important feature of remodeling wounds. We have previously postulated that establishing the gelatinase A/MT1-MMP proteolytic axis changes

Figure 5.2 MMP cleavage of MCP-1, -2, and -4.

Electrospray mass spectrometry and Tris-tricine gel analysis of MCP-1, -2, and -4 cleavage products produced by recombinant MMP activity. APMA-activated MMP (1 ng) was incubated with 1 μg MCP in MMP assay buffer at 37 °C for 8 hours. Reaction was stopped by the addition of SDS-PAGE sample buffer. The mass of the molecular weight markers is indicated.


MMP - 1 2 3 7 8 9 13 14 -
14.5
5.8
MCP-1
8665
8665 + 8271
8665
8665 + 8271
8665
8665 + 8271
8665
8665
8665
8665
Mass
(Da)
MMP - 1 2 3 7 8 9 13 14 -
14.5
5.8
MCP-2
8894
8894
8894
8894 + 8486
8894
8894
8894
8894
8894
Mass
(Da)
MMP - 1 2 3 7 8 9 13 14 -
14.5
5.8
MCP-4
8580
8185 + 7843
8580
8273 + 8185
8580
8580
8580
8580
8580
8580
Mass
(Da)
MMP-1, -3
MCP-1 Q-P-D-A-I-N-A-P-V-T-C-C...
-3
MCP-2 Q-P-A-S-V-S-I-P-I-T-C-C...
-1, -2, -3, -13, -14
MCP-3 Q-P-V-G-I-N-T-S-T-T-C-C...
-1, -3
-1, -3
-1
MCP-4 Q-P-D-A-L-N-V-P-S-T-C-C...
1
10

MMP	k_{cat}/K_m ($M^{-1}s^{-1}$)	Mass[1] of MCP-3 after incubation with MMP
MMP-1	2,600	8,574 Da
MMP-2	8,000	8,574 Da
MMP-3	2,700	8,574 Da
MMP-7	0	8,935 Da
MMP-8	0	8,935 Da
MMP-9	0	8,935 Da
MMP-13	2,600	8,574 Da
MMP-14	6,000	8,574 Da

[1]Mass of full length (uncleaved) MCP-3 = 8,935 Da.
A mass of 8,574 Da corresponds exactly to MCP-3 (5-76) with a cleavage site at Gly^4-Ile^5.

Table 5.1 Kinetic analysis of MMP cleavage of MCP-3.

Eight MMPs were assayed for their efficiency at processing MCP-3. Graphical analysis of time course reactions up to 18 h that monitored production of cleaved MCP-3 determined the k_{cat}/K_m values. Also shown is the mass of the reaction products as determined by electrospray mass spectometry.

the proteolytic profile of stromal cells from collagenolytic to gelatinolytic (Overall and Sodek, 1990; Overall, 2000). These processes are likely to predominate at different phases in the inflammatory and repair cycle. Thus, focal proteolysis of a MCP-3 by membrane-bound enzymes in both phases is likely to be augmented by several secreted MMPs, but only during tissue resorption. Therefore, because both gelatinase A and MT1-MMP are effective at proteolytically processing MCP-3, continued disruption of the chemokine gradient has the potential to occur late into the connective tissue formative phase. Thus, the presence of a general CCR-1, -2, and -3 antagonist at these times has the potential to stabilize the new matrix by restricting new leukocyte migration.

Previous protein engineering studies by us and others (Gong and Clark-Lewis, 1995; Gong *et al.*, 1996, Hemmerich *et al.*, 1999) have demonstrated that modification of the N-terminus of MCP chemokines alters receptor binding and activation. Therefore, pathophysiological cleavage of MCPs by MMPs has the potential to generate natural antagonists. To address this, we compared the ability of full length and MMP-cleaved MCP-3 to elicit a cellular response in THP-1 monocytes that express CCR-1 and -2. In response to chemokines that bind to these receptors, this cell line releases cytosolic free Ca^{2+}, and migrates toward a chemotactic gradient. Figure 5.3 shows that the MMP-cleaved form not only lost receptor agonist activity (Figure 5.3A, bottom scan, first arrow indicates treatment with MMP-cleaved MCP-3 showing no change in fluorescence) but also

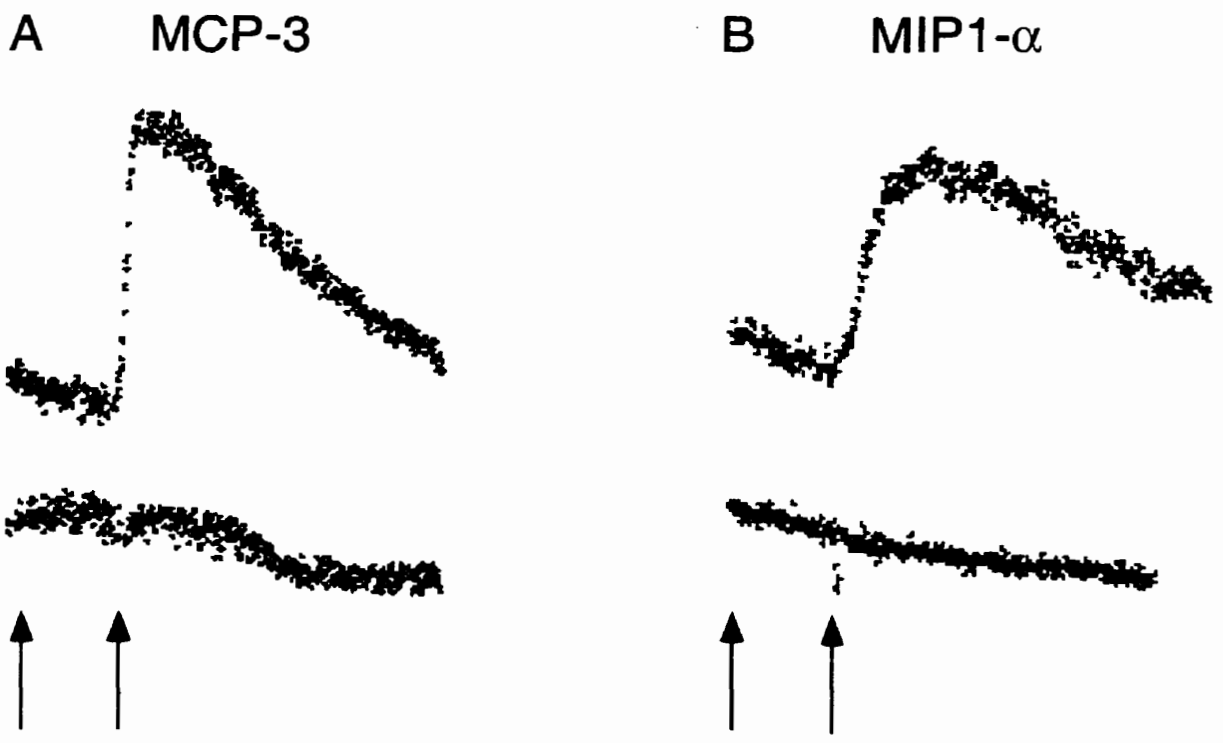


Figure 5.3 Receptor activity of full length and MMP-cleaved MCP-3.

A; Loss of intracellular calcium induction by MCP-3 following MMP cleavage and antagonist activity of the processed MCP-3. First arrow indicates pre-treatment of Fluo-3AM loaded monocytes with buffer control (top scan) or 300 nM MMP-cleaved MCP-3 (bottom scan). Second arrow indicates treatment of monocytes with 10 nM full length MCP-3. **B**; As in **A** except with full length MIP-1α as second arrow treatment.

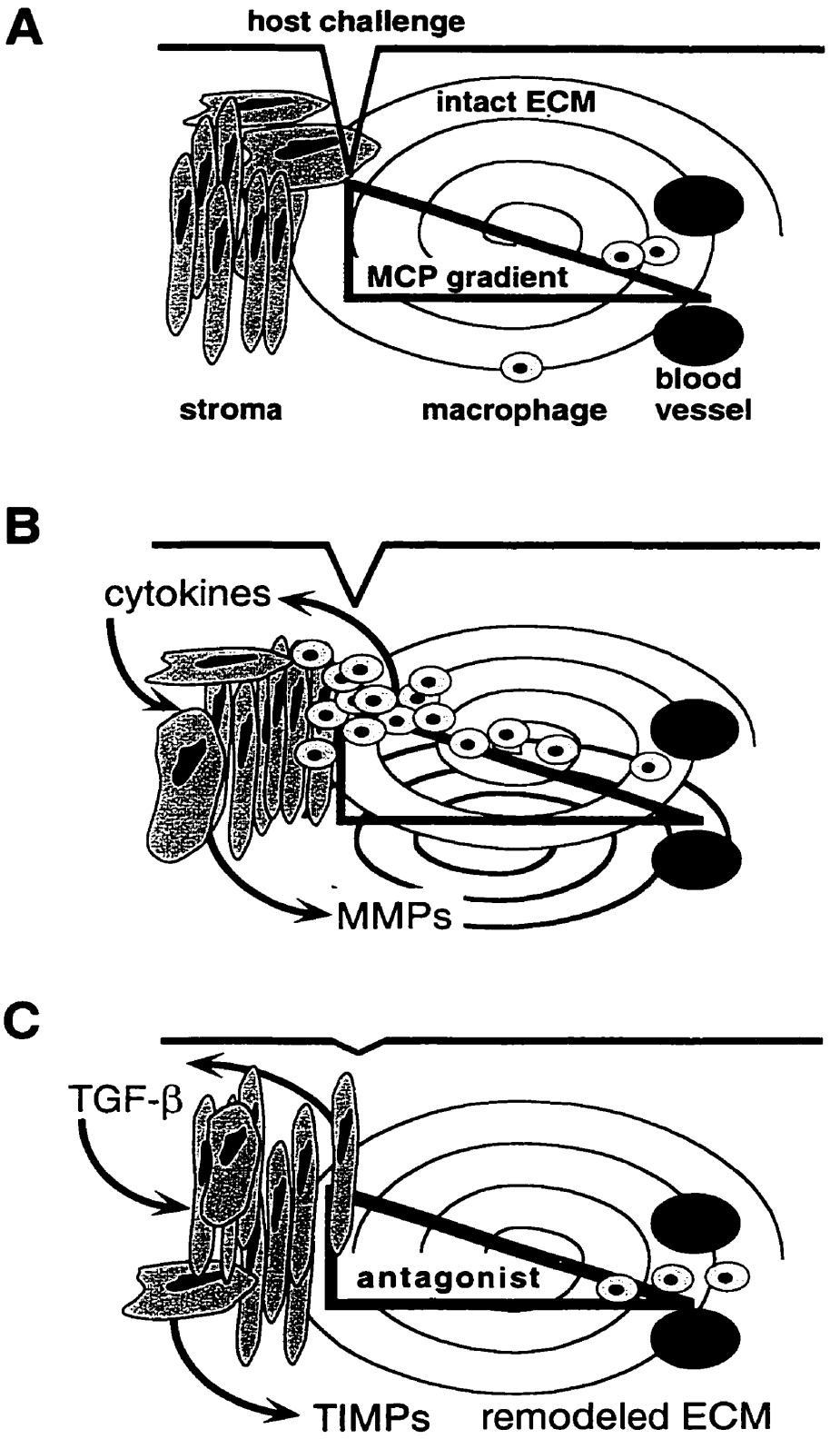
antagonized the subsequent response to full length MCP-3 (Figure 5.3A bottom scan, second arrow indicates treatment with full length MCP-3). MMP-cleaved MCP-3 also antagonized the effects of both MIP1α (Figure 5.3B, bottom scan), which binds CCR-1, and MCP-1 (not shown), which binds CCR-2.

Chemokine processing has been observed in cell cultures following treatment with cytokines, growth factors (Proost *et al.*, 1998a, b), and ConA (McQuibban *et al.*, 2000), conditions which model aspects of inflammation. Characterization by Proost *et al.* revealed their N-terminal truncations to be MCP-1 (5-76 and 6-76), MCP-2 (6-76), and C-terminal truncations MCP-1 (1-69) and MCP-2 (1-74). Thus, it is possible that production of MCP-1 (5-76) in their conditions is due to MMP activity. Here, we have characterized the involvement of the MMP family in the processing of MCP-1, -2, -3, and -4, and its implications in the inflammatory response and tissue resolution. The pattern of MMP proteolysis of the MCP family is striking, most notable is the inability of the primarily leukocyte enzymes collagenase-2 and gelatinase B to process these chemokines efficiently. We propose the following model (see Figure 5.4) describing the connection between the activity of chemokines and MMPs in the stages that define the inflammatory reaction.

1] As a result of tissue injury or infection, blood leukocytes extravasate from the blood vessel. This process is a defined sequence of slow rolling, adhesion, and migration. MCP chemokines play a pivotal role in initiating this to attract the

Figure 5.4 Model of MMP and chemokine action during inflammation.

A; In response to host challenge, a chemotactic gradient is laid down in the matrix for the attraction of leukocytes from the vasculature. **B**; Infiltration requires matrix proteolysis such as leukocyte-derived collagenase-2 and gelatinase B. As the cellular infiltrate builds, growth factors and cytokine concentrations increase following release from the infiltrate. Stromal cells respond to cytokines by increasing MMP expression. **C**; MMP activity converts MCP chemoattractant gradients to antagonistic gradients of CCR-1, -2, and -3, in addition to clearance of matrix fragments. With time, general MMP expression is suppressed with increased TIMP levels under the influence of TGF-β1, which orchestrates the final remodeling of the extracellular matrix.


A
host challenge
intact ECM
MCP gradient
stroma
macrophage
blood vessel
B
cytokines
MMPs
C
TGF-β
antagonist
TIMPs
remodeled ECM

leukocytes to the site of challenge. Chemokine gradients are laid down in the stromal matrix surrounding the blood vessels, enhanced by their high affinity for heparan sulfate, a major component of the extracellular matrix. Concurrently, cytokine expression, notably interleukin-1 and TNF-α by infiltrating cells induce the expression and secretion of MMPs (Mackay *et al.*, 1992).

2] Chemoattractant-directed leukocytes secrete MMPs to assist in cell migration to reach the site of tissue damage (McCawley and Matrisian, 2000). Leukocytes almost exclusively secrete gelatinase B and neutrophil collagenase 2, enzymes that degrade the type IV collagen component of the basement membrane and stromal collagens, respectively, but are not competent for MCP cleavage. Basal expression of gelatinase A, the other major type IV collagen degrading MMP, is notably low or absent in leukocytes (Schwartz *et al.*, 1998). Hence, selective MMP proteolysis favors leukocyte migration along the cognate MCP gradients that are not disrupted by leukocytic MMPs, allowing for the accumulation of effector cells in the inflammatory infiltrate.

3] As the inflammatory reaction progresses, the stromal cells respond to the influx of leukocytes. In addition to secreting proteases, infiltrating monocytes, macrophages and basophils release many growth factors and pro-inflammatory cytokines. In response, stromal fibroblasts adapt a matrix-degradative phenotype. Proteinase secretion contributes towards the bulk removal of matrix in the wound prior to tissue resolution. Following matrix substrate depletion, we

propose that MMPs then efficiently destroy the MCP chemotactic gradients. MMP processing creates CCR-1, -2, and -3 antagonist gradients, resulting in the depletion of cellular infiltrates by leukocytes expressing these 3 receptors. In the tissue resolution phase the inflammatory reaction slows, transforming growth factor (TGF)-β represses general MMP expression and elevates TIMP and extracellular matrix component expression allowing for the deposition and stabilization of new matrix. Of note, TGF-β1 stimulates gelatinase A expression (Overall *et al.*, 1991), the most efficient MMP we found to cleave and inactivate MCP-3. Thus, gelatinase A may be considered an effector of TGF-β action to perform the final clearance and remodeling of the extracellular matrix whilst maintaining a CCR antagonist gradient of cleaved MCP-3.

There are over 50 human chemokines presently characterized and we have initiated a high-throughput screen of MMP-mediated processing of these. It is difficult to predict which other chemokines will be processed by MMP activity since we have found that both scissile bond sequence and precise domain structure contribute to the proteolytic susceptibility of the chemokine (see Chapter 4). Thus, simple scanning of databases for MMP-preferred scissile bonds is unlikely to be particularly insightful since not all scissile bonds will be surface exposed and proteinase accessible. Overall, our data strongly suggest that MMP proteolysis of chemokines may be a general mechanism to modulate inflammation and potentially other immune responses in a variety of physiological

and pathological processes.

Chapter 6 Conclusions and Perspectives

I consider that this thesis advances the MMP field with novel data in two main areas. First, I show strong evidence that MMPs, in addition to playing an important and pivotal role in effecting inflammation, can also regulate this process (Chapter 3). Second, I have dissected the key role of substrate-binding exosites, outside the catalytic center, in contributing towards specificity and efficiency of catalysis. I will discuss these separately, then consider the future directions of this project.

MMPs in inflammation

MMPs have long been known to be important mediators of tissue destruction during processes that involve an inflammatory response. MMP expression and secretion is critical in facilitating leukocyte transmigration through the basement membrane and extracellular matrix. Our discovery that the gelatinase A hemopexin C domain interacts with MCP-3 casts a new light on MMP activity in inflammation. The fact that gelatinase A did not degrade the chemokine, but precisely processes it to remove a tetrapeptide from the N-terminus hinted that this was an important regulatory process. Indeed, three other MCPs were similarly processed by other MMPs suggesting the evolution of a specific and potentially widespread function in regulating chemokine activity. Our data

demonstrating the creation of a general chemokine receptor antagonist from the agonist, along with a literature that supports the importance of the N-terminal region of the chemokine, established that this was likely a very important biological activity. Indeed, we were able to demonstrate *in vivo* the efficacy of this finding in both mice and humans (McQuibban *et al.*, 2000; Chapter 3).

Chemokine inactivation has also been well demonstrated by the cell membrane-bound serine protease CD26 that has dipeptidyl-peptidase activity. Despite its initial characterization over 20 years ago and an important report revealing the potential involvement of CD26 in HIV fusion and entry into T-cells (Broder *et al.*, 1994) there is still much speculation regarding the role of this proteinase in biology. Most reports only characterize the ability of recombinant soluble CD26 to remove dipeptides from the substrate, when a proline is the penultimate residue, and describe the resultant effects on the activity of the substrate. While there is no direct *in vivo* evidence for CD26-mediated chemokine processing, this proteinase is likely important in the regulation of circulating hormones and chemokines.

The implications of MMP inactivation of chemokines agrees nicely with the general understanding that MMPs are involved in biological processes that are both beneficial and detrimental to the organism. That inhibiting MMP activity can be problematic has been demonstrated in initial human trial results with general MMP inhibitors. At higher doses, patients developed inflammatory polyarthritis

(Wojtowicz-Praga *et al.*, 1998), most likely the result of prolonged and/or de-regulated inflammatory responses. Our data would support that hypothesis and provides a potential explanation for the development of chronic inflammation following MMP inhibitor administration. This has important implications on the future rationale of MMP drug design (see below) and therapeutic applications.

In terms of MMP proteolytic specificity of chemokine processing, in particular the lack of MMP-8 proteolytic specificity in processing MCPs, this is consistent with the general timetable of events during an inflammatory reaction. This has been discussed briefly at the end of Chapter 5, but merits additional comment here. There are two important aspects of MMP activity that will most likely facilitate a strong infiltration of leukocytes at the onset of inflammation, but reduce the immune response as tissue regeneration begins. Since MMP activity is induced by secretion from the effector cells and also from the surrounding stroma under the influence of various growth factors, predominantly IL-1 and TNF-α, MMPs are immersed in massive quantities of matrix substrates. It is our hypothesis that the "early" MMPs (-8 and -9 predominately) have little proteolytic activity towards the low levels of chemokines present in comparison to the matrix support molecules that must be degraded for transmigration of the leukocytes. It is at the later stages, when the majority of tissue destruction has occurred that chemokines will begin to be processed in biologically significant amounts.

As the chemokine gradients are disrupted in the degraded matrix, the

chemokines will be released and more accessible for processing by MMPs. I believe this timing of events will be orchestrated under the influence of TGF-β. TGF-β is considered a "wet-blanket" for inflammation, "putting out the fire", and allowing for regenerative and reparative processes to resolve the immune response. Interestingly, TGF-β expression is well correlated with the suppression of most MMP production, with the exception of gelatinase A. I believe that this selective gelatinase A expression at the end of inflammatory responses will ensure many chemokine gradients will be attenuated since gelatinase A was most efficient at creating the wide-acting antagonist MCP-3 (5-76). In general, the activity of MMPs as a chemokine processing enzymes at the later stages of inflammation may explain why these enzymes are required for an appropriate physiological response to tissue injury.

The complexity and need for a tightly regulated immune response may explain why the chemokine and MMP families are so redundant, or as more optimistic researches have termed, robust. It could be argued that this multiplicity points to the extreme importance of these two systems for the proliferation of the organism. A system in which the organism has great dependence for proliferation would by necessity have built-in back-up mechanisms to ensure fidelity of operation in the absence of any particular individual member. Indeed, for both the chemokine and the MMP fields, transgenic studies in mice would support this idea. The most beneficial aspect of

robustness is that the relative output of the system will be quite insensitive to alterations in the biochemical parameters (Hartwell, 1997). A good example for instilling robustness into a vital system is that natural polymorphism's that occur will have little effect on the overall outcome of the pathway. I believe an inflammatory reaction is a perfect example for the need for robustness. The number of players involved is amazingly high when one takes into consideration the cells involved, growth factors produced, chemokines, MMPs, and immune response molecules. This multiplicity will ensure the organism the proper output.

The importance of exosites

Chapter 4 in this thesis presents strong evidence for the requirement of the gelatinase A hemopexin C domain in directing efficient proteolysis of the chemokine substrate. It is our hypothesis that the hemopexin C domain contributes towards both the specificity of chemokine processing and catalytic efficiency. We demonstrated that both the gelatinase A and the MT1-MMP hemopexin C domain bound only to MCP-3 (cf. MCP-1, -2, -3, and -4), which matches the processing specificity of these enzymes. The MCP mutant and chimera data strongly support our hypothesis. We showed that changing the scissile bond sequence while maintaining the hemopexin C domain interaction resulted in reduced yet significant processing. In contrast, when the scissile bond sequence was placed on a background of negligible hemopexin C domain

interaction, processing was prevented. This data provides compelling evidence of the importance of substrate-binding exosites outside the catalytic domain in directing proteolysis. It has been shown for collagen degradation that there exist important regions on collagenases, including the hemopexin C domain (Murphy *et al.*, 1992), flexible hinge (Knäuper *et al.*, 1997), and loop region near the active site (Chung *et al.*, 2000) that direct or are required for the collagen degrading activity. Our data is important in that this is the first demonstration of exosite interactions on MMP cleavage of non-collagenous substrates.

There are still important kinetic issues to be resolved regarding our exosite data. We have shown that a hemopexin C domain-truncated gelatinase A (N-gelatinase A) still cleaves MCP-3 with relative efficiency (k_{cat}/K_m 500 M^1/s^{-1} as compared to 8,000 $M^{-1}s^{-1}$). This is strong evidence that the hemopexin C domain improves the turnover rate. I would expect there to be some binding interactions between the catalytic domain (presumably near the active site groove) and the N-terminal region of the chemokine. This would explain why N-gelatinase A could still process the chemokine. What was surprising was the extent to which the chemokine chimera MCP-3 (1-10)/MCP-1 (11-76) was such a poor substrate. I would have expected this substrate to have similar kinetics in that it reflects a similar situation as the N-gelatinase A experiment. It could be that there is a repulsive interaction between the MCP-1 domain and the hemopexin C domain. This may not allow the natural substrate (the N-terminal region of MCP-3) to

interact with and be cleaved by the catalytic region of the enzyme. We are presently attempting to resolve this by use of a peptide substrate based on the MCP-3 N-terminal sequence.

Implications for drug design

My work showing the importance of the gelatinase A hemopexin C domain has important ramifications on the design of MMP inhibitors. I believe it would be possible to design or screen for small peptide analogues that would disrupt one aspect of MMP function yet allowing the enzyme to fulfill other biological roles. Indeed, a recent report has characterized a chemical that selectively disrupts the gelatinase A hemopexin C domain interaction with a cell surface integrin (Silletti *et al.*, 2001). This group demonstrated that this inhibitor would reduce the amount of angiogenesis in their model system of metastasis whereas other aspects of gelatinase A function, namely proper and timely activation, remained. I believe that gelatinase A cleavage of MCP-3 and MMP cleavage of chemokines in general represents a beneficial aspect of MMP activity during inflammation. Thus, I would not suggest the design of an inhibitor that disrupted this activity. As the degradome of MMP proteinases becomes identified, I believe many of the substrates will show dependence on substrate-binding exosites outside the catalytic center, be it the hemopexin C domain, the collagen-binding domain, or the other MMP domains which have been described. Thus, mapping these sites

may provide leads for new drugs in different pathologies.

Future Directions

In many ways this project is just beginning. The yeast two-hybrid system has proved very useful in identifying at least one new MMP substrate. It is likely that with additional genetic screens, in particular with a variety of libraries, additional protein partners for the gelatinase A hemopexin C domain would be identified, some of which would surely be substrates. Indeed, 26 other positive clones representing putative gelatinase A hemopexin C domain interacting clones await further characterization. Two-hybrid screens could be done with other domains of the enzyme as described in Chapter 2. If only substrates are desired, a successful strategy may be to engineer a catalytically inactive enzyme (with MMPs an active site glutamic acid to alanine mutation) which should theoretically maintain binding interactions with potential substrates.

Beyond the almost limitless two-hybrid screening potentials, much work could be initiated based on the discovery of MCP-3 (5-76) as a biologically active chemokine receptor antagonist. Our experiments in mice demonstrated the activity of the MMP-cleaved chemokine in two established models of inflammation. It should be confirmed in other systems, for example the MRL-lpr mouse model of arthritis, that the MCP-3 antagonist serves to modulate the development of disease (C.M. Overall, in progress—Canadian Arthritis Network).

The relevance of MCP chemokine derivatives from MMP processing in human pathology could also be extended.

The neo-epitope antibodies that were generated would be extremely useful in detailing where, and at what levels, and in which human pathologies, the chemokine receptor antagonists were present. Additionally, design and production of antibodies based on the creation of new epitopes upon MMP cleavage of other chemokines could be initiated. If it became established that chemokine receptor antagonists were important players in human diseases, the design or screening of peptide analogues that would mimic this activity could be pursued. Several studies have begun this type of characterization of antagonists (Proudfoot *et al.*, 2000 for review), indicating the scientific and industrial interest in this type of endeavor.

Chapter 7 References

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