Biological Function of Mast Cell Chymase

In vitro and in vivo studies: a thorny pathway

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Abstract


Mast cells (MCs) are key effector cells in various types of inflammatory conditions. The MC secretory granules contain inflammatory mediators such as histamine, heparin proteoglycan (PG), cytokines and various heparin-binding proteases, including tryptases, chymases and carboxypeptidase A.

Previously, a mouse strain with a defect in its heparin biosynthesis was produced by targeting the gene for NDST-2 (N-deacetylase/N-sulfotransferase-2). These mice showed reduced levels of MC inflammatory mediators such as histamine and various heparin-binding proteases, including chymases, tryptases, and carboxypeptidase A. By using this mouse strain, we found that chymase in complex with heparin PG degraded fibronectin, suggesting a role for chymase in the regulation of connective tissue composition. Further, we found that chymase/heparin PG complexes degraded and thereby inactivated both thrombin and plasmin, suggesting an additional role for chymase in regulation of extravascular coagulation and fibrinolysis. However, although our findings implicated chymase in these processes, it was not possible to exclude the contribution to the observed activities by other MC components that are influenced by the knockout of NDST-2.

Out of the different mouse chymases, mouse MC protease 4 (mMCP-4) has the most similar tissue distribution, heparin-binding and angiotensin I-converting properties as the only identified human chymase. Thus, mMCP-4 may be the closest homologue to human chymase and we therefore chose to target the gene for mMCP-4. A mouse strain with a targeted inactivation of the mMCP-4 gene was generated. This mouse strain displayed defects in the regulation of thrombin and in fibronectin turnover, demonstrating a key role for mMCP-4 in these processes.

To address the role of MC proteases in the activation of matrix metalloproteases (MMPs) we used both the NDST-2 and mMCP-4 deficient mice. An analysis of peritoneal cells and tissue extracts from these mice revealed the presence of both pro-MMP-9 and active enzyme (MMP-9) in WT mice, but only the proform of MMP-9 was found in knockout mice. We also found that mMCP-4 can regulate the activation of pro-MMP-2 in the same manner. Our findings suggest that mMCP-4 plays a critical role in the activation of both pro-MMP-2 and pro-MMP-9 in vivo. The mMCP-4 knockout led to the accumulation of the fibronectin and collagen, resulting in fibrotic signs in the skin of the mMCP-4−/− mice. Possibly, mMCP-4-mediated activation of pro-MMP-2 and -9 may provide a link between MCs and processes that are regulated by MMPs.

Keywords: mast cell, serine protease, chymase, heparin, N-deacetylase/ N-sulfotransferase, fibronectin, thrombin, matrix metalloproteinase.

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To Life

Per aspera ad astra --
"Through hardships to the stars"
"Через тернии к звёздам"
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Papers I-IV

The present thesis is based on the following papers, which will be referred to by their Romanian numerals:


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Abbreviations

The following abbreviations are used in the text:

Ang  Angiotensin
BMMC  Bone marrow-derived mast cell
CD  Cluster of differentiation
ChSy  Chondroitin sulfate polymerase
CLP  Cecal ligation and puncture
CPA  Carboxypeptidase A
CS  Chondroitin sulfate
CTMC  Connective tissue mast cell
DPPI  Dipeptidyl peptidase I
DS  Dermatan sulfate
ES  Embryonic stem
GAG  Glycosaminoglycan
GalNAc  N-acetylgalactosamine
GlcA  Glucuronic acid
GlcNAc  N-acetylglucosamine
GM-CSF  Granulocyte-macrophage colony-stimulating factor
HDC  L-histidine decarboxylase
HIV  Human immunodeficiency virus
HS  Heparan sulfate
IdoA  Iduronic acid
Ig  Immunoglobulin
IL  Interleukin
LPS  Lipopolysaccharide
LT  Leukotriene
MC  Mast cell
MHC  Major histocompatibility complex
MIP-1α  Macrophage inflammatory protein-1α
MMC  Mucosal mast cell
mMCP  Mouse mast cell protease
MMP  Matrix metalloproteinase
MT1-MMP  Membrane type 1 metalloproteinase
NDST  N-deacetylase/N-sulfotransferase
NGF  Nerve growth factor
PAF  Platelet-activating factor
PG  Prostaglandin
PMSF  Phenylmethanesulfonyl fluoride
rMCP  Rat mast cell protease
SCF  Stem cell factor
SG  Serglycin
SLPI  Secretory leukocyte proteinase inhibitor
STAT  Signal transducer and activator of transcription
TGF-β  Transforming growth factor-β
TIMP  Tissue inhibitor of metalloproteinase
TLR  Toll-like receptor
TMT  Transmembrane tryptase
TNF-α  Tumor necrosis factor-α
<table>
<thead>
<tr>
<th>Acronym</th>
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<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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<tr>
<td>WT</td>
<td>Wild type</td>
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<td>Xyl</td>
<td>Xylose</td>
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Introduction

Mast cells

There is a great variety of infectious agents in the environment that continuously try to attack our organism. Our immune system protects us from the outer world. Various microorganisms (bacteria, viruses, protozoa, parasites) invade our body and induce specific immune responses. The principle components of the immune system are lymphocytes (B cells, T cells, large granular lymphocytes, monocytes (giving rise to macrophages), granulocytes (neutrophils, eosinophils, basophils, mast cells (MCs)) and platelets. Each type of cell produces and secretes an array of highly specific mediators, which are, in many cases, vitally important for our organism.

Itching, sneezing and wheezing are typical symptoms of allergy and other inflammatory events. The major troublemakers in these processes are MCs. These cells were first described by Paul Ehrlich in his doctoral thesis in 1878 (Ehrlich, 1878). He detected these cells in various organs of several animal species and introduced the term "mastzellen" meaning cells filled in their cytoplasm granules.

Development of mast cells

MCs arise from hematopoietic stem cells in the bone marrow. They circulate in blood as precursors (Galli, 2000; Metcalfe, Baram & Mekori, 1997). Mature MCs are not found in peripheral blood (Nakano et al., 1985) (Figure 1). These cells completely mature in peripheral mucosal or connective tissue microenvironments under the influence of specific growth factors (Metcalfe, Mekori & Rottem, 1995). The latter include stem cell factor (SCF), the ligand for tyrosine kinase c-kit receptor, interleukin (IL)-3 both in humans and rodents and nerve growth factor (NGF) in humans only. Other mediators such as IL-4, IL-9, IL-10 can also regulate MC differentiation (Galli, 2000; Wedemeyer & Galli, 2000).

To understand the MC biological functions, it is important to clarify the interactions of MCs with extracellular matrix components and other cells. Mast cells adhere to extracellular matrix through the binding to the matrix components, namely to laminin, fibronectin and vitronectin, via different integrins (Dastych et al., 1991; Thompson, Burbelo & Metcalfe, 1990; Walsh et al., 1991). The interaction of matrix components with MCs stimulates various processes in MCs including protein phosphorylation, histamine release and cell motility. For instance, membrane-bound SCF presented by fibroblasts interacts with c-kit on MCs, facilitating the aggregation and promoting the differentiation and survival of MCs (Adachi et al., 1992). In addition, activated MCs aggregate with T lymphocytes, suggesting a functional relationship between the cells (Oh & Metcalfe, 1996).

Both MC and basophil development is associated with almost exclusive expression of FcεRI receptor on their surface. FcεRI is the high affinity receptor for immunoglobulin E (IgE) and the member of the antigen receptor superfamily.
responsible for the connection of pathogen- or allergen-specific IgE with cellular immunologic functions. FcεRI expression occurs when the cells are still undifferentiated and intracellular granules cannot be detected (Tkaczyk & Gilfillan, 2001). Mature MCs are characterized by condensed nuclei and the abundance of granules in the cytoplasm.

Fig. 1. Development of mast cells from pluripotent progenitor cells. Differences in the mast cell granule components in human and mouse mast cell subtypes.

Mast cell activation

MC can be activated in different ways. Stimulation of the MC activation, initiated either by interaction of the antigen specific antibodies or the antigen with the corresponding MC receptors, is referred to as immunologic activation. Alternatively, the stimulation, induced by substances such as neuropeptides, basic compounds, cytokines, and certain drugs, is called nonimmunologic activation (Figure 2). Both immunologic and nonimmunologic stimulations produce morphologically similar degranulation events. However, biochemical processes preceding the degranulation are different.

**FcεRI**

The classical MC activation during inflammatory reaction occurs through the high-affinity receptor FcεRI. FcεRI is a tetrameric complex, where an extracellular α chain binds the Fc portion of IgE, whereas a transmembrane β chain along with disulfide-linked transmembrane γ chains participate in the signal transduction. Cross-linkage of IgE molecules bound to FcεRI on MCs with allergen/antigen causes MC degranulation. The cascade of signal transduction pathways triggers solubilization of the granule contents, granule swelling, membrane ruffling, fusion of the perigranular and plasma membranes, and, finally, leading to the exocytosis of granule content (Metzger, 1992; Turner & Kinet, 1999).
Degranulation of the mouse MCs can be also triggered by aggregation of the surface-expressed IgG receptors, \textbf{FcRII} and \textbf{FcRIII}. These low-affinity receptors may regulate high-affinity IgE receptor-mediated activation (Miyajima \textit{et al.}, 1997).

\textbf{Complement}

Complement-dependent signals are important components of the mechanisms by which MCs are activated during infections. MCs express \textbf{multiple receptors for the complement components} (anaphylatoxins) C3a, C4a and C5a. These receptors include CD11b (CR3), CD11c (CR4), which are up-regulated during systemic mastocytosis, and C5aR (Nilsson \textit{et al.}, 1996). It was shown that both C3- and C4-deficient mice demonstrated increased susceptibility to infection and reduced MC activation (Prodeus \textit{et al.}, 1997).

\textbf{TLR}

The TLR (\textit{Toll-like receptor}) family of pattern-recognition receptors has an important role in many host defense mechanisms. Different members of the TLR family are activated by pathogen-associated or endogenous proteins. The MC uses selected TLRs to respond to pathogens. For example, MCs respond to lipopolysaccharide (LPS) through TLR4. In contrast, activation by peptidoglycan from gram-positive bacteria and the yeast cell-wall component zymosan are mediated through the TLR2 receptor (Supajatura \textit{et al.}, 2002).

\textbf{Other activators}

MC activation can be triggered by other different agents. It was demonstrated that a number of cytokines (IL-1, IL-3) cause the release of histamine, SCF and macrophage inflammatory protein-1\(\alpha\) (MIP-1\(\alpha\)) activates the degranulation of MCs \textit{in vitro} and \textit{in vivo} (Mekori and Metcalfe, 2000).
Dextrans and lectins appear to activate MCs through a multipotent interaction with the cell membrane and a cross-linking of glucose receptors on the membrane. Other compounds that can directly activate MCs include calcium ionophores, substance P (neuropeptide), compound 48/80, and drugs such as morphine, codeine and synthetic adrenocorticotropic hormone, adenosine and endothelin (Metcalfe, Baram & Mekori, 1997).

Mast cell diversity

There is strong evidence that the morphological and histochemical diversity of MCs and, correspondingly, MC mediator functions are controlled by tissue local environment. Traditionally, two MC subtypes are distinguished in rodents: connective tissue MC (CTMC) localized in the sub-mucosa of the gastrointestinal tract, in the skin and in the peritoneum (Aldenborg & Enerback, 1985), mucosal MC (MMC), mainly found in the mucosa of the gastrointestinal system and in the lamina propria of the respiratory tract (Enerback, 1966; Metcalfe, Baram & Mekori, 1997). Beside the localization, there are also phenotypical differences between these MC subtypes, including size, histamine, protease and proteoglycan content (Figure 1).

In humans MCs are classified according to the protease content. Some human MCs (MC_TC), found in connective tissues, contain trypase, chymase, a cathepsin G-like protease, and carboxypeptidase. Another MC subclass (MC_T), found in mucosal tissues, contain only trypase (Irani, A.A. et al., 1986; Irani, A.M. et al., 1989). In comparison with rodents, human MCs have the major differences in protease content but not in histamine and proteoglycans. In rodents, MC granules are electron-dense and homogenous. MMCs have small granules with variable size, while CTMCs have large granules with uniform size (Amihai et al., 2001). Ultrastructural evaluation of MCs revealed a heterogeneity of the MC-granule structure in humans. Granules of MC_T have a scroll-rich morphology, whereas granules of MC_TC have either a grating or lattice-like structure with distinct periodicity (Weidner & Austen, 1991).

Characterization of mast cell mediators

Newly (de novo) synthesized mediators

Leukotrienes, prostaglandins and platelet-activating factor

The activation of MCs initiates de novo synthesis of lipid-derived mediators and some cytokines. De novo synthesized MC lipid mediators are the cyclooxygenase and lipoxygenase metabolites of arachidonic acid, which have potent inflammatory activity. The major lipoxygenase products are leukotrienes (LTs). Sulfidopeptide LT (LTC4) is converted to LTD4 and LTE4 after the release into extracellular space. Leukotrienes stimulate prolonged bronchoconstriction, bronchial mucus secretion, increased venular permeability and induce constriction of arterial and intestinal smooth muscle (Drazen & Austen, 1987; Robinson, 2004). Human MCs can produce LTB4 that has chemotactic activity for neutrophils, eosinophils and enhances lysosomal enzyme release (Lewis, Austen & Soberman, 1990).
The major cyclooxygenase product is prostaglandin (PG) D₂. PGD₂ acts as a bronchoconstrictor and vasodilator and increases vascular permeability (Goetzl, 1981). PGD₂ is an inhibitor of platelet aggregation, and it mediates accumulation of neutrophils, and together with some other PGs, has antiproliferative activity against tumor cells (Harris et al., 2002; Sasaki & Fukushima, 1994).

**Platelet-activating factor** (PAF) is a potent proinflammatory phospholipid that aggregates and degranulates platelets and leads to systemic hypotension, mediating systemic anaphylaxis (Stafforini et al., 2003).

Cytokines, chemokines and growth factors
Both mouse and human mast cells are a source of many cytokines, chemokines and growth factors, which are different in the two MC phenotypes. The mediators include: IL-1, IL-3, IL-4, IL-5, IL-6, IL-8, IL-10, IL-11, IL-13, IL-16, tumor necrosis factor-α (TNF-α), granulocyte-macrophage colony-stimulating factor (GM-CSF), SCF, vascular endothelial growth factor (VEGF), transforming growth factor-β (TGF-β) and chemokines, such as MIP-1α and monocyte chemoattractant protein-1 (Bradding et al., 1995; Galli, 2000) (more details about cytokines and growth factors function in different processes are described below).

Interestingly, peritoneal MCs were actually the first cell type that was shown to constitutively transcribe TNF-α and contain large amounts of TNF-α bioactivity (Gordon & Galli, 1990). Both the production and storage of TNF-α enables prolonged effects of the cytokine. TNF-α is important in many patho- and physiological conditions, including neutrophil recruitment in peritonitis and protection from endotoxic shock (Echtenacher, Mannel & Hultner, 1996; Malaviya et al., 1996). It upregulates the expression of E-selectin and intracellular adhesion molecule 1 on endothelial cells, thus facilitating the trafficking of both eosinophils and neutrophils to the inflammatory site (Gordon & Galli, 1991; Klein et al., 1989).

De novo synthesized MC mediators have important biological roles in inflammation, hematopoiesis, tissue remodeling and other biological processes.

**Preformed mediators**
MCs contain mediators that are stored preformed in the cytoplasmic granules. During activation they are released into the extracellular space. They include histamine, cytokines, proteoglycans and proteases.

**Histamine**
Histamine is a well-known MC mediator. It is a biogenic amine, which is formed after decarboxylation of histidine. During MC degranulation, histamine dissociates from the proteoglycan-protein complex. Histamine is released and metabolized within minutes, suggesting that its functional activity is present near the site of release. The multiple biological activities of histamine are attributed to its activation of four receptors (H₁, H₂, H₃, and H₄). For example, the interaction of
histamine with H1 receptors causes the contraction of airway, gastrointestinal smooth muscle and vascular permeability (White, Slater & Kaliner, 1987).

Important information about histamine function was gained after the creation of histamine deficient mice, by knocking out a histamine-forming enzyme (L-histidine decarboxylase, HDC) (Ohtsu et al., 2001). Using HDC-deficient mice, three major functions of histamine were evaluated: the significance of histamine for gastrointestinal action, vascular permeability and neurotransmission. There are also newer functions of histamine, disclosed by studies on HDC-deficient mice. The HDC-deficient mice demonstrated a delay in the development of angiogenesis. It was also shown that histamine plays a role in the negative regulation of neutrophil infiltration via H2 receptors in the allergic inflammation, in the suppression of the symptoms in systemic anaphylaxis reaction. Histamine is involved in the accumulation of eosinophils into the airways after allergic challenge. After stimulation of bacterial peritonitis, HDC−/− mice were faster in the clearance of the bacteria than wild type (WT) mice, suggesting that histamine inhibits bacterial exclusion (Ohtsu & Watanabe, 2003).

Proteoglycans

MCs contain different types of granule proteoglycans. The proteoglycans consist of polysaccharide chains, termed glycosaminoglycans (GAGs), which are covalently attached to a core protein.

Heparin and chondroitin sulfate (CS) are members of this group and are synthesized as GAG sidechains of proteoglycans. Heparin is found only inside of secretory granules of CTMCs, while the closely related heparan sulfate (HS) proteoglycans are expressed and secreted by most, if not all, mammalian cells. In contrast to CTMCs, MMCs synthesize chondroitin-4-sulfate (CS-A), chondroitin-4, 6-sulfate (CS-E) and also dermatan sulfate (DS) or (CS-B) (Enerback et al., 1985; Kolset & Gallagher, 1990; Stevens, R.L. et al., 1986). In humans, both types of MCs contain heparin and CSs (Lidholt, Eriksson & Kjellen, 1995; Stevens, R.L. et al., 1988).

The type of GAGs may vary, whereas the core protein may be the same. The serglycin (SG) core protein is the dominant proteoglycan found in the secretory granule of most haemotopoietic cell types, including MCs. Recently, SG knockout mice were generated. It was demonstrated that SG inactivation resulted in major defects in CTMCs, including affected morphology and ability to store MC secretory granule proteases (Abrink, Grujic & Pejler, 2004).

In general, biosynthesis of proteoglycans occurs in the Golgi compartment, and can be divided into three steps: chain initiation, polymerization and modification (Lindahl, Kusche-Gullberg & Kjellen, 1998). During the chain initiation, linkage tetrasaccharides (xylose (Xyl)-galactose (Gal)-Gal-glucuronic acid (GlcA)) are assembled on serine residues in the core protein. The polymerization is initiated by the work of N-acetylglucosaminotransferase (GlcNAc transferase) in the case of heparin/HS and N-acetylgalactosaminotransferase (GalNAc transferase) in the case of CS/DS respectively. Further, the polymerization is achieved through the activities of chondroitin sulfate polymerase (ChSy) or heparan sulfate polymerase (EXT1
family). Heparin/HS and CS/DS chains are composed of variably sulfated disaccharide units of iduronic acid (IdoA) IdoA\(\beta\)-1,4-GlcNAc\(\alpha\)-1,4/GlcA\(\beta\)-1,4-GlcNAc\(\alpha\)-1,4 and GlcA\(\beta\)-1,3-GalNAc\(\beta\)-1,4/IdoA\(\beta\)-1,3-GalNAc\(\beta\)-1,4, respectively (Esko & Selleck, 2002). As the chains grow, they are modified by a series of enzymes (Figure 3).

The differences in composition and extent of sulfation make heparin more charged than HS. Heparin is a unique CTMC product, suggesting specific functions for the CTMCs, in which it plays an important role. The first and key modification reaction in heparin/HS biosynthesis, is catalyzed by glucosaminyl N-deacetylase/N-sulfotransferase (NDST). It removes acetyl groups from GlcNAc residues, which are then sulfated through the N-sulfotransferase activity. There are four isoforms of NDST (NDST-1-4) (Grobe et al., 2002). NDST-3 and -4 transcripts are predominantly expressed during embryonic development (Aikawa & Esko, 1999; Aikawa et al., 2001). Targeting of the genes for NDST-2 and NDST-1 demonstrated that NDST-2 is essential for heparin biosynthesis while NDST-1 is essential for heparan sulfate (Fan et al., 2000; Forsberg et al., 1999; Humphries et al., 1999; Ringvall et al., 2000). Importantly, heparin deficient mice (NDST-2\(-/-\)) have defective CTMCs, namely an altered morphology and drastically reduced amounts of histamine and MC proteases in secretory granules. Thus, negatively charged heparin proteoglycan is involved in the packing and storage of positively charged proteins and histamine. Moreover, heparin proteoglycan can modulate MC protease activity, being in complex with protease, and facilitating the interaction with other proteins, especially with heparin-binding proteins, without changing the actual enzymatic activity (Kolset, Prydz & Pejler, 2004). Heparin is also needed in MCs for stabilization of the tryptase tetramer and it is required in the formation of the active tryptase tetramer (Hallgren et al., 2000;

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**Fig. 3.** A general scheme of the synthesis of chondroitin sulfate, dermatan sulfate, heparin sulfate and heparin glycosaminoglycans. (*) marks enzymes from knockout mice.
The NDST-2−/− mice were used as a model to study the role of MC proteases in different biological processes.

Heparin has been used for many years as a potent anticoagulant, an effect mediated by its ability to interact with anti-thrombin III via a specific pentasaccharide sequence (Lindahl et al., 1984). However, many of the other biological properties of heparin are unrelated to its anticoagulant activity. For example, it was shown that heparin is able to reduce the allergen and PAF-induced eosinophil infiltration in animals (Seeds, Hanss & Page, 1993). Heparin is also able to inhibit the recruitment of leukocytes to tissues such as the skin and gut by the direct inhibitory effects on the adhesion of leukocytes to vascular endothelium (Becker, Menger & Lehr, 1994; Xie et al., 1997). It was reported that heparin influences the fibroblast growth and collagen synthesis (Ferrao & Mason, 1993). Further, heparin is known to act as a competitive inhibitor of enzymes of the heparanase family, which are important in cell diapedesis and trafficking into tissues (Vlodavsky et al., 1994). The ability of heparin to modulate different aspects of the inflammatory response, including a role in the reduction of the accumulation of leukocytes in the lung, allowed to use it in the treatment of asthma and other inflammatory diseases.

Targeting of the gene for NDST-1 demonstrated a critical role of this enzyme in HS biosynthesis. Lack of the NDST-1 results in neonatal lethality. The newborn pups die shortly after birth due to a condition resembling respiratory distress syndrome (Fan et al., 2000; Ringvall et al., 2000). There are a number of other knockout animal models with targeted genes for the enzymes in heparin/HS biosynthesis. They include EXT-1, C5 epimerase, 2-O sulfotransferase and 3-O-sulfotransferase-1 (Bullock et al., 1998; Li et al., 2003; Lin et al., 2000; Shworak et al., 2002). These animal models are characterized by lethal phenotypes and abnormalities found during embryonic development, supporting the importance of HS in the different biological processes.

The main modification steps in the CS biosynthesis include 6-O-GalNAc sulfation (catalyzed by 6-O-GalNAc sulfotransferase) and 4-O-GalNAc sulfation (catalyzed by 4-O-GalNAc sulfotransferase). They result in the formation of CS-A (4-O-GalNAc sulfation) and CS-E (4-O- and 6-O-GalNAc sulfation), whereas in CS-B biosynthesis, 2-O-IdoA sulfation additionally takes place. The targeting of the gene for 6-O-GalNAc sulfotransferase revealed that the level of chondroitin GalNAc-6-O-sulfate was almost undetectable, while the level of CS-E (with GalNAc-4-O- and 6-O-sulfate residues) was unchanged. These results suggested that the 6-O-sulfation reaction leading to the disulfated GalNAc residues in CS-E is catalyzed by a separate enzyme, whereas the single sulfation of the GalNAc residues is catalyzed by 6-O-GalNAc sulfotransferase. The deletion of the 6-O-GalNAc sulfotransferase revealed decreased numbers of the naive T-lymphocytes (Uchimura et al., 2002).

Interestingly, hyaluronic acid (hyaluronan) is a nonsulfated GAG and is not covalently attached to core protein. Hyaluronan was found in rodent and human skin MCs (Eggli & Graber, 1993). It was demonstrated that MC granules are a rich source of hyaluronan, suggesting the involvement of hyaluronan in pathological
processes such as asthma and allergy (Knutson et al., 1993; Soderberg et al., 1989).

Neutral proteases
Another important group of preformed, cytoplasmic granule-associated mediators is the neutral proteases. Because the amount of space in the cytoplasm of MC limits the number of secretory granules it can retain, MCs have evolved post-translational control mechanisms that ensure that only biologically active proteins are targeted to the granules in defined molar ratios. Proteases in granules have a positively charged proteoglycan-binding face on the surface of the folded protease that resides away from the catalytic site (Sali et al., 1993). The positively charged face on the surface of each MC protease is made up of non-contiguous residues, which ensures that only properly folded proteases are bound to proteoglycans and thereby targeted to the granule. It was suggested that granule proteases and SG proteoglycans form macromolecular complexes in the trans region of the Golgi apparatus just before they are targeted to the secretory granule.

MC granule proteases are translated as inactive zymogens, containing a hydrophobic signal peptide followed by a propeptide that consists of 2 to 94 amino acids. The signal peptide is removed before the translated protease leaves the endoplasmic reticulum. The site of protease activation from zymogen precursors is not known. It may occur in a pre-secretory compartment, such as the Golgi apparatus, avoiding premature activation of the proteases, which could cause serious damage to the cell (Caughey, 2002).

One of the neutral proteases is carboxypeptidase A (CPA) found in CTMCs (in both human and rodent). CPA is a Zn$^{2+}$-dependent metalloprotease with exopeptidase activity, preferably cleaving substrates with C-terminal aromatic or aliphatic residues. It is stored in the secretory granules of MCs, where it forms macromolecular complexes with proteoglycans (Goldstein et al., 1989). Granule-bound CPA is fully active. It was suggested that the enzyme responsible for the processing of pro-CPA might be a thiol protease since treatment of MCs with selective thiol-protease inhibitors significantly increased amounts of pro-CPA (Springman, Dikov & Serafin, 1995). Recent data showed that the processing of pro-CPA to its active form requires the presence of heparin (Henningsson et al., 2002). In addition, CPA forms a complex with another MC protease, mMCP-5, indicating the necessity of this tandem for efficient storage of CPA and possible involvement in various biological processes (Huang, Sali & Stevens, 1998). Recently, it was shown that CPA together with mouse MC protease 4 (mMCP-4) plays a key role in the formation and degradation of angiotensin II (Ang II) (Lundequist et al., 2004).

Dipeptidyl peptidase I (DPPI), also known as cathepsin C, is a cysteine protease of the lysosomal group, found in cytotoxic lymphocytes, neutrophils and in MCs. DPPI plays an important role in cell growth (Thiele, McGuire & Lipsky, 1997) and in the activation of platelet factor XIII (Lynch & Pfuegger, 1988), MC proteases and neutrophil-derived serine proteases. It also regulates the development of acute experimental arthritis (Adkinson et al., 2002) but is also essential for activation of MC proteases. Human tryptase and chymase are synthesized as inactive zymogens
and are then activated by removal of NH₂-terminal dipeptides by DPPI (Murakami, Karnik & Husain, 1995; Sakai, Ren & Schwartz, 1996). To determine whether DPPI is essential for their activation in vivo, DPPI⁻/⁻ mice were created. It was found that MCs from these mice contain normal amounts of at least two chymases (mMCP-4 and -5), but these and other chymases are completely inactive. Moreover, the MCs contained reduced amounts of normally processed, active tryptase mMCP-6, suggesting that DPPI is required for the activation of chymases, but not tryptases in mice (Wolters et al., 2001). The activation of tryptase is a more complicated process, which requires additional processing of the enzyme (Sakai, Ren & Schwartz, 1996).

**Serine proteases**

Almost one-third of all proteases can be classified as **serine proteases**. The serine proteases are a class of proteolytic enzymes characterized by the presence of a uniquely reactive serine side chain. This mechanistic class was originally distinguished by the presence of the an aspartic acid, histidine and serine residue (Asp-His-Ser) "charge relay" system or "catalytic triad" (Carter & Wells, 1988). Serine proteases are endopeptidases. The potential sites of interaction of the substrate with the enzyme are designated P, and corresponding binding sites on the enzyme are designated S. The scissile bond (the bond to be cleaved) is the reference point. Residues on the amino-terminal side of the scissile bond are labeled P₁, P₂, P₃, and so forth, indicating their positions in relation to the scissile bond. By analogy, residues on the carboxyl side of the scissile bond are labeled P₁', P₂', P₃', and so forth. The corresponding enzyme sites are referred to as S₁, S₂ etc. and S₁', S₂' etc. (Schechter, I. & Berger, 1967) (Figure 4). The specificity of the serine proteases can be determined through recognition of the P₁ side-chain. S₁ specificity generally dictates the selective hydrolysis of naturally occurring protein substrates and, therefore, is the most important factor contributing to the limited proteolysis (Czapinska & Otlewski, 1999).

![Fig. 4. The definition of protease-substrate interactions. The amino acid residues toward the N-terminal end from the cleavage site are designated P₁, P₂, P₃ etc. The residues in the C-terminal end are designated P₁', P₂', P₃' etc. The corresponding binding sites on the enzyme are called S₁, S₂, S₃…and S₁', S₂', S₃' respectively. The scissile bond (in bold) is the reference point.](image)

**Chymotrypsin-like serine proteases: catalytic mechanism and specificity**
The chymotrypsin-like serine proteases catalyze the hydrolysis of proteins with selectivity for peptide bonds on the carboxyl-side of the aromatic side chains of Tyr, Trp, Phe, and hydrophobic residues such as Met. Trypsin-like protease and elastase are variations of the chymotrypsin-like proteases with difference in the substrate specificity. In the pocket of the substrate binding site of trypsin, a Ser is replaced by an Asp. This Asp in the pocket of trypsin can form a strong electrostatic bond with a basic Lys or Arg side-chain of a substrate. In elastase, the two Gly residues (as in chymotrypsin) are replaced by the much bulkier Val and Thr, giving the possibility to form the bond with small hydrophobic residues (Ala and Val) (Hedstrom, 2002).

In chymotrypsin and other serine proteases, highly reactive Ser\textsuperscript{195} plays a critical role. The first step of the hydrolysis of a peptide is acylation, the formation of a covalent acyl-enzyme intermediate. The acyl group of the substrate is linked to the oxygen atom of Ser\textsuperscript{195}. A proton is transferred from Ser\textsuperscript{195} to His\textsuperscript{57}. The positively charged His is stabilized by electrostatic interaction with negatively charged Asp\textsuperscript{102}. After attack by the oxygen atom of the hydroxyl group of Ser\textsuperscript{195} on the carbonyl carbon of the peptide bond, the carbon-oxygen bond of this carbonyl group becomes a single bond, along with formation of a transient tetrahedral intermediate. It is stabilized by hydrogen bonds between the negatively charged carbonyl oxygen (oxyanion) and two main-chain NH groups (oxyanion hole). In the second step, deacylation, the acyl-enzyme intermediate is hydrolyzed by water. Finally, the acid component of the substrate is released, ready for another round of catalysis (Carter&Wells, 1988; Kossiakoff&Spencer, 1981).

**Mast cell serine proteases**

The serine proteases of the MCs are stored in secretory granules in the mature form. The low pH of the granules helps to prevent the autolysis of the proteases (Huang,Sali&Stevens, 1998; Lagunoff&Rickard, 1983). They are positively charged at low pH, and form a tight complex with highly negatively charged proteoglycan chains. This interaction results in a decrease of electrostatic repulsion, allowing dense packaging of proteases in the granules (Schwartz et al., 1981). During degranulation, the MC serine proteases are released into pH 7.0 environment. After exocytosis most MC proteases remain in macromolecular complex with their proteoglycans. In some cases the large size of the exocytosed protease/proteoglycan complexes physically hinders the diffusion of the proteases in tissues. The different MC subtypes express different serine proteases. The most important types of serine proteases found in MC granules are chymases and tryptases (Table 1).
Table 1. Serine proteases in the different populations of mast cells

<table>
<thead>
<tr>
<th></th>
<th>MMC (mouse)</th>
<th>CTMC (human)</th>
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<tbody>
<tr>
<td>chymase</td>
<td>mMCP-1, mMCP-2, mMCP-9</td>
<td>mMCP-4, mMCP-5</td>
</tr>
<tr>
<td>tryptase</td>
<td>α-tryptase, β-tryptase, hTMT</td>
<td>mMCP-6, mMCP-7, mMTP</td>
</tr>
<tr>
<td>cathepsin</td>
<td>human cathepsin G</td>
<td>human cathepsin G</td>
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The first member of a novel subfamily of mouse MC serine proteases, mMCP-8, was found in MCs. A high degree of homology with mouse granzyme B in the critical regions for substrate specificity was observed. A comparative analysis of the amino acid sequence of mMCP-8 revealed a close relationship to cathepsin G and T cell granzymes. MMCP-8 expression is low in normal tissues and is completely absent in MMCs, whereas it is abundantly present in the lungs of *Schistosoma mansoni* infected mice. It was concluded that mMCP-8 belongs to the MC proteases but is distinct from both chymases and tryptases (Lutzelschwab et al., 1998). MMCP-9, another member of the family of serine proteases, is selectively expressed in uterine MCs under normal conditions. MMCP-9 was also observed in the jejunal submucosa of *Trichinella spiralis* infected mice. Although mMCP-9 exhibits a high degree of homology with other chymases, it has a unique substrate binding cleft, suggesting the involvement of this protease in the degradation of a distinct groups of proteins (Hunt et al., 1997).

**Cathepsin G**

Cathepsin G is one of the major serine proteases in neutrophils, with both tryptic and chymotryptic specificities (Polanowska et al., 1998). It has also been found in the human MC
TC, but not in MC
T. Cathepsin G plays an important role in innate host defense against infection, confirming the similar functions for MC
TC (Schechter, N.M. et al., 1990).

**Tryptases**

Tryptases are a family of serine proteases with trypsin-like substrate specificity. Tryptases are a diverse group of proteases that include α (αI and αII isoenzymes), β (βIa, βIb, βII and βIII isoenzymes) and human transmembrane tryptase/ tryptase γ (γI and γII isoenzymes) isoforms in humans and three tryptases (mMCP-6, mMCP-7 and mouse transmembrane tryptase) in mice (Caughey, 1996; Pallaoro...
et al., 1999). β-tryptases are 98 to 99% identical to each other by amino acid sequence, while α-tryptases are 93% identical to β-tryptases in amino acid sequence (Caughey et al., 2000). As it was already mentioned, tryptases cleave substrates at the carboxyl side of Arg and Lys residues (Hedstrom, 2002). Tryptases are translated as zymogens with a 10-12 amino acids long N-terminal propeptide. One of the possible candidates to activate tryptase zymogen is DPPI. It was suggested that DPPI acts in the second phase of a two-step process. The first step is an autocatalytic intermolecular cleavage by tryptase, which occurs optimally at acidic pH and in the presence of heparin. The resulting product is a monomer, which is about 50 times less active than the final tetramer. The second step involves the removal of the remaining precursor dipeptide by DPPI, thus allowing the mature peptide to form the active tetramer. This process also requires heparin (Sakai, Ren & Schwartz, 1996).

The analysis of the structure of the human α1-tryptase revealed that a single amino acid residue near the active site, Asp216, in α-tryptases is replaced by a Gly residue in β-tryptases. This fact is the reason for the low proteolytic activity of α-tryptase (Huang et al., 1998; Marquardt et al., 2002). α-tryptase is not stored in secretory granules, but instead is constitutively secreted by MCs and is the predominant form of tryptase found in blood in both healthy subjects and those with systemic mastocytosis under nonacute conditions (Sakai, Ren & Schwartz, 1996). During rheumatoid arthritis α-tryptase appeared to be the major form in synovial fluid (Buckley et al., 1997).

Human β-tryptase is the most abundant mediator stored in MC granules and it is the main active form of tryptase. Human β-tryptase was shown to be stabilized as an enzymatically active tetramer by association with heparin (Schwartz & Bradford, 1986). Further, it was established that it is a ring-like tetramer with the active sites facing a central pore. Heparin chains stabilize the complex by binding to an elongated patch of positively charged residues spanning two adjacent monomers (Pereira et al., 1998). This unique organization explained its resistance to macromolecular inhibitors, such as α-proteinase inhibitor, α2-macroglobulin and aprotinin (Alter et al., 1990), and explains the fairly narrow substrate specificity of tryptase. There are just a few known macromolecular substrates for tryptase, e.g., fibrinogen (Schwartz et al., 1985), fibronectin (Lohi, Harvima & Keski-Oja, 1992), but many low molecular weight peptides (vasoactive intestinal peptide, calcitonin gene-related peptide) (Sommerhoff et al., 2000). Recent data demonstrated that β-tryptase may dissociate into active monomers with properties that are distinct from the tetrameric counterpart (Fajardo & Pejler, 2003a). In addition, tryptase, being a potential activator of pro-matrix metalloproteinase-3 (proMMP-3) (Gruber et al., 1989), also possesses gelatinolytic activity (Fajardo & Pejler, 2003b). Human β-tryptase plays an important role in different biological processes. Tryptase is a potent growth factor for epithelial cells, airway smooth muscle cells and fibroblasts (Cairns & Walls, 1996; Gruber et al., 1997). β-tryptase activates protease activated receptor 2, followed by G-protein activation and intracellular signalling (Berger et al., 2001). It is involved in airway homeostasis, gastrointestinal smooth muscle activity, intestinal transport, and coagulation. Moreover, β-tryptase levels in serum are elevated in most subjects with systemic anaphylaxis of sufficient severity to cause hypertension (Schwartz et al., 1987).
In rodents, CTMCs contain tryptase mMCP-6 (78% identity to the human β-
tryptase). Studies on recombinant mMCP-6 established that formation of
enzymatically active mMCP-6 from inactive monomers requires the presence of
heparin. In the absence of heparin, mMCP-6 was present in monomeric inactive
form (Hallgren et al., 2000). The importance of heparin for tryptase activity was
confirmed by experiments in vivo, where mMCP-6 in the presence of heparin
displayed pro-inflammatory properties. Further, it was suggested that tryptase
tetramer formation involved the bridging of tryptase monomers by heparin or other
highly sulfated polysaccharides of appropriate length.

MMCP-7 is another tryptase stored in secretory granules of CTMCs. MMCP-7 is
not expressed in C57BL/6 mice, and thus its expression is strain dependent (Hunt
et al., 1996). In the acidic granule, mMCP-7 interacts ionically with serglycin
proteoglycan via its positively charged His residues. The proteoglycan-binding
face on the surface of mMCP-7 loses much of its positive charge when it is
exocytosed into a neutral pH environment that allows the rapid dissociation of the
tryptase from the macromolecular complex (Matsumoto, R. et al., 1995). Thus,
prolonged retention of exocytosed mMCP-6 in the extracellular matrix around
activated MCs suggested a local action, while mMCP-7, being dissociated from
heparin, easily moves from tissues and cleaves proteins located at more distant
sites. This tryptase exhibits anticoagulant activity in vivo and in vitro due to its
ability to degrade fibrinogen in the presence of a diverse array of protease
inhibitors in plasma, suggesting that fibrinogen is a physiological substrate for
mMCP-7 (Huang et al., 1997).

Transmembrane tryptase (TMT)/ tryptase γ is a membrane-bound serine protease
stored in the secretory granules of human and mouse lung MCs. Mouse TMT
(mTMT) is 74% identical to human TMT (hTMT) (Wong et al., 1999). Pro-γ-
tryptase is activated directly from its catalytically inactive zymogen form by a
tryptic protease, which will separate the propetide from the catalytic domain,
allowing it to adopt an enzymatically active conformation. Mature, active γ-
tryptase, unlike known tryptases, may be a two-chain protein, with part of the
propetide remaining covalently attached via a disulfide linkage involving Cys26.
One of the most distinctive features of TMT is the transmembrane segment located
at its COOH terminus (Caughey et al., 2000). TMT reaches the external face of
the plasma membrane when MCs are induced to degranulate. There it can cleave or
interact with proteins/ peptides residing in the extracellular matrix and on the
surface of those cells that MCs contact. TMT, therefore, functions outside of
activated MCs. TMT induces airway hyperresponsiveness in lungs by activating
an IL-13/ IL-4Rα/ signal transducer and activator of transcription 6 (STAT6)-
dependent pathway (Wong et al., 2002).

Chymases

MC chymases were first identified and localized in MC granules in 1959
(Benditt&Arase, 1959). As mentioned above, they possess chymotrypsin-like
substrate specificity. These proteases cleave substrates with an aromatic P1 amino
acid residue (Powers et al., 1985).
Activation of chymase

Chymases are synthesized as inactive precursors but are stored in secretory granules as active enzymes. Chymase activation in MC granules occurs by the removal of an acidic 2-residue propeptide by DPPI. The macromolecular complex of inactive zymogen and heparin proteoglycan is transported in small vesicles to the larger-sized storage granule. The negatively charged GAG successfully competes for the positively charged face on the protease. This process causes the liberated propeptide to be susceptible to DPPI as soon as the complex achieves the granule. After removal of the propeptide by DPPI, the processed chymase undergoes a conformational change that activates the chymase. In addition, the protease is no longer susceptible to further processing by DPPI (Murakami, Karnik & Husain, 1995). Recently, it was suggested that after activation heparin would promote the dissociation of chymase from DPPI (Reiling et al., 2003) (Figure 5).

![Fig. 5](image.png)

**Fig. 5.** The activated chymase in complex with heparin proteoglycan. After cleavage of prochymase N-terminus by DPPI, the newly formed N-terminus of chymase is rapidly captured by the activation groove. It prevents further degradation of N-terminus by DPPI and causes chymase activation.

Classification of chymases and characterization regarding angiotensin processing

In mice, mMCP-1, -2 and -9 in MMC and mMCP-4 and -5 in CTMC were originally identified as chymases, whereas human MCs (of the MC_{TC} subclass) express only a single chymase. An analysis of chymase structure by molecular modeling revealed on the surfaces of CTMC chymases two regions with strong positive electrostatic potentials. These regions are located far from the substrate binding cleft. Thus, these regions may interact with heparin proteoglycan in granules. In contrast, chymases from MMCs, with CS in granules, lack one of these regions and have lower charge density in the other (Sali et al., 1993). Previously, based on phylogenetic analysis, chymases were divided into two groups: α and β chymases (Chandrasekharan et al., 1996). In mammalian MCs α-
chymases are widely expressed across species. In humans only one chymase gene, belonging to the α-family, has been identified. In contrast, rodents have been shown to express a number of β-chymase genes, including mMCP-1, -2, -4 and -9 and one α-chymase, mMCP-5. Recent studies established that exchange of a residue Gly216 to Val in the active site of human chymase dramatically alters its substrate specificity into being elastase-like (Solivan et al., 2002). Since all rodent α-chymases contain Val in this position, it was suggested that mMCP-5 has elastase-like rather than chymotrypsin-like substrate specificity (Kunori et al., 2002). Earlier, the functional studies revealed a difference in substrate selectivity of α- and β-chymases regarding Ang I conversion into Ang II (Chandrasekharan et al., 1996). Ang II is a powerful vasoconstrictor but also participates in the inflammatory response, acting as a proinflammatory mediator (Ruiz-Ortega et al., 2001). The Ang II is formed from Ang I (1DRVYIHPFHL10) by cleavage of the Phe8-His9 bond. Originally, α-chymases were shown to cleave the Phe8-His9 bond to form Ang II whereas the β-chymases cleave both the Phe8-His9 and Tyr4-Ile5 bonds degrading the Ang II (Chandrasekharan et al., 1996). Later, the involvement of mMCP-1 in the generation of Ang II and hydrolysis at Tyr4-Ile5 by α-chymase (i.e., canine) was demonstrated (Saito et al., 2003). Moreover, the recent studies revealed a cooperation between mMCP-4 and CPA in the formation and degradation of Ang II and suggested a primary role for mMCP-4 in the extravascular formation of Ang II (Lundequist et al., 2004). In addition, Lys48 contributes to human chymase’s remarkable preference for Ang II generation over destruction (Muilenburg et al., 2002).

**Inhibitors**

In the cytoplasmic secretory granules of MCs, chymase is tightly bound to proteoglycans. Upon degranulation chymase and proteoglycans remain bound to each other. After secretion, chymase is surrounded by extracellular fluid, which contains protease inhibitors, such as α1-antitrypsin, α2-macroglobulin, α1-antichymotrypsin and eglin C. The concentrations of these inhibitors are likely to increase at sites of MC activation, since histamine increases endothelial permeability and induces leakage of plasma proteins. It was found that chymase in complex with proteoglycans is more resistant to high molecular weight protease inhibitors present in blood plasma, specifically to α1-antitrypsin and α2-macroglobulin and to a lesser extent (about 50%) to α1-antichymotrypsin and eglin C. At the same time there was no resistance to the synthetic or nonphysiological low molecular weight protease inhibitors, including phenylmethanesulfonyl fluoride (PMSF), chymostatin and Soybean Bowman-Birk protease inhibitor (Lindstedt, Lee & Kovanden, 2001). Earlier studies demonstrated the resistance of rat mast cell chymase 1 (rMCP-1)/heparin proteoglycan complex to macromolecular protease inhibitors α1-antichymotrypsin and α1-protease inhibitor, while the small synthetic serine inhibitor PMSF inhibited chymase independently from heparin proteoglycan. Thus, proteoglycans create a steric hindrance that limits the accessibility of the chymase to the inhibitors (Pejler & Berg, 1995). It is important to note that some macromolecular inhibitors serve better as substrates for purified human skin chymase than as inhibitors (Schechter, N.M. et al., 1989). The secretory leukocyte proteinase
inhibitor (SLPI) is a serine proteinase inhibitor present in human mucus secretions and tissues where MCs are located. It binds heparin. The chymase-SLPI interactions revealed the inhibition of chymase and resulted in the reversible formation of their complex. Remarkably enhanced inhibition of chymase activity was achieved in the presence of heparin (Walter, Plotnick & Schechter, 1996). This could be explained by binding of heparin to SLPI, and that this interaction enhances protease-inhibitor association (Faller et al., 1992). These observations suggest that the regulation of chymases by protease inhibitors is strongly dependent on the presence of proteoglycans.

Different approaches in the study of the biological functions of MC proteases

The most definitive approach for characterizing the importance of a single potential effector cell or its mediator in a biological response is to attempt to elicit the response in animals that differ solely in having or lacking the element of interest. MC deficient mice are one of the most important models in studies of the physiological and pathological functions of MCs. Since MCs produce a lot of different granule components, it is impossible to elucidate the role of certain mediators in one or another biological process by using MC deficient mice only. To address the in vivo functions of MC proteases and confirm previous in vitro studies, animal models with a lack of different MC proteases are therefore needed.

In vitro, human chymase has been demonstrated to induce apoptosis of vascular smooth muscle cells (Leskinen, M. et al., 2001) and to inhibit mitogen-induced proliferation of human airway smooth muscle cells (Lazaar et al., 2002). Human chymase can activate the potent inflammatory cytokine IL-1β (Mizutani et al., 1991) and can cleave membrane bound SCF resulting in its release from cells (de Paulis et al., 1999). Albumin has been established as a substrate for human chymase as well (Raymond et al., 2003). Further, the extracellular matrix-associated latent TGF-β1 was released and activated by human chymase (Taipale et al., 1995). The intradermal injections of human chymase resulted in skin swelling and eosinophil accumulation, suggesting a role for chymase in pathogenesis of chronic dermatitis (Tomimori et al., 2002). NC/Nga mice develop skin lesions similar to atopic dermatitis (Watanabe et al., 2002). Human MC chymase induces the accumulation of neutrophils and other inflammatory cells in vivo (He & Walls, 1998a) and contributes to the increase of microvascular permeability (He & Walls, 1998b). Chymase may also play a role in heart remodeling by increasing Ang II formation and activating MMP-9, and by regulating collagen I gene expression (Chen et al., 2002). In vitro experiments also demonstrated the ability of mMCP-4 to directly activate pro-MMP-9 (Coussens et al., 1999). The ability of chymase to activate procollagenase (proMMP-1) (Saarinen et al., 1994) and prostromelysin (proMMP-3) (Suzuki et al., 1995) suggested a role for chymase in tissue remodeling. In addition, α-chymase has been shown to cleave and inactivate tissue inhibitor of metalloproteinase-1 (TIMP-1) (Frank et al., 2001). Further, human chymase and rMCP-1 have the ability to cleave fibronectin (extracellular matrix protein) (Leskinen, M.J. et al., 2003; Vartio, Seppa & Vaheri, 1981). In vitro studies revealed that rMCP-1 is involved in the regulation of extravascular
coagulation by degrading thrombin (Pejler & Karlstrom, 1993). In fact, a lot of in vitro studies indicated the involvement of heparin proteoglycan in the cleavage of different substrates by chymases. Chymase has the potential ability to promote angiogenesis in hamster sponge granulomas through local chymase-dependent and angiotensin-converting enzyme-dependent Ang II generation (Muramatsu et al., 2000). Human chymase is also implicated in the development of bleomycin-induced pulmonary fibrosis in mouse model (Tomimori et al., 2003). Moreover, the development of skin fibrosis in a mouse model for scleroderma (Tsk mice) was accompanied by upregulation of mMCP-4 (Kakizoe et al., 2001).

To address the in vivo functions of MC proteases, transgenic mouse models have been generated. MMCP-1 is expressed constitutively, but levels increase in the bloodstream and intestinal lumen of parasitized mice and are maximal at the time of worm expulsion (Huntley et al., 1990). Mice lacking mMCP-1 demonstrated a significantly delayed expulsion of the nematode *Trichinella spiralis* and increased deposition of muscle larvae. This shows, for the first time, that MC proteases have a role in the expulsion of parasites (Knight et al., 2000). The mMCP-5 deficient mice were also created. The inactivation of mMCP-5 gene was accompanied by reduced levels of stored CPA (Stevens et al., 1996). However, there are no reports so far demonstrating biological consequences of the mMCP-5 disruption. Another potential model for studies of functions of CTMC proteases is the NDST-2 deficient mouse strain, where the storage of mMCP-4, -5, -6 and CPA was affected (Forsberg et al., 1999; Humphries et al., 1999). Finally, mMCP-4 deficient mice were successfully created and some biological functions of mMCP-4 have already been described (see "Present investigations").

**Pathological and physiological conditions: a role for mast cells**

MCs are widely distributed in the body, especially at serosal and mucosal surfaces of the gastrointestinal tract, surface of airways and resident cells of submucosal and dermal connective tissue. They keep a certain distance to each other and are always prepared to repulse to invaders breaking through the first defense line. According to their distribution it is easy to suggest that MCs play an important role in host defense against bacterial and parasite infections by activation and releasing different mediators (innate and acquired immunities). However, it is important to remember that MCs are implicated in different pathological conditions as well. Thus MCs possess both pathological and physiological functions.

The most definitive approach for characterizing the importance of MCs in a biological response is to attempt to elicit the response in animals that differ solely in lacking MCs. Because of the mutation at c-kit, which encodes the receptor for a pleiotropic growth factor that represents a major MC survival and developmental factor, SCF, adult Kit⁺/Kit⁺ mice virtually lack tissue MCs (Galli & Kitamura, 1987). SL/Sl⁺ mice have a mutation in the gene encoding SCF. Bone marrow from SL/Sl⁺ mice reconstitutes the MC population of Kit⁺/Kit⁺ mice, but SL/Sl⁺ mice themselves do not respond to bone marrow transplantation because there is no ligand for the SCF-dependent MC development (Galli, Tsai & Wershil, 1993). MC-
deficient mice have been extensively used to characterize the role of MCs in numerous pathological conditions but also physiological functions of MCs.

*In vivo* studies employing MC-deficient mice have shown that MCs are essential for mounting efficient innate immune responses against bacterial infections. The MC-deficient mice are less efficient in clearing and surviving experimentally induced enterobacterial infections (*Klebsiella pneumoniae*) as compared with WT mice. It was directly correlated with fewer neutrophils at the sites of infection, most likely as a result of lower levels of the MC-derived polymorphonuclear granulocyte chemotactic activity induced by TNF-α in these mice (Malaviya *et al.*, 1996). It was also found that MC-deficient mice exhibit dramatically increased mortality after cecal ligation and puncture (CLP, a model for acute septic peritonitis), compared with WT mice (Echtenacher, Mannel & Hultner, 1996). Peritoneal MCs also express complement receptors. In the CLP model, mice lacking complement C3 exhibited diminished degranulation of peritoneal MCs and showed increased mortality, further suggesting a role for MCs in bacterial infections (Prodeus *et al.*, 1997). MCs use selected TLRs (family of pattern-recognition receptors) to respond to pathogens. The reconstitution of Kit^+/Kit^-v^mice with TLR4-deficient bone marrow-derived MCs (BMMC) during bacterial peritonitis demonstrated increased mortality and a defective neutrophil recruitment compared with controls. It was therefore suggested that TLR4 on MCs has a role in MC-mediated clearance of bacteria (Supajatura *et al.*, 2001). MCs may also be involved in human immuno-deficiency virus (HIV-1) infection. The HIV-1 glycoprotein gp120 stimulates IL-4 and IL-13 release from MCs. In addition, the chemokine receptors CCR3 and CXCR4, co-receptors for HIV-1, are expressed by FcrRI^+^ cells (Marone *et al.*, 2001).

MCs are also important players in acquired immunity. They are capable of phagocytizing and presenting antigens to T cells and of modulating T- and B-cell responses such as lymphocyte growth, recruitment and Ig production. IgE production is substantially upregulated during immune responses to infections with intestinal parasites, where MC populations undergo considerable expansion. Previously, it was shown that MCs participate in the expulsion of helminthes, such as *Trichinella spiralis* and *Strongyloides ratti* (Nawa *et al.*, 1985; Oku, Itayama & Kamiya, 1984). The MCs are also capable of phagocytosis of diverse micromolecular and particulate materials, but this ability is decreased with the increasing maturity of the cells, indicating a close ontogenetic relationship of MC to myeloid phagocytes (Czarnetzki, 1982). The expression of major histocompatibility complex (MHC) class I molecules by MCs, and their ability to express MHC class II molecules under certain circumstances (Frandji *et al.*, 1993) have indicated MCs as antigen presenting cells (Tedla *et al.*, 1998).

Tissue remodeling is another process where MCs play a key role. The studies on Kit^+/Kit^-^mice demonstrated that the hair follicle cycling, which is associated with enormous architectural changes of the skin, including proteolysis, angiogenesis, and nerve supply rearrangement, is significantly impaired in the absence of MCs (Maurer *et al.*, 1997). An analogous situation was described for bone remodeling (Cindik *et al.*, 2000). MCs have been shown to participate in the physiological wound repair. They influence the wound healing process by
increasing fibroblast migration and proliferation, which are mediated by histamine and IL-4 (Kupietzky & Levi-Schaffer, 1996). Angiogenesis is dependent on the balance between different angiogenic modulators and requires the activities of angiogenic factors, extracellular matrix proteins, adhesion receptors and proteolytic enzymes (Pepper, 1997). MCs are a rich source of cytokines and growth factors that induce or modulate angiogenesis. The most important of these mediators are TNF-α, IL-8, fibroblast growth factor 2, VEGF, heparin, histamine and MC proteases (Grutzkau et al., 1998; Norrby, 1993; Qu et al., 1998).

It has been suggested that MCs, under physiological conditions, may form a functional unit with sensory nerves. MCs and sensory nerves may be presented as a functional unit, having similar responses to stimulation (degranulation), common localization (Botchkarev et al., 1997) and activation by products from each other (Botchkarev et al., 1995; Steinhoff et al., 2000). Finally, MCs and sensory nerves have been suggested to cooperate in number of physiological processes such as hair follicle cycling and development, wound healing (Gottwald et al., 1998), stress response (Singh et al., 1999), and in the pathogenesis of inflammatory diseases (Bienenstock et al., 1991).

Many studies have indicated a contribution of MCs in the development of pathological conditions. In allergies, apart from the classical role in the early phase, MCs also have an important function in its late and chronic stages through the release of cytokines, chemokines and other mediators that can influence the recruitment and activation of inflammatory cells such as eosinophils (De Monchy et al., 1985), T cells, basophils (Guo et al., 1994), neutrophils (Koh et al., 1993) and macrophages (Calhoun et al., 1993). MCs participate in the pathogenesis of asthmatic inflammation. They are localized at the interface of the internal and external environment within the lung where they respond to exogenous stimuli. MC preformed mediators (tryptase, chymase, and histamine) are important in the pathogenesis of asthmatic inflammation, whereas the newly formed mediators cause even stronger effects. PGD_2 acts as a bronchoconstrictor and vasodilator. LTC_4 is converted extracellularly into its receptor-active metabolites LTD_4 and LTE_4, both potent mediators of asthma (Austen, 1995).

MCs participate in fibrotic processes. MCs are found in fibrogenic lesions in various tissues in human diseases, such as pulmonary fibrosis (Pesci et al., 1993), cardiovascular diseases (Matsumoto, T. et al., 2003), hepatic cirrhosis (Armburst et al., 1997), scleroderma and keloid (Craig, DeBlois & Schwartz, 1986). Fibrosis also occurs during atopic dermatitis (Watanabe et al., 2002). MCs serve as a source of proteases (tryptase and chymase) (Ruoss, Hartmann & Caughey, 1991) and a number of cytokines (IL-4, IL-6, IL-13, TGF-β, TNF-α), which are mitogenic and chemotactic for fibroblasts and stimulate the production of extracellular matrix by fibroblasts (Gordon & Galli, 1994). Furthermore, MCs themselves produce components of the extracellular matrix (Thompson et al., 1991). MCs produce the latent form of MMP-9, which is further activated by chymase (Tanaka & Matsuda, 2004). Since MMP-9 hydrolyzes basement membrane collagen types IV, V, VII, fibronectin and elastin, this may be a mechanism, by which MC cause connective tissue degradation under inflammatory conditions (Goetzl, Banda & Leppert, 1996).
Angiogenesis and the production of angiogenic factors are fundamental for tumor progression in the form of growth, invasion and metastasis. Tumors induced in Kit$^\text{W}/\text{Kit}^\text{v}$ display both reduced angiogenesis and ability to metastasize. Angiogenesis is restored after local reconstitution of MCs (Starkey, Crowle & Taubenberger, 1988). MCs are recruited and activated via different factors secreted by tumor cells (Gruber, Marchese & Kew, 1995). Thus, MCs are directly involved in tumor angiogenesis by releasing several angiogenic factors from their secretory granules. MCs are important in the pathogenesis of atherosclerotic disease. Again, the secretion of pro-inflammatory cytokines and enzymes propagate the inflammatory response and destabilize atheromatous plaques. MCs can facilitate foam cell formation by promoting cholesterol accumulation (Kovanen, 1995).

In addition, MCs have been implicated in several autoimmune diseases, including, bullous pemphigoid, experimental vasculitis and rheumatoid arthritis (Benoist & Mathis, 2002). A study on the animal model of multiple sclerosis, experimental allergic encephalomyelitis in Kit$^\text{W}/\text{Kit}^\text{v}$ mice, indicated an essential role for MCs in this neurologic inflammation (Secor et al., 2000).

In conclusion, MCs have multiple functions in many different physiological and pathological processes, although it seems that MCs are not essential for life. They provide important information about these processes, provoking the continuation of the study of the role of MCs and their mediators.
Aim and results of present investigations

Aim of the study

The general aim of this study was to investigate the biological functions of mast cell proteases both \textit{in vitro} and \textit{in vivo}. The possible physiological substrates for chymase, connective tissue type mast cell protease 4 (mMCP-4), were defined by using heparin deficient mice (NDST-2) and mMCP-4 deficient mice. The following aspects were investigated:

- study of the consequences of the lack of functional heparin by analysis of peritoneal cells from NDST-2 deficient mice and elucidation of the possible roles of CTMC proteases (paper I)
- study of the consequences of heparin/MC protease deficiency for extravascular coagulation and fibrinolysis (paper II)
- creating of mMCP-4 deficient mice (paper III) to study the biological role and confirm previous investigations concerning MC chymase (mMCP-4)
- study of the importance of MCs and MC chymase in the regulation of connective tissue homeostasis (paper IV)

Paper I. Altered processing of fibronectin in mice lacking heparin. A role for heparin-dependent mast cell chymase in fibronectin degradation

Previously, a mouse strain was generated with a defect in its heparin biosynthesis by targeting the gene for \textit{N}-deacetylase/\textit{N}-sulfotransferase-2 (NDST-2). NDST-2 is an enzyme involved in the initial stages of the heparin modification process. In the absence of functional NDST-2, heparin remains as the unmodified nonsulfated precursor. MCs from NDST-2\textsuperscript{−/−} mice showed drastically reduced levels of inflammatory mediators, such as histamine and various CTMC proteases, including tryptase mMCP-6, chymases mMCP-4, -5 and CPA. Thus, it indicated the importance of heparin in the storage of these mediators. Finally, NDST-2 deficient mice had altered morphology of MC granules with large "empty" vacuoles instead of the normal electron-dense granule.

In this study peritoneal cells were harvested from normal and NDST-2\textsuperscript{−/−} mice. After incubation of cells, SDS-PAGE analysis of cell culture media revealed the presence of an \textasciitilde 250 kDa protein in the medium from knockout mice, but not in normal mice. Peptide microsequencing revealed that the identity of this protein was fibronectin. Fibronectin is multi-functional glycoprotein and one of the components of extracellular matrix. Fibronectin is known to play a role in various biological processes, including cell binding, migration, matrix assembly, embryonic development and tissue remodeling (Hynes, 1990). Moreover, altered fibronectin processing has been found in numerous pathological conditions, such as cancer, thrombosis and wound healing (Homandberg, 1999). Thus, inflammatory conditions are often associated with fibronectin degradation.
To confirm the alteration of fibronectin processing, Western blot analysis of media from NDST-2<sup>−/−</sup> and normal cells was performed. The results demonstrated the presence of intact fibronectin in the NDST-2<sup>−/−</sup> cell medium, whereas fibronectin degradation products were clearly detected in the medium from normal mice. Further, MC-depleted peritoneal cells were reconstituted with purified MCs, resulting in increased fibronectin degradation in a dose-dependent manner. Hence, the dependence on MCs was established. Experiments with inhibitors for different classes of proteases revealed a strong inhibition of fibronectin degradation in cell culture from normal mice by a specific inhibitor of serine proteases. Furthermore, a macromolecular inhibitor of chymase prevented inhibition of degradation, suggesting that chymase, rather than tryptase, is responsible for this processing. Addition of heparin antagonist in a similar experiment resulted in inhibition of proteolysis in a dose-dependent fashion, indicating that this process is highly dependent on heparin. Experiments in a purified system by using rat chymase rMCP-1 (rat homologue to the mouse chymase mMCP-4) confirmed the increase in the rate of chymase-catalyzed degradation of fibronectin by heparin proteoglycan. Finally, N-terminal sequences of fibronectin fragments, obtained by rMCP-1 cleavage, demonstrated that all cleavage sites contain an aromatic amino acid residue at the P<sub>1</sub> position. These data are in agreement with the known substrate specificities of chymotrypsin-like enzymes.

Summarizing the present study the following conclusions can be made:

- intact fibronectin is present in the media from NDST-2<sup>−/−</sup> mice, whereas fibronectin degradation products are present in the media from normal mice
- degradation of fibronectin observed in cell cultures from NDST-2<sup>+/+</sup> mice is catalyzed by MC chymase, suggesting that fibronectin may be a natural substrate for chymase
- degradation of fibronectin by chymase is strongly dependent on heparin. Under physiological conditions, chymase occurs in macromolecular complex with heparin proteoglycan. This study thus indicates that the association with heparin proteoglycan is required for optimal chymase activity towards fibronectin, and possibly towards other substrates
- proteolysis of fibronectin by chymase could unleash activities of fibronectin fragments that might potentially be important components in the regulation of e.g. inflammatory responses.

**Paper II. Regulation of extravascular coagulation and fibrinolysis by heparin-dependent mast cell chymase**

Here we studied the consequences of heparin/ MC protease deficiency for extravascular coagulation and fibrinolysis. Although the nature of the various potential substrates for MC proteases is variable, it is noteworthy that several of the known MC protease substrates belong to the coagulation system (Huang et al., 1997; Pejler & Karlstrom, 1993). Thus, a role for MC proteases in the regulation of
extravascular coagulation processes and the associated plasminogen activation/plasmin system might be addressed.

In the present investigation, the NDST-2/- mice were used as model for studying the biological consequences of heparin deficiency for the macrophage prothrombinase. Macrophages are thought to be the main cell type responsible for initiation of coagulation at extravascular sites by expressing various coagulation factors, including tissue factor, Factor VII, Factor X and Factor V. It was shown that peritoneal macrophages can assemble these coagulation factors into active Factor X-activating and prothrombinase complexes (Pejler, 1999; Pejler, Lunderius & Tomasini-Johansson, 2000).

Addition of prothrombin to peritoneal cells, a mixture of macrophages, lymphocytes and MCs, resulted in formation of thrombin but the accumulation of thrombin occurred faster in the NDST-2/- cells than in normal controls. Further, the generated thrombin was subsequently inactivated in the NDST-2/- cell cultures but not in the NDST-2/+ cells, indicating that regulation of thrombin activity in this system is strongly heparin-dependent. Protamine, a polycationic heparin antagonist, was added to the normal cell culture and resulted in acceleration of the accumulation of thrombin and abolishment of subsequent thrombin inactivation. Previous publications have shown that thrombin is a substrate for MC chymase (Pejler & Karlstrom, 1993; Pejler & Maccarana, 1994). Since peritoneal MCs from heparin deficient mice completely lack MC chymase, it was reasonable to address a role for chymase in the regulation of thrombin. Indeed, addition of α1-antichymotrypsin to normal cells resulted in an increase in the rate of thrombin formation and a subsequent decrease of thrombin inactivation.

In analogous experiments thrombin was added directly to NDST-2/+ and NDST-2/- cells. Thrombin inactivation was blocked by inhibition of chymase α1-antichymotrypsin or by protamine, suggesting the contribution of chymase and heparin proteoglycan in the regulation of thrombin. The processing of prothrombin was studied in peritoneal cell cultures by immunoblot analysis as well. Rapid formation of thrombin was observed. However, generated thrombin was reduced over time in normal cell cultures, whereas the accumulation of thrombin was occurred in cell cultures from knockout mice.

While the extravascular coagulation was affected by chymase and heparin proteoglycan at the level of inactivating thrombin, no significant differences were found in the level of Factor Xa in both cultures. Thus, Factor Xa did not seem to be affected by chymase-heparin complexes.

It was also reasonable to have a mechanism that would regulate the generated thrombin to prevent the formation of excess amounts of fibrin deposits. The extravascular plasmin/plasminogen activator system is another potential system to study the influence of heparin proteoglycan. Dissolution of the extravascular fibrin deposits requires plasmin, which may be formed by action of the macrophage urokinase plasminogen activator (uPA), provided that plasminogen is available. Addition of plasminogen to peritoneal cells from NDST-2/+ and NDST-2/- mice resulted in a high rate of plasminogen activation in the cells from heparin deficient mice compared with normal mice. The generated plasmin was, similar to
thrombin, inactivated by MC chymase-heparin proteoglycan complexes in the NDST-2^{+/+} but not in the NDST-2^{-/-} cells. Plasminogen processing was investigated by immunoblot analysis. Plasminogen activation was present in both cell cultures, but with further inactivating chymase-catalyzed cleavages occurring mainly in the NDST-2^{+/+} cultures. Further, it was established that plasminogen processing is MC dependent. Addition of specific inhibitors to the normal cell cultures established that plasminogen activator, especially urokinase plasminogen activator was responsible for formation of plasmin. In addition, the rate of inactivation of plasmin was increased upon the activation of MCs.

It was also found that protamine does not cause any major release of chymase activity from the cells into the medium. Moreover, protamine had only a slight direct inhibitory effect on the cell surface-associated chymase activity toward chromogenic substrates, in agreement with the notion that protamine does not affect the active site of chymase but rather interacts with heparin. After stimulation of MCs, chymase-heparin proteoglycan complexes were still located on the cell surface as well as slightly released into the medium. Thus, the newly exocytosed complexes tend to reassociate with the MC surface and hence be prevented from escaping into the surrounding tissue.

**Fig. 6.** A model for the regulation of thrombin and plasmin activities by mast cell chymase.

In conclusion, we found that:

- inactivation of NDST-2, and thus of the heparin/ MC protease system, may affect the regulation of extravascular coagulation and fibrinolysis
- MC chymase-heparin proteoglycan complexes may modulate extravascular coagulation and the plasminogen activator/ plasmin system by cleaving thrombin and plasmin, respectively, into inactive fragments
• the heparin moiety of the chymase-heparin proteoglycan complex is necessary to obtain optimal substrate cleavage. Binding of thrombin and plasmin are mediated by their heparin-binding regions. Heparin acts by attracting heparin-binding substrates, facilitating contact between them and chymase, and thus accelerating the rate of chymase-catalyzed proteolysis of both thrombin and plasmin (Figure 6).

• chymase-heparin proteoglycan complexes may have the capacity to modulate processes where the plasminogen activator/plasmin system is involved, e.g. tumor metastasis and wound healing.

Paper III. The chymase, mouse mast cell protease 4, constitutes the major chymotrypsin-like activity in peritoneum and ear tissue. A role for mouse mast cell protease 4 in thrombin regulation and fibronectin turnover

Papers I and II suggested a crucial role for MC chymase in different biological processes. Peritoneal MCs are known to express at least two different chymases, β-chymase mMCP-4 and α-chymase mMCP-5 while in humans there is only one chymase. However, because no reagents that specifically block either mMCP-4 or -5 activity were available, it was not possible to distinguish whether these processes were dependent on mMCP-4 or mMCP-5. In addition, of the known mouse β-chymases, mMCP-4 has the most similar tissue distribution to human chymase and has also similar heparin-binding and Ang I-converting properties, suggesting the closest relationship to the human chymase. To confirm the significance of chymase (most probably mMCP-4) in these processes it was necessary to exclude an influence of other factors which are affected by absence of the functional heparin.

We created the mMCP-4 knockout mouse strain, in order to gain further insight into the biological function of MC chymase. A DNA fragment of the mMCP-4 gene was isolated from a genomic library. Next, we characterized the structure of the mMCP-4 gene and identified suitable restriction sites for construction of the target vector for homologous recombination in embryonic stem (ES) cells. The ES cell work was performed using a standard protocol. A PCR based strategy was designed and used for the screening of possible positive clones. One of the positive clones was chosen for injection into blastocysts, followed by implantation into pseudo-pregnant mice and generation of chimeras. Further, obtained heterozygous animals were crossed to get animals homozygous for the mutation.

Homozygous null animals were mated and were found to be fertile. First, analysis on two of the most CTMC-rich tissues, the peritoneum and ears, was done. Immunoblot analysis, using specific antibodies to different CTMC proteases, revealed full absence of immunoreactive mMCP-4 protein in mutant mice. Moreover, the presence of other CTMC proteases, mMCP-5, -6 and CPA was not affected in mMCP-4−/− mice, despite the fact that early reports demonstrated that the lack of one MC mediator may influence the storage of others (Ohtsu et al.,...
Inactivation of the mMCP-4 gene did not affect the number of MCs in the peritoneum, nor MC morphology or response to MC-degranulating agents. Importantly, mMCP-4 showed a major contribution to the total amount of stored chymotrypsin-like activities in tissues. At the same time, no difference in trypsin-like or CPA activities in peritoneum or ear tissue was detected.

To confirm previous results, the contribution of mMCP-4 in the regulation of thrombin activity was studied. The results showed that thrombin was rapidly inactivated in mMCP-4+/+ cell cultures, especially after addition of degranulating agents, whereas the similar process was much slower in mMCP-4−/− cell cultures.

To study the contribution of mMCP-4 in fibronectin turnover, conditioned media from mMCP-4+/+ and mMCP-4−/− peritoneal cells were analyzed. The level of intact fibronectin was much higher in medium from mMCP-4+/+ cells than in mMCP-4−/−. The effect was even more pronounced after stimulation of cells. Similar experiments on older mice demonstrated that the content of intact fibronectin in normal cultures versus knockout was much more pronounced. This was in line with our observation that the level of surface-associated chymase activity increases with increasing age of the animals.

To sum up the present investigation, we have shown that:

- the inactivation of mMCP-4 did not affect the storage of other MC proteases, and did not affect the number of MCs or MC morphology
- mMCP-4 inactivation resulted in complete loss of chymotryptic activity in the peritoneum and in ear tissue, indicating that mMCP-4 is the main source of stored chymotrypsin-like protease activity at these sites
- the mMCP-4 null cells were, in contrast to WT cells, unable to perform inactivating cleavages of thrombin, indicating a role for mMCP-4 in regulating the extravascular coagulation system
- fibronectin present in connective tissues may be one of the main physiological targets for MC chymase.

**Paper IV. A key role for mast cell chymase in the activation of pro-matrix metalloproteases-9 and -2**

Numerous studies have suggested a significant role for MC chymase in various pathophysiological events. However, it has not been possible to determine the mechanism by which chymase influences these processes, i.e. the physiological substrate(s) for chymase has not been identified.

MMPs hydrolyze components of the extracellular matrix. These proteases play a central role in many biological processes, such as embryogenesis, tissue remodeling, wound healing, angiogenesis, arthritis, cancer and tissue ulceration. The MMPs are synthesized as inactive zymogens with an N-terminal propeptide that needs to be removed in order to activate the latent form.
MMP-2 and -9 or gelatinases A and B, respectively, play an important role in angiogenesis and tumor metastasis (Coussens et al., 2000; Hiratsuka et al., 2002). Early in vitro studies identified a number of proteases as potential activators of pro-MMP-9, including MMP-2, MMP-3, MMP-13 and plasmin. (Van den Steen et al., 2002), whereas membrane type 1 MMP (MT1-MMP) and TIMP-2 are required for pro-MMP-2 processing in vivo (Strongin et al., 1995; Wang, Juttermann & Soloway, 2000). Importantly, MCs are often found at the sites where MMPs function. Moreover, MC chymase, according to previous investigations, can directly activate pro-MMP-9 in vitro (Coussens et al., 1999; Fang et al., 1997).

The importance of MCs in the processing of endogenous pro-MMP-9 was investigated by culturing of normal peritoneal cells depleted of MCs or with MCs present, followed by gelatin zymography analysis. The active form of MMP-9 appeared in the presence of MCs, while supernatants from MC-depleted cells showed only the pro-form. Peritoneal cells from heparin deficient mice (NDST-2-/- mice) were used, in order to determine whether MC proteases participate in pro-MMP-processing. These experiments showed that pro-MMP-9 processing occurred in WT cells but not in cultures from NDST-2-/- mice, suggesting a role for MC proteases. To address the function of chymase in this process, mMCP-4 deficient mice were used. Analysis of peritoneal cell cultures and extracts from ears, lungs and hearts showed only the pro-form of MMP-9 in mMCP-4-/- samples, whereas both forms were found in WT samples. Gelatin zymography analysis of tissues for MMP-2 showed the presence of pro- and active forms of MMP-2 in both WT and mMCP-4-/- samples. The level of active MMP-2 was higher in WT that in mMCP-4-/- tissues, indicating that MC chymase is important but not essential for the activation of pro-MMP-2 in vivo. In situ gelatin zymography was used to confirm these findings. Indeed, strong gelatinolytic activity was found in the dermis region of ear tissue, in the vicinity of blood vessels and bronchioli in lungs of WT, but not in chymase deficient samples.

Because MMPs have been implicated in various connective tissue remodeling processes we next studied whether the lack of mMCP-4 and the consequent reduction in active MMPs caused alterations in connective tissue composition. Histochemical analysis of ear tissues revealed an increase in collagen deposition accompanied by a significant increase in ear thickness in mMCP-4 null animals. A similar tendency was observed in lung tissues. Immunohistochemical analysis for fibronectin, a prominent connective tissue component, showed a markedly stronger staining in mMCP-4 null lung- and ear tissues as compared with control, indicating a higher degree of fibronectin deposition.

Finally, summarizing the present results we found that:

- mMCP-4 has a central role in regulation of MMP-2 and -9 activities. Since mMCP-4 most likely represents the functional homologue to the only human chymase identified, we may propose a similar role for the human chymase in the regulation of the corresponding human MMPs.
• accumulation of connective tissue proteins in the mMCP-4−/− tissues, which are controlled by active MMPs, may be explained by reduced levels of active MMPs

• since MMP-2 and -9 have been strongly implicated in similar disorders as those that have been linked to chymase, mMCP-4-mediated activation of pro-MMP-2 and -9 may provide a link between MCs and processes that are regulated by MMPs.
Concluding remarks and outlook

MCs possess the unique ability to help the organism to fight with different outer "enemies". Probably, the mediators that are released following MC degranulation play important roles in the defence mechanism. Each of them has an important function, but different mediators can be of importance, depending the particular insult to the organism. Previously, the majority of studies on MC protease functions were performed in vitro. Lately, a few knockout animal models for MC mediators have been described. A major problem with some of these knockouts (mMCP-5, CPA and HDC) is that the deletion of one MC component may indirectly alter the levels of other MC mediators.

At the beginning of this project the aim was to look for possible functions of CTMC proteases, focusing on the heparin deficient mice that, in turn, were almost completely devoid of a number of CTMC proteases. After the implication of MC chymase in the certain biological processes, the challenge was to create the knockout animal model for the protease of our interest, mMCP-4. The obtained transgenic animals were devoid of mMCP-4 without affecting of other MC mediators. Such a model allowed us not only to confirm previous observations related to chymase by indirect evidences, but will also help us to address the potential functions of this protease in numerous patho- and physiological processes (Figure 7).

In conclusion, a number of aspects can be lit up in the near future:

- The influence of targeting of mMCP-4 on the processing of other MC granule components, in particular those that, according to previous investigations, might be directly or indirectly influenced by chymase (e.g., TNF-α, TGF-β, SCF and IL-1)
- The influence of targeting of mMCP-4 on the activation of other MMPs, such as MMP-1 and MMP-3, but also the finding of new physiological substrates
• Assessment of the role of mMCP-4 in different pathological conditions, where MCs have been implicated (rheumatoid arthritis, dermatitis, asthma model, multiple sclerosis and tumor angiogenesis)

Thus, there are still a lot of unsettled questions and enigmas as regards the function of MC chymase. Hopefully, the results and conclusions presented in this thesis will help to understand the function of this MC protease and inspire to future exciting studies.
References


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