Biological Functions and Regulation of Serglycin-dependent Mast Cell Proteases

Anders Lundequist
Faculty of Veterinary Medicine and Animal Science
Department of Molecular Biosciences
Uppsala

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Abstract


Mast cells (MCs) are immune cells that release preformed mediators from granules when activated by e.g. bacteria, viruses and allergens. Among the granule components are proteoglycans, histamine and proteases (chymase, tryptase and carboxypeptidase A). The proteases are bound to serglycin proteoglycans in the granules, and some remain attached to the proteoglycans after expulsion from the secretory granules. The importance of the interaction between proteases and proteoglycans is exemplified by MCs lacking an enzyme essential for heparin biosynthesis, N-deactylase/N-sulfotransferase-2 (NDST-2). Mice with this deficit display impaired storage of many MC granule components, e.g. proteases and histamine.

We could show that polycationic compounds inhibited chymase (mMCP-4/rMCP-1) by disrupting its interaction with heparin. Furthermore, these polycations inhibited human tryptase (recombinant βI-tryptase) and made the otherwise resilient enzyme susceptible to macromolecular inhibitors. Our findings may be of therapeutic interest when treating pathologies associated with these enzymes.

Mast cells may take part in regulation of blood pressure and angiogenesis, and this could occur through metabolism of the peptide angiotensin I (AngI). We confirmed the MC involvement in AngI processing using the NDST-2 deficient mice. We subsequently elucidated that the responsible proteases were chymase (mMCP-4) and carboxypeptidase A (CPA), and that they acted in concert on AngI, producing both blood pressure elevating peptides and blood pressure reducing peptides.

Mast cells are implicated in connective tissue regulation, and a suggested pathway is through activation of matrix degrading enzymes, e.g. the matrix metalloproteases (MMPs). We could show that activation of proMMP-9 is dependent on MCs, and especially the chymase mMCP-4, as shown by impaired processing of proMMP-9 in peritoneal cell cultures from mice lacking NDST-2 or mMCP-4. Investigation of tissues from mMCP-4−/− revealed increased deposits of extracellular matrix components and reduced levels MMP-9. Additionally, we could show that proMMP-2 processing was completely abolished in peritoneal cell cultures from mice lacking serglycin. Furthermore, we showed that the formation of MMP-2 was abrogated in the presence of serine protease inhibitors and when cell cultures were depleted of MCs. The latter study reveals a novel pathway for proMMP-2 processing, which could be of relevance in connection with MMP2-associated pathological conditions such as cancer.

Keywords: mast cell mediator, protease, chymase, matrix metalloprotease, proteoglycan, allergy, arthritis, cancer

Authors address: Anders Lundequist, Department of Molecular Biosciences, SLU, Box 575, S-751 23 Uppsala, Sweden
To my loving family

“If I have seen further it is by standing on the shoulders of giants.”

_Sir Isaac Newton_
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The present thesis is based on the following papers, which will be referred to by their Roman numerals:


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### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>Ang</td>
<td>Angiotensin</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CPA</td>
<td>Carboxypeptidase A</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
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<tr>
<td>CS</td>
<td>Chondroitin sulfate</td>
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<tr>
<td>CTMC</td>
<td>Connective tissue type MC</td>
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<tr>
<td>DPPI</td>
<td>Dipeptidylpeptidase</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>ET-1</td>
<td>Endothelin-1</td>
</tr>
<tr>
<td>GAG</td>
<td>Glycosaminoglycan</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
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<tr>
<td>ICAM</td>
<td>Intracellular adhesion molecule</td>
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<tr>
<td>IFN</td>
<td>Interferon</td>
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<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>ITAM</td>
<td>Immune receptor tyrosine-based activation motif</td>
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<tr>
<td>ITIM</td>
<td>Immune receptor tyrosine-based inhibitory motif</td>
</tr>
<tr>
<td>LFA</td>
<td>Leukocyte function-associated antigen</td>
</tr>
<tr>
<td>LTC₄</td>
<td>Leukotriene C₄</td>
</tr>
<tr>
<td>MC</td>
<td>Mast Cell</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte chemoattractant peptide-1</td>
</tr>
<tr>
<td>MIP</td>
<td>Macrophage inflammatory protein</td>
</tr>
<tr>
<td>MMC</td>
<td>Mucosal type MC</td>
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<tr>
<td>mMCP</td>
<td>Mouse mast cell protease</td>
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<tr>
<td>MMP</td>
<td>Matrix metalloprotease</td>
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<tr>
<td>MS</td>
<td>Multiple Sclerosis</td>
</tr>
<tr>
<td>NDST</td>
<td>N-deactylase/N-sulfotransferase</td>
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<tr>
<td>PCA</td>
<td>Passive cutaneous anaphylaxis</td>
</tr>
<tr>
<td>PGD₂</td>
<td>Prostaglandin D₂</td>
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<tr>
<td>PG</td>
<td>Proteoglycan</td>
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<tr>
<td>rMCP</td>
<td>Rat mast cell protease</td>
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<tr>
<td>TIMP</td>
<td>Tissue inhibitor of metalloproteases</td>
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<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
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<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
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<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
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<tr>
<td>VIP</td>
<td>Vasoactive intestinal peptide</td>
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</table>
Introduction

The immune system

Our environment contains matters essential for our existence, such as air, water and food. These components also hold organisms, which are possibly harmful, e.g. viruses, bacteria and parasites. In order to keep these pathogens out, we have the natural barriers of skin and mucosal surfaces. Once the foreign organisms penetrate these barriers, their presence activates cells of the immune system.

The immune system comprises two components, the innate response and the acquired/adaptive response. The innate part of the immune system is common to all humans, and enables us to mount a defense toward disease even on the first encounter with the pathogen. The acquired immune system produces pathogen strain-specific antibodies and the specificity increases upon repeated exposure to the pathogen.

The immune system comprises several different cell types, of which the majority belong to the leukocyte family. One group of leukocytes is the lymphocytes, which consists of T-cells and B-cells. The B-cells produce antibodies, whereas the T-cells, among other things, govern the type of antibody that the B-cell produces. The second leukocyte group is the phagocytic cells (mononuclear phagocytes, polymorphonuclear neutrophils and eosinophils), which can utilize the antibodies produced by B-cells to bind, engulf and neutralize pathogens. The phagocytic cells can recognize pathogens by other means than antibodies, and present pathogen specific antigens to T-cells, thereby influencing the acquired immune response. Additionally, there are mast cells (MCs), basophils and platelets, commonly referred to as auxiliary cells. Their purpose is e.g. to induce inflammation to attract leukocytes to the site of infection, but the release of different mediators also affects the development of the immune reaction.

Mast cells

MCs are highly granulated cells situated in the interface between the interior and the exterior environment, e.g. the skin, airways and gastro-intestinal tract. They were given their name by Paul Ehrlich in 1877 who, using aniline dyes, described a cell type that looked “well-fed” due to granules filling the cytoplasm. His more descriptive name of these cells was “granular cells of the connective tissue”. These cells have gained increased attention in recent decades as evidence of their involvement in several physiological and pathological conditions accumulates. The MC exerts its function partly by components stored in the granules that Ehrlich described, but also through substances produced in response to activation. The immediate surroundings influence the composition and response of the MC.
Mast cell heterogeneity

Although the MCs have many common features, regardless of species and distribution, they are not a homogenous population. Hardy and Westbrook described tissue-dependent morphological differences in rat MCs as long ago as 1895, but it was not until 1966 that these findings were further substantiated (Enerbäck, 1966). MCs arise from bone-marrow and differentiate into circulating CD34+/CD13+/c-kit+/CD14- progenitors in humans (Kirshenbaum et al., 1999), whereas the mouse MC progenitor is characterized as c-kit+/Thy-1lo and lacks FcrRI (Rodewald et al., 1996). The progenitors migrate into tissues, where the microenvironmental influence gives rise to MC subtypes. A broad division of MCs in the mouse is connective tissue type mast cells (CTMCs) and mucosal type mast cells (MMCs), a classification based on tissue location, proteases and proteoglycan repertoire. The CTMCs locate to the skin and peritoneal cavity, express chymase, tryptase, CPA (Carboxypeptidase A), heparin proteoglycan and high levels of histamine. MCs situated in the lamina propria of the intestine and airways display an MMC phenotype, with expression of chymase, chondroitin sulfate proteoglycan (CSPG) and low levels of histamine.

Human MCs are also divided into two groups; MC_T, expressing tryptase, and MC_TC, expressing chymase, cathepsin G and CPA in addition to tryptase. Further characterization of these subsets shows that they can be classified according to the cytokines and eicocsanoid mediators that they produce; MC_TCs preferentially produce IL-4, IL-13 and PGD₂, whereas MC_Ts preferentially synthesize IL-5, IL-6 and LTC₄ (Macglashan et al., 1982; Lawrence, I. D. et al., 1987; Bradding et al., 1995; Anderson et al., 2001). The MC_T subset is situated in the lamina propria of the airways and gastrointestinal tract, while the MC_TC subtype is found in the subepithelium and dermis.

Table 1: Heterogeneity of MCs in man and mouse

<table>
<thead>
<tr>
<th></th>
<th>Mouse</th>
<th>Human</th>
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<tbody>
<tr>
<td></td>
<td>CTMC</td>
<td>MMC</td>
</tr>
<tr>
<td>Chymase</td>
<td>mMCP-4/mMCP-5</td>
<td>mMCP-1/mMCP-2</td>
</tr>
<tr>
<td>Tryptase</td>
<td>mMCP-6/mMCP-7/mMNT</td>
<td>-</td>
</tr>
<tr>
<td>CPA</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Cathepsin G</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>Proteoglycan</td>
<td>Heparin/Chondroitin</td>
<td>Heparin/Chondroitin</td>
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Mast cell activation

The MCs are highly versatile in their response mechanisms as they are able to respond to many different stimuli. The response is governed by the manner in which they become activated, i.e. through the specific type of receptors that are engaged. On MCs, the FcεRI and its ligand, IgE, are among the most extensively studied means of activation. There are several non-IgE mediated ways of activating MCs and in the following paragraphs, IgE-dependent and IgE-independent activation mechanisms will be discussed.

IgE-dependent mast cell activation

In order to accomplish MC activation through its IgE-receptors, interaction between IgE-molecules bound to the receptors has to occur in a process called cross-linking where an antigen interacts with at least two IgE-molecules. The MC expresses and presents the high affinity receptor for IgE, FcεRI, which consists of three subunits; FceRIα, FceRIβ and FceRIγ. The receptor is assembled in its tetrameric structure (αβγε) in the endoplasmic reticulum before transfer into the Golgi network and transport to the plasma membrane. In this tetramer, the subunits have different tasks. The α-chain is responsible for binding the Fc part of IgE (Hakimi et al., 1990; Blank, Ra & Kinet, 1991; Lin, S. et al., 1996), thereby tethering IgE to the cell surface. The β-chain amplifies the signal by interaction with the kinase, Lyn, which phosphorylates the β- and γ-chains upon receptor activation (Jouvin et al., 1994; Lin, S. et al., 1996). The γ-chains, once phosphorylated by Lyn, are responsible for conveying the signal into the cell through activation of Syk (Jouvin et al., 1994; Shiue, Zoller & Brugge, 1995). An alternative to Lyn-mediated activation is through a Fyn-dependent pathway (Parravicini et al., 2002), but whether these pathways display any cross-talk is not yet elucidated.

The tetrameric assembly of FcεRI is essential for presentation on the cell surface in rodents, whereas in humans there is an isoform of FcεRI that lacks the β-chain. The distribution of FcεRI is also not limited to MCs and basophils in humans, but is also found on monocytes, dendritic cells and eosinophils, although only in its trimeric configuration (αγε)(Maurer, D. et al., 1994; Maurer, D. et al., 1996; Sihra et al., 1997). An interesting finding is that binding of monomeric IgE, i.e. non-crosslinked, to FcεRI on MCs results in an increased number of FcεRI at the cell surface (Furuichi, Rivera & Isersky, 1985; Yamaguchi et al., 1997), partly through protection against receptor break-down and partly through increased receptor translation. The interaction between receptor and its ligand also primes the effector function of the MC by upregulating synthesis of mediators such as cytokines (Yamaguchi et al., 1997; Yano et al., 1997).
There are at least three sub-types of IgG-receptors present on MCs under certain conditions; the activating receptors, FcγRI and FcγRIII and the inhibiting receptor, FcγRII. The high affinity receptor FcγRI, consisting of one IgG-binding α-chain and the homodimer of γ-chains with ITAM-motifs found in the FcεRI-receptor, is found on human MCs under the influence of interferon-γ (Okayama, Y., Kirshenbaum & Metcalfe, 2000). It seems to preferentially interact with IgG1 rather than IgG2, IgG3 or IgG4, and engagement leads to degranulation, prostaglandin synthesis and cytokine production (Woolhiser, Brockow & Metcalfe, 2004).

Although FcεRI and FcγRI share γ-subunits (Tkaczyk et al., 2004), and it is expected that they would have similar activation pathways in MCs, some differences have been described. Following FcγRI activation, the mRNA and/or protein levels for IL-1β, IL-5 and TNF-α are higher compared to FcεRI activation although degranulation, arachidonic acid metabolite release and number of cytokines synthesized was similar (Okayama, Yoshimichi, Hagaman & Metcalfe, 2001; Woolhiser et al., 2001). The finding that MCs express FcγRI and degranulate upon IgG cross-linking implicates them in Th1 types of immune responses.
Human and rodent MCs appear to differ in the expression of IgG-receptors, as the FcγRI has yet to be detected on murine MCs. In the mouse, the low affinity IgG receptor, FcγRIII, is found on MCs of the connective tissue type, but not on mucosal type MCs (Katz & Lobell, 1995). Its structure of one α-chain and a homodimer of γ-subunits is analogous to that of the FcγRI receptor and the FcγRIII is responsible for IgG-mediated degranulation of MCs in the absence of FcγRI (Katz et al., 1992).

The third IgG-receptor subtype, FcγRII, is involved in restricting the MC response. FcγRII consists of a single α-chain with an intracellular tail containing an ITIM-motif, which upon phosphorylation activates phosphatases that neutralize degranulation-inducing mediators like inositol-3-phosphate (Ono, M. et al., 1996), thereby modulating the strength of the MC response. The FcγRII receptor not only negatively regulates IgG-mediated MC activation, but it also affects activation accomplished through IgE cross-linking by down-regulating the secretory response (Daeron & Vivier, 1999; Kepley et al., 2000), making it important in both Tn1 and Tn2 reactions. A fourth receptor subtype, belonging to the immunoglobulin superfamily, is the gp49 family. This subgroup, which is preferentially expressed on MCs and mononuclear macrophages, also contains ITIM motifs, suggesting a function similar to FcγRII (Katz et al., 1996).

**Complement mediated activation**

Activation of MCs through interaction with C3a and C5a, components of the complement system, has long been known (Johnson, Hugli & Muller-Eberhard, 1975). In rodents, it appears to be only CTMCs that can respond to these mediators since MMCs do not express the appropriate receptors (Mousli et al., 1994). Human MCs also have differential expression of complement receptors. The receptor for C5a is found on human skin MCs, but they do not seem to present any receptors for C3-derived components during base-line conditions (Fureder et al., 1995). In contrast, human lung MCs do not respond any of these stimuli. However, both receptors can be detected in patients with systemic mastocytosis (Nunez-Lopez et al., 2003).

**Toll-like receptors**

In its defensive role against bacteria, the MC is able to recognize parts of the bacterial cell such as the gram-negative cell wall component, lipopolysaccharide (LPS). This recognition is attributed to the Toll-like receptors (TLRs), which is a family comprising eleven members identified so far (Rock et al., 1998; Takeuchi et al., 1999; Du et al., 2000; Chuang & Ulevitch, 2001; Zhang et al., 2004). The common features of this family are a leucine-rich extracellular domain and a Toll/IL-1 receptor-domain in their intracellular region (Medzhitov, Preston-Hurlburt & Janeway, 1997). MCs express TLR-2, 3, 4, 6, 7, 8 and 9 (Takeda, Kaisho & Akira, 2003; Matsushima et al., 2004), as determined on cultured rodent MCs. Deletion of the gene coding for TLR-4 showed that this receptor is responsible for LPS-recognition, and hence recognition of gram-negative bacteria (Hoshino et al., 1999). Peptidoglycan, a constituent of gram-positive bacteria,
elicits a response through binding to TLR-2 (Supajatura et al., 2002), as does a
variety of pathogen-derived lipoproteins as well as the fungal element, zymosan
(Takeda, Kaisho & Akira, 2003). In contrast to TLR-4, which is functional by
itself, TLR-2 seems to be dependent on TLR-1 and -6 for correct recognition of
microbial components (Ozinsky et al., 2000). In the defense against viruses, the
MC produces TLR-3 which recognizes dsRNA (Kulka et al., 2004), and gives rise
to type I IFN transcripts of IFNα and β. The interaction between the pathogen-
derived components and TLRs on MCs causes an activation, which leads to
expression and release of cytokines, leukotrienes and prostaglandins (Marshall,
King & Mccurdy, 2003; Okumura, S. et al., 2003; Varadaradjalou et al., 2003).
In contrast to IgE-mediated activation, TLR-induced MC activation usually does
not lead to degranulation, but to release of mediators in a process referred to as
piece-meal release.

Miscellaneous mast cell activators

In addition to the ways of activating MCs already listed, other mechanisms may
apply. Peptides such as substance P, neurotensin and vasoactive intestinal peptide
(VIP), which are released by neurons, are able to provoke adjacent MCs (Bauer &
Razin, 2000). Certain cytokines and chemokines are also able to elicit a response,
which has been shown for IL-1, IL-3, SCF, macrophage inflammatory protein-1α
(MIP-1α) and monocyte chemoattractant peptide-1 (MCP-1) (Alam et al., 1994;
Mekori & Metcalfe, 2000). Furthermore, cell-to-cell interactions can activate MCs;
activated T-cells can induce degranulation and cytokine production through the
interaction between intracellular adhesion molecule-1 (ICAM-1) and leukocyte
function-associated antigen-1 (LFA-1) (Bhattacharyya et al., 1998; Inamura et al.,
1998). Substances that directly activate MCs include opiates, calcium ionophores,
compound 48/80, endothelin and adenosine (Metcalfe, Baram & Mekori, 1997). In
addition to compound induced activation, there is also activation due to
physiological stress such as changes in osmolarity and pressure.

Mast cell mediators

MCs express a multitude of mediators. Some are stored in secretory granules and
can be released upon stimulation (histamine, neutral proteases, proteoglycans and
preformed cytokines) and others that are expressed only upon MC activation.

De novo-synthesized mediators

Prostaglandins and Leukotrienes

Prostaglandins (PGs) and leukotrienes (LTs) are lipid mediators derived from a
common precursor, arachidonic acid, which is released from the plasma membrane
by the action of cytosolic phospholipase A2 (cPLA2) (Uozumi et al., 1997).
LTs encompass the unstable LTA4, LTB4 and the cysteinyl-leukotrienes (cys-LTs)
LTC4, LTD4 and LTE4. Neutrophils preferentially form LTB4 (Evans, Dupuis &
Ford-Hutchinson, 1985), whereas MCs, basophils and eosinophils predominantly
form LTC4 through conjugation of glutathione and LTA4 by LTC4 synthase (Lam
et al., 1994). LTC4 is further processed extracellulary into LTD4 (Carter et al.,
1998), which in turn is metabolized into LTE4 (Lee, C. W. et al., 1983).
To exert their effect, cys-LTs interact with two receptors, CysLT$_1$ and CysLT$_2$. These receptors have somewhat overlapping tissue-distribution, and both are found on human MCs. Their affinity towards cysLTs differ; CysLT$_1$ has the highest affinity to LTD$_4$ and bind LTC$_4$ and LTE$_4$ with less affinity, CysLT$_2$ binds LTC$_4$ and LTD$_4$ with equal affinity but show weaker interaction with LTE$_4$ (Kanaoka & Boyce, 2004). The physiological effects of cysLTs with their receptors include bronchoconstriction (Dahlén et al., 1980; Davidson et al., 1987), attracting eosinophils and neutrophils (Laitinen et al., 1993), induction of cytokine expression (Mellor, E. A., Austen & Boyce, 2002; Mellor, Elizabeth A. et al., 2003), vascular leakage (Beller et al., 2004b), endothelial activation, dendritic cell migration (Machida et al., 2004) and tissue repair (Peters-Golden et al., 2002; Beller et al., 2004a).

Prostaglandins are produced through the activity of cyclooxygenase (COX) on arachidonic acid. The major COX product in MCs after allergen induced activation is PGD$_2$ (Lewis et al., 1982), which exerts its effect through the receptors DP and chemoattractant receptor-homologue molecule expressed on T$_{h2}$ cells (CRTH2) (Narumiya, Sugimoto & Ushikubi, 1999; Hirai et al., 2001). Mice lacking DP have reduced T$_{h2}$ cytokine production and reduced cellular infiltration in an allergen-induced asthma model, indicating a role for PGD$_2$ and its receptors (Matsuoka et al., 2000).

**Cytokines**

One way for MCs to influence their environment is through expression and release of cytokines. Some cytokines are stored in the secretory granules, ready to be released upon activation, thereby being classified as one of the preformed mediators. One proinflammatory cytokine associated with MC granules is TNF-α (Young et al., 1987; Walsh et al., 1991), which is a potent multi-functional cytokine involved in leukocyte extravasation through up-regulation of endothelial leukocyte adhesion molecule-1 (Messadi et al., 1987). TNF-α is also involved in maturation and migration of dendritic cells (Cumberbatch, Fielding & Kimber, 1994) and the induction of inflammatory mediator responses in macrophages, neutrophils and MCs (Sirois et al., 2000; Coward et al., 2002). Apart from the TNF-α already present in granules, additional expression of this cytokine is accomplished upon MC activation via several of the previously discussed pathways, *i.e.* FceRI, FcγRI, TLR, substance P (Gordon, J. R. & Galli, 1990; Gordon, J. & Galli, 1991; Cocchiara et al., 1999; Okayama, Yoshimichi, Hagaman & Metcalfe, 2001; Matsushima et al., 2004).

Other cytokines produced by MCs include IL-3, IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, GM-CSF bFGF-2 and TGF-β1 (Razin, Leslie & Schrader, 1991; Okayama, Y. et al., 1995a; Okayama, Y. et al., 1995b; Qu et al., 1998; Ishizuka et al., 1999a; b; Kanbe et al., 1999; Kobayashi, Ishizuka & Okayama, 2000). IL-3 is important in MC development and is used to differentiate bone marrow cells into functional MCs (Razin et al., 1984). IL-4, another T$_{h2}$ cytokine, is involved in modulating MC responses through its regulation of receptor expression (FceRI,
Preformed mediators

Histamine

Histamine was first found in 1910, and has since been regarded as one of the most important biogenic amines. It is produced in many cell types through decarboxylation of histidine, but the main sources of histamine are MCs (Riley & West, 1952) and basophils (Ishizaka et al., 1972). Histamine is associated with smooth muscle contraction, increased vascular permeability, gastric acid secretion, neurotransmission, immunomodulation and proliferation. It exerts its effect through interaction the histamine receptors H₁, H₂, H₃, H₄ which are G-protein coupled receptors with wide and differential tissue distribution (Haaksma, Leurs & Timmerman, 1990; Hill, 1990). H₁ is found on blood vessels, smooth muscles, in heart and CNS, H₂ is found in gastric mucosa, heart, uterus and CNS and H₃ is located in the airways, the GI tract and the CNS (Bachert, 2002). In allergic diseases, the interaction of histamine and its H₁-receptor causes symptoms associated with allergy, including vascular permeability, smooth muscle contraction and vasodilatation (Howarth, 1990; White, 1990).

In addition to being a source of histamine, MCs also express histamine receptors H₁, H₂ and H₄ (Yanni et al., 1997; Lippert et al., 2004), indicating an autocrine/paracrine activation mechanism. In order to further clarify the biological function of histamine, a mouse strain lacking the enzyme responsible for its biosynthesis, histidine decarboxylase (HDC), was developed. Deficiency in HDC caused alterations in MC morphology and granule content; the knock-outs have fewer MCs, and both the proteoglycan and protease amount in the MC granules are drastically decreased (Ohtsu et al., 2001). This strain has been used to show the influence of histamine in contact dermatitis (Seike et al., 2005), gastric acid secretion (Tanaka, S. et al., 2002) and angiogenesis (Ghosh et al., 2002).

Proteoglycans

Proteoglycans (PGs) are essential components of MC granules. They are made up of a protein core with glycosaminoglycan (GAG) side chains. GAGs are long, unbranched sugar chains with repeating disaccharide units attached to the core protein via a linker consisting of a xylose followed by two galactose residues and one glucuronic acid (GlcUA). There are many species of PGs, defined both by their core protein as well as by their side chains. It is suggested that the major core protein in MCs is serglycin (Stevens, Richard L. et al., 1988; Kjellen et al., 1989; Åbrink, Grujic & Pejler, 2004), whilst its GAG chain differs in different MC subtypes. Rodent connective tissue type MCs exclusively store PGs with
heparin side chains (Yurt, Leid & Austen, 1977), whereas the MCs of mucosal subtype have PGs with chondroitin sulfate (CS) side chains (Enerbäck et al., 1985). In contrast, human MCs contain both heparin and CS regardless of MC subtype (Stevens, R. L. et al., 1988).

The biosynthesis of GAG chains is intricate and several enzymes are involved in the elongation and modification that takes place in the Golgi apparatus. Once the linker region (Xylose-Galactose-Galactose-GlcUA) is attached to a serine residue in the core protein, the chain is elongated by alternating addition of N-acetylgalactosamine (GalNAc) and GlcUA by chondroitin sulfate polymerase in the case of CS. Heparin and heparan sulfate chains consist of alternating N-acetylgalactosamine (GalNAc) and GlcUA residues added by the enzymes EXT1 and EXT2, which need to form heterodimers in order to reach maximal activity (Lind et al., 1998; McCormick et al., 2000). Knock-out mice devoid of EXT1 are embryonic lethal and heterozygote individuals show diminished heparan sulfate synthesis (Lin, X. et al., 2000). After EXT1/EXT2 have added the sugar residue, sulfate groups are introduced at various positions.

Figure 2: Biosynthesis of heparin/heparan sulfate and chondroitin sulfate

The first modification in heparin synthesis is carried out by glucosaminyl N-deactylase/N-sulfotransферases (NDSTs), which replaces the acetyl group in N-acetylgalactosamine with a sulfate group. This alteration is essential for subsequent modifications as they can only take place in the vicinity of N-sulfate groups. Four
isoforms of NDSTs have been characterized in mouse; NDST-1 and -2 are ubiquitously expressed in embryonic and adult tissue, but at varying levels. In contrast, NDST-3 and -4 are predominantly expressed during embryogenesis although NDST-3 is also found in adult heart and brain (Humphries, Lanciotti & Karlinsky, 1998; Kutsche-Gullberg et al., 1998; Toma, Berninsone & Hirschberg, 1998; Aikawa & Esko, 1999; Aikawa et al., 2001). Crystallization of the sulfotransferase domain of NDST-1, and modeling of the other three isoymes based on the NDST-1 structure revealed differences in substrate binding pockets, implying differences in substrate specificity (Aikawa & Esko, 1999; Kakuta et al., 1999). Biochemical studies showed that NDST-2 was able to generate longer GAG chains with higher N-sulfate content compared to NDST-1 (Cheung et al., 1996; Pikas, Eriksson & Kjellen, 2000). Mice deficient in NDST-2 had a less severe phenotype than expected, as the effect was restricted to CTMCs despite its wide distribution. There are fewer MCs in this strain, and the storage of neutral proteases and histamine is impaired as a consequence of the absence of heparin (Forsberg et al., 1999). Apparently, CTMCs are restricted to express NDST-2, whereas the majority of cell types express at least two isoymes, which would explain the observed phenotype due to a lack of compensatory isozyme in CTMCs. Deficiency of NDST-1 causes embryonic or neonatal death due to e.g. lung- and skull-defects (Fan et al., 2000; Ringvall et al., 2000) and analysis of organs revealed large reduction of heparan sulfate (HS) in basement membranes implying that NDST-1 has an important role in HS synthesis.

Further modifications of the sugar residues, after the step catalyzed by NDSTs, involve conversion of GlcUA into iduronic acid (IdoUA), 2-O-sulfation of GlcUA and IdoUA, as well as 6-O- and 3-O-sulfation of the aminosugar residues. Mice strains unable to convert GlcUA to IdoUA or to perform 2-O-sulfation are neonatally lethal following lung and kidney failure (Bullock et al., 1998; Li et al., 2003).

Proteases
Some of the major MC components expelled upon degranulation include proteases that are active at neutral pH; as much as 35% of total protein content in the granules is made up of these proteases (Schwartz et al., 1987a; Schwartz et al., 1987b). This group consists of e.g. tryptases, chymases and carboxypeptidase, which are stored in their active forms in the granules in complex with proteoglycans (Forsberg et al., 1999; Åbrink, Grujic & Pejler, 2004). MC granule proteases are synthesized as preproproteins, containing a hydrophobic signal peptide and a 2 to 94 amino acid long propeptide. The signal peptide is removed in the endoplasmic reticulum, whereas the location for propeptide removal is yet to be elucidated.

Carboxypeptidase A
Carboxypeptidase A (CPA) is a Zn²⁺-dependent exopeptidase found in CTMC/MCₜᵣᵩ subsets (Everitt & Neurath, 1980; Goldstein, S. M. et al., 1987; Reynolds et al., 1989; Irani et al., 1991). Activation of this protease is suggested
to take place within the MC granules (Dikov et al., 1994; Springman, Dikov & Serafin, 1995) and cathepsin E is involved in this process (Henningsson et al., 2005). The mature CPA carries a high positive charge, and its positively charged residues provide a means by which it may interact with a PG, to which CPA remains bound once exocytosed (Serafin et al., 1987; Goldstein, S. et al., 1992). The importance of proteoglycan binding is evident in mice lacking heparin proteoglycan in MCs as a consequence of gene targeting of the enzyme NDST-2 (Forsberg et al., 1999), where the storage of several proteases is impaired. The activation of CPA seems to be dependent on the species of proteoglycan present in MCs; Henningsson et al. showed that MCs lacking heparin proteoglycan had impaired CPA activation and that chondroitin sulfate proteoglycan could not substitute for heparin proteoglycan in this context (Henningsson et al., 2002). Being a carboxy exopeptidase, CPA processes peptides from their C-terminal and displays preferential proteolysis after aliphatic or aromatic amino acid residues (Everitt & Neurath, 1980; Goldstein, S. M. et al., 1987).

The biological role of MC CPA has remained elusive, but it has been suggested that CPA may act in concert with chymase since they are localized to the same macromolecular proteoglycan complex (Goldstein, S. et al., 1992). Further support of this cooperativity was described in a study of MC involvement in processing of the vasoactive peptide, endothelin-1 (ET-1), where use of selective inhibitors as well as purified components showed that CPA and chymase could indeed cooperate in the processing of ET-1 (Metsarinne et al., 2002). More evidence of the interdependence of CPA and chymase is displayed by CPA knock-out mice, in which the lack of CPA also causes mMCP-5 deficiency without affecting the mRNA levels for this chymase (Feyerabend et al., 2005).

Matrix Metalloproteases

Matrix metalloproteases (MMPs) comprise a family of Zn$^{2+}$ and Ca$^{2+}$ dependent metalloproteases with 25 members described in vertebrates so far (Nagase & Woessner, 1999; Lohi et al., 2001). These members can be divided into eight groups based on their domains. The minimal domain MMPs consist of the common denominators for the family; a signal peptide, an inhibiting propeptide and a catalytic domain with a Zn$^{2+}$-binding site. Members of this subgroup are matrilysin (MMP-7) and matrilysin-2 (MMP-26). A structure common to most MMPs, except for minimal domain MMPs and MMP-23, is the hemopexin-like domain, which connects to the catalytic domain through a linker region of variable length. The hemopexin domain is involved in protein-protein interactions, such as substrate binding and binding of tissue inhibitors of metalloproteases (TIMPs). Mutational studies show that the hemopexin domain in collagenase-1 (MMP-1) is involved in local unwinding of the collagen triple helix, which enables proteolysis of the individual collagen chains (Murphy et al., 1992; Sanchez-Lopez et al., 1993; Overall, 2001). The linker region, although it is absent in MMP-21 (X MMP), seems to be of importance in autoactivation and substrate specificity (Knauper et al., 1997). Although most MMPs are secreted, some remain bound to the cell membrane, either through a transmembrane domain with a cytoplasmatic tail (MT-MMPs), or through a glycoprophatidyl inositol (GPI) anchor.
Members of the subgroup referred to as gelatinases (MMP-2 and MMP-9) have a collagen binding domain made up of three fibronectin type II like repeats, which facilitates interaction with collagenous substrates and elastin (Steffensen, Wallon & Overall, 1995), fatty acids (Berton et al., 2001) and thrombospondins (Bein & Simons, 2000).

Figure 3: Schematic view of MMP composition
There are several levels of regulation of MMP activity, including transcription, secretion, enzyme activation and inhibition. Most MMPs, with the exception of the constitutively expressed MMP-2 (Fini & Girard, 1990; Birkedal-Hansen et al., 1993), are regulated at the transcriptional level. Factors that influence transcription include phorbol esters (Mackay et al., 1992), integrin-derived signals (Wize et al., 1998; Xie, Laouar & Huberman, 1998), cell stress, changes in cell shape (Kheradmand et al., 1998; Tyagi et al., 1998) and cytokines (Ogata et al., 1992). The mediators for several of these inducers are activator-protein-1 sites within the MMP gene promotor-regions (Lin, C. W., Georgescu & Evans, 1993).

Stability of the mRNA also affects expression of MMPs. Phorbol esters can increase the stability of transcripts of MMP-1 and MMP-3, whereas TGF-β, destabilizes mRNA for e.g. MMP-13 (Delany et al., 1995; Vincenti, 2001; Iyer, Pumiglia & Dipersio, 2005). Most MMPs are secreted once translated, but some, such as MMP-8 in neutrophils and MMP-9 in differentiating granulocytes, are retained in granules until the cells become activated (Christner et al., 1982; Hibbs & Bainton, 1989; Borregaard et al., 1995).

Like most proteolytic enzymes, MMPs translate into zymogens, and their latency depends on the interaction between a cysteine in the propeptide and the active site Zn⁺ ions. This interaction can be disrupted through proteolysis of the propeptide, or through the action of reactive oxygen species (Rajagopalan et al., 1996; Owens et al., 1997; Meli, Christen & Leib, 2003). Many MMPs are secreted as zymogens, but some that contain a furin-like enzyme recognition motif can be activated before secretion. Extracellular activation can be accomplished by already activated MMPs as well as by several different serine proteases. An interesting activation mechanism is described for MMP-2; a TIMP-2 complexed with MT1-MMP binds the hemopexin domain of proMMP-2, which is then activated by an adjacent MT1-MMP (Deryugina et al., 2001).

Besides general protease inhibitors such as α₂-macroglobulin, there is a group of reversible inhibitors with higher specificity towards different MMPs. This group of tissue inhibitors of metalloproteases has four members (TIMP-1, -2, -3, -4), which inhibit MMPs through interaction of the TIMP N-terminus with the active site of MMP (Gomis-Ruth et al., 1997).

Initially, MMPs were recognized for their ability to degrade the components of the extracellular matrix. It is now established that these enzymes have several non-matrix proteins as substrates, including cytokines, chemokines, inhibitors and receptors (Sternlicht & Werb, 2001; Folgueras et al., 2004). MCs can be a source of certain MMPs. Mouse MCs express MMP-9 as progenitors and this synthesis is down-regulated by SCF (Tanaka, A. et al., 1999). Expression of this MMP is also described in dog (Fang et al., 1996) and human MCs (Kanbe, N. et al., 1999), but in contrast to the murine system, the expression is up-regulated upon c-kit stimulation (Fang et al., 1999). Addition of TGF-β1 to dog MCs abolishes expression of MMP-9, and decreases the SCF-induced expression. MCs have an autocrine regulation of MMP-9 expression; TNF-α released upon MC stimulation activates T-cells, and the activated T-cells stimulate MCs to express
MMP-9 through interaction of LFA-1 and ICAM-1 (Baram et al., 2001; Brill et al., 2004). MC expression of MMP-9 is also affected by direct stimulation, e.g. through FcεRI (Tanaka, A. & Matsuda, 2004) and TLRs (Tanaka, A., Yamane & Matsuda, 2001; Ikeda & Funaba, 2003). Expression of the collagenase MMP-1 is also detected in human MCs (Di Girolamo & Wakefield, 2000; Edwards et al., 2005).

Tryptase

Tryptases are serine proteases with trypsin-like substrate specificity, i.e. cleavage after basic amino acids. Human MCs have two major tryptase groups; α-tryptases consisting of αI and αII subtypes (Miller, Westin & Schwartz, 1989; Pallaoro et al., 1999) and β-tryptases with βI, βII and βIII subtypes (Miller, Moxley & Schwartz, 1990; Vanderslice et al., 1990). Precursors of α- and β-tryptases are secreted constitutively, but α-tryptase is the predominant form detected in serum under normal conditions (Schwartz et al., 1995; Schwartz et al., 2003). Active β-tryptases, however, are retained in the MC granules. Furthermore, α-tryptase has much lower activity than β-tryptase due to an amino acid substitution in the active site as well as structural differences in the substrate binding domain (Huang et al., 1999; Marquardt et al., 2002). There appears to be a redundancy of α-tryptases, with as much as 29% of the population lacking this subtype with no apparent clinical effects (Soto et al., 2002). Additional members of this serine protease family include the δ-tryptases (δI and δII) (Wang et al., 2002) and the membrane-associated γ-tryptases (γI and γII) (Caughey et al., 2000). Tryptases so far described in murine MCs include mMCP-6, mMCP-7, mouse transmembrane tryptase (mTMT) and mMCP-11. The mouse tryptase most similar to human β-tryptase is mMCP-6, both in amino acid composition and substrate specificity (Huang, C. et al., 2001). Expression of tryptases differs between human and rodents in that all human MCs contain tryptase, whereas tryptase storage in murine MCs seems to be restricted to CTMCs.

Activation of tryptase may be accomplished in two steps, where the first step is an autocatalytic cleavage. The second step, which might involve dipeptidylpeptidase (DPPI), removes the remaining dipeptide to form the mature monomers (Sakai, Ren & Schwartz, 1996; Wolters et al., 2001). Active tryptase is stored in MC granules in a tetrameric structure in complex with heparin, with the active sites of the monomers facing a central pore (Schwartz, Lewis & Austen, 1981; Schwartz & Bradford, 1986; Pereira et al., 1998). The assembly and stabilization of the tetramers is dependent on acidic pH, since the interaction of heparin and tryptase is mediated by histidines that become uncharged at neutral pH, thereby disrupting the ionic interaction (Hallgren et al., 2000; Hallgren, Spillmann & Pejler, 2001; Hallgren et al., 2004; Hallgren, Lindahl & Pejler, 2005). Because of the organization of the tetramer, macromolecular active site inhibitors and large substrates are excluded from the active sites, giving tryptase its specificity towards substrates that are small enough to enter the central pore.

Tryptase is suggested to have anticoagulant activity, since it can degrade fibrinogen, thereby limiting blood clot formation (Schwartz et al., 1985).
Increased vascular permeability through activation of prekallikrin and production of bradykinin from kinogen is also attributed to tryptase activity (Imamura et al., 1996; Kozik et al., 1998). Tryptase may induce increased bronchial responsiveness due to degradation of bronchodilating neuropeptides such as VIP, peptide histidine-methionine and (Walls et al., 1992) calcitonine gene-related peptide (Caughey et al., 1988; Tam & Caughey, 1990; Walls et al., 1992). Degradation of these neuropeptides is associated with asthma, and tryptase may further contribute to this condition by causing thickening of airway walls through increased proliferation of fibroblasts, smooth muscle cells and epithelial cells (Ruoss, Hartmann & Caughey, 1991; Brown et al., 1995). It was recently discovered that active tryptase monomers can be formed and that these monomers are able to process fibronectin, a protein otherwise excluded from the tetramer active site (Hällgren, Spillmann & Pejler, 2001; Fajardo & Pejler, 2003). However, the existence of active tryptase monomers in vivo remains to be proven.

**Cathepsin G**

Human MC\textsubscript{TCs} express Cathepsin G, which is a serine protease that has both trypic and chymotryptic substrate specificity (Schechter et al., 1990). Its presence in rodent MCs requires further investigation, since contradictory results exist. Spleen-derived mouse MCs contain transcripts for Cathepsin G, while CTMCs collected from rat peritoneum do not (Lutzelschwab et al., 1997; Jippo et al., 1999).

**Chymase**

This family of serine proteases with chymotrypsin-like substrate specificity was first described in 1959 (Benditt & Arase, 1959). Chymases preferentially cleave substrates at sites following aromatic residues, in a manner similar to chymotrypsin (Powers et al., 1985). MCs store active chymases in their granules, but synthesize them as inactive precursors. The zymogen has an acidic dipeptide that is removed by DPPI (Wolters et al., 2001). Removal of the propeptide causes a conformational change in the chymase that results in an active protease (Murakami, Karnik & Husain, 1995).

Classically, the chymases are divided into two groups based on phylogenetic analysis and the proteolysis pattern of the peptide angiotensin I (AngI) (Chandrasekharan et al., 1996). Chymases that preferentially produce angiotensin II (AngII) by cleaving the Phe\textsuperscript{5}-His\textsuperscript{6}-bond are classified as \(\alpha\)-chymases, whereas those that degrade AngI by proteolysis of the Tyr\textsuperscript{5}-Ile\textsuperscript{5} bond belong to the \(\beta\)-chymase family. Only one chymase-expressing gene is identified in humans and it translates into an \(\alpha\)-chymase (Caughey, Zerweck & Vanderslice, 1991), whereas rodents express a number of chymases. Mouse mucosal MCs express the \(\beta\)-chymases mMCP-1, mMCP-2 and mMCP-9 whereas the CTMCs contain the \(\beta\)-chymase mMCP-4 and the \(\alpha\)-chymase mMCP-5. The functional classification regarding angiotensin I processing has been re-evaluated as the \(\beta\)-chymases mMCP-1 and mMCP-4, as well as dog \(\alpha\)-chymase, cleaves at both Tyr\textsuperscript{5}-Ile\textsuperscript{5} and Phe\textsuperscript{5}-His\textsuperscript{6} (Caughey, Raymond & Wolters, 2000; Saito et al., 2003). Mutational studies of human \(\alpha\)-chymase revealed that Gly\textsuperscript{216} is responsible for its
chymotrypsin-like specificity, since substitution of this residue into valine switched the specificity to one resembling elastase (Solivan et al., 2002). Interestingly, rodent α-chymases have Val instead of Gly in that position, implying that they are elastase-like proteases, a notion confirmed by phage-display analysis (Kunori et al., 2002; Karlson et al., 2003).

There are a number of ways to regulate the biological activity of chymase. Several endogenous inhibitors, such as α₁-antichymotrypsin and α₂-macroglobulin, can interact with the protease, thereby limiting the chymase-associated proteolysis (Fukusen et al., 1987; Schechter et al., 1989). Chymase is stored in complex with proteoglycans and some remain attached to the GAG chains after degranulation. This interaction makes chymase less susceptible to inhibition by the large macro-molecular inhibitors mentioned, but it does not protect against low molecular weight inhibitors such as chymostatin and phenylmethanesulfonyl fluoride (Pejler & Berg, 1995; Lindstedt, L., Lee & Kovanen, 2001). The proteoglycan can also act as a scaffold that mediates interaction between chymase and its substrates. This could be through neutralization of positive charges on chymase and substrate that would otherwise disturb their interaction, as demonstrated using a charged chromogenic substrate (Pejler & Sadler, 1999). The proteoglycan can also increase the substrate availability by sequestering it from the solution and presenting it to the protease (Pejler & Sadler, 1999; Tchougounova & Pejler, 2001).

*In vitro* studies have implicated chymase in processing a wide array of proteins. Human chymase can activate the highly potent inflammatory cytokine, IL-1β (Mizutani et al., 1991), release cell-associated SCF and latent TGF-β1 (Taipale et al., 1995; De Paulis et al., 1999) and degrade albumin (Raymond et al., 2003). In a physiological context, chymase might be associated with tissue remodeling through activation of TGF-β₁, proMMP-1, proMMP-3, proMMP-9 and degradation of fibronectin and TIMPs (Vartio, Seppa & Vaheri, 1981; Saarinen et al., 1994; Suzuki et al., 1995; Fang et al., 1996; Fang et al., 1997; Frank et al., 2001; Lindstedt, K. A. et al., 2001; Leskinen et al., 2003; Okumura, K. et al., 2004). Additional evidence for chymase being involved in tissue regulation is displayed in a model of fibrosis, where chymase inhibitors reduced the fibrotic response towards bleomycin (Tomimori et al., 2003; Sakaguchi et al., 2004). In addition, the development of fibrosis is accompanied by increased levels of chymase in a model of scleroderma, and the development is ameliorated by a chymase inhibitor (Kakizoe et al., 2001; Shiota et al., 2005).

A possible role in coagulation is proposed as chymase can degrade thrombin and plasmin (Pejler & Karlström, 1993; Tchougounova & Pejler, 2001). Furthermore, chymase may contribute to the pathology of e.g. atopic and chronic dermatitis as illustrated in studies where injection of chymase provoked similar symptoms to those experienced in these conditions (Tomimori et al., 2002; Watanabe et al., 2002). The injections induced eosinophil and neutrophil influx following increased vascular permeability (He & Walls, 1998a; b).
To further elucidate chymase’s in vivo role, transgenic mice lacking individual chymases have been developed. Targeted disruption of the gene encoding the MMC β-chymase mMCP-1 showed for the first time that chymase is involved in defense against parasites. This chymase is found in the circulation, and its level is increased upon infection with e.g. Trichinella spiralis, peaking as the parasite is expelled (Huntley et al., 1990). A strain deficient in mMCP-1 displays delayed remission and increased larve deposition when infected with T. spiralis (Knight et al., 2000). A strain that lacks the α-chymase mMCP-5 exists and these animals have less severe muscle injuries following ischemia-reperfusion (Abonia et al., 1998). However, the results obtained using this strain are compromised because the mMCP-5 deficiency causes a lack of CPA. The β-chymase mMCP-4 is the major chymotryptic protease present in skin, as deduced from analysis of a strain that is devoid of this protease. This strain also exhibits impaired fibronectin, plasmin and thrombin degradation, supporting the in vitro findings (Tchougounova, Pejler & Åbrink, 2003).

The mast cell in its physiological context

The tissue distribution of MCs in the gastrointestinal tract, skin and airways suggests a role in defense against invading pathogens. Helminths, such as nematodes and schistosomes, induce a Th2 response, which involves secretion of IL-4, IL-5, IL-6, IL-10 and IL-13 followed by eosinophilia, mastocytosis and IgE production (Madden et al., 1991; Finkelman et al., 1997; Maizels & Holland, 1998). Signaling through the IL-4 receptor (IL-4R) seems to be important in nematode clearance, since blocking antibodies against IL-4Rα abrogate expulsion of Nippostrongylus brasiliensis (Urban et al., 1998). Studies using MC-deficient mice (W/W) show that MCs are important, but not essential, for parasite clearance, since the recovery of MC-deficient mice infected with Strongyloides venezuelensis or Trichinella spiralis is delayed compared to normal mice (Ha, Reed & Crowle, 1983; Lantz et al., 1998). Different MC mediators contribute to elimination of different nematodes. The β-chymase mMCP-1 is involved in expulsion of T. spiralis but does not affect clearance of N. brasiliensis (Knight et al., 2000). Additional investigations of mMCP-1−/− mice reveal that neutrophil infiltration and TNF-α levels are decreased compared to wild type mice after T. spiralis infection (Lawrence, C. E. et al., 2004).

As previously discussed, MCs express TLRs that recognize bacterial and viral components. In addition to releasing mediators following recognition, the MCs can phagocytose bacteria and function as antigen-presenting cells (Banovac et al., 1989; Malaviya et al., 1994; Frandji et al., 1995; Arock et al., 1998; Tkaczyk et al., 1999). In contrast to normal mice, MC-deficient mice show a reduced ability to fight bacterial infections and, highlighting the significance of MCs in host defense against these pathogens (Echtenacher, Mannel & Hultner, 1996). The protection may be conferred through release of e.g. TNF-α, tryptase, LTC₄, MIP-1α, MIP-1β, MCP-1 and IL-8 (Malaviya et al., 1996; Maurer, M. et al., 1998;
Malaviya & Abraham, 2000; Huang, Chifu et al., 2001; Krishnaswamy et al., 2001).

In some ways, the protective mediators can be used against the host by the bacteria. An example of this is *Shigella dysenteriae*, which secretes shiga toxin and triggers excessive release of LTC4, causing the diarrhea associated with this pathogen (Cruz et al., 1995; Pulimood, Mathan & Mathan, 1998). Yet another bacteria-associated pathology, where MCs might be the culprit, is *Helicobacter pylori*-induced gastritis. There is an increase of MC numbers in the mucosa following *H. pylori* infection and some products from this bacteria can cause MCs to degranulate, thereby inducing a sustained inflammatory process (Plebani et al., 1994; Nakajima et al., 1997; Yamamoto et al., 1999).

The immune system is designed to be protective against harmful entities, but sometimes it reacts towards seemingly harmless matter and manifests itself e.g. as allergies and allergic asthma. MCs play a major role in the early phase of allergic inflammation, which is clearly demonstrated in a model for type I hypersensitivity, passive cutaneous anaphylaxis (PCA). It is not possible to detect a PCA reaction in mice devoid of MCs (W/Wv), whereas reconstitution with MCs restores the anaphylaxis (Wershil et al., 1987).

Functional studies have revealed that MCs are involved in several pathological conditions. One group of diseases is the autoimmune disorders, such as multiple sclerosis (MS) and rheumatoid arthritis (RA). Gene-array experiments reveal evidence for MC involvement in MS progression; the number of MC-derived or MC-associated transcripts increases in sclerotic brain lesions (Lock et al., 2002). Furthermore, in a mouse model for MS, experimental autoimmune encephalomyelitis (EAE), the absence of MCs resulted in a delayed onset of the disease and also a decreased severity of the symptoms (Secor et al., 2000).

The influence of MC on arthritic development is even more dramatic. Mice devoid of MC (W/Wv and Sl/Sl) do not develop antibody-induced arthritis (Lee, D. M. et al., 2002). Moreover, MC degranulation exclusively in the joints is the first detectable reaction after administration of arthritogenic antibodies to wild type and MC-reconstituted mice. Other arthritis models substantiate these results, e.g. in collagen-induced arthritis where MCs accumulate and degranulate in the paws of affected animals (Malfait et al., 1999).

MCs are also associated with cancer and they often accumulate in the vicinity of tumors. It is likely that MCs contribute to the growth and progression of tumors, as the MCs are rich sources of growth factors and angiogenic cytokines. Additionally, there is a correlation between MC numbers and a poor prognosis in e.g. Hodgkin’s lymphoma (Molin et al., 2002).
Present investigations

Aim

The purpose of the present investigations is to elucidate the functional aspects of MC proteases and to understand how proteoglycans regulate their activity and storage.

Results and discussion

Paper I: Polycationic peptides as inhibitors of mast cell serine proteases.

Heparin affects the activity of chymase towards certain substrates as well as its susceptibility to endogenous inhibitors. Furthermore, the interaction between tryptase and heparin proteoglycan is essential for proper assembly and activation of this tetrameric serine protease, and the association with heparin stabilizes tryptase tetramers. Additional interactions that stabilize tryptase tetramers include hydrophobic and ionic interactions. We thus examined whether repeats of the heparin-binding motifs, XBBXBX and XBBBBXXBX (X; hydrophobic or uncharged, B; basic amino-acid), and homopolymers of arginine and lysine have the ability to inhibit tryptase and chymase activity.

Inhibitory studies revealed that the peptides constructed from heparin-binding motifs are inefficient inhibitors of heparin-free human β1-tryptase, but show potency towards the chymase, rMCP-1. Polymers of lysine and arginine are potent inhibitors of both tryptase and chymase, with IC_{50}-values in the nanomolar range. The homopolymers are competitive inhibitors of chymase and since the polymers have very little inhibitory effect on heparin-free chymase, it is likely that they inhibit this protease by displacing it from heparin. Previous studies show that thrombin is inactivated by chymase released from rodent peritoneal cells, and that this process is largely heparin-dependent (Pejler & Karlström, 1993; Pejler, Söderström & Karlström, 1994; Tchougounova & Pejler, 2001). To assess the efficacy of the polycations in a setting more closely resembling the in vivo situation, we tested if they could inhibit thrombin inactivation in this cellular system. Both poly-Lys and poly-Arg could inhibit the proteolysis of thrombin at nM concentrations, but polymers of the heparin-binding motifs had no effect.

Poly-Lys and poly-Arg display differential inhibitory modes of action regarding tryptase; poly-Arg appears to be a strict competitive inhibitor of tryptase, whilst poly-Lys acts with mixed competitive and non-competitive inhibition kinetics. Because the tryptase preparation used in this study was devoid of heparin, the inhibitors must interact directly with the protease. Moreover, we addressed the issue of whether polycationic inhibition of tryptase is reversible, and if the interaction between enzyme and inhibitor causes a conformational alteration in the tetramer. Addition of an excess of heparin to the enzyme-inhibitor mix partly restored the activity that was lost due to the inhibitors, but this ability decreased over time. A prominent trait of the tryptase tetramer is its resilience to
macromolecular inhibitors (Pereira et al., 1998; Sommerhoff et al., 1999), a feature we explored, to see if the rescued tetramer had an altered conformation. Addition of bovine pancreatic trypsin inhibitor (BPTI) does not affect naïve tryptase, but had an impact when added to polycation-treated, heparin-rescued tryptase. The latter finding indicates that the polycationic substances induce a rearrangement in the tetramer, making it more open and thereby more susceptible to inhibitors.

The results in this study suggest that polycationic compounds can be of therapeutic interest when treating pathologies associated with MC serine proteases. A possible limitation for their use is their size, which probably prevents them from exerting their effects on the intended pathways i.e. they remain in the circulation if injected intravenously. However, administration of the macromolecular inhibitor lactoferrin successfully prevents bronchoconstriction in allergic sheep (Elrod et al., 1997), which indicates that it is possible to use high molecular weight substances in therapeutic intervention.

Paper II: Cooperation between Mast Cell Carboxypeptidase A and the Chymase Mouse Mast Cell Protease 4 in the Formation and Degradation of Angiotensin II.

MCs are associated with angiogenesis and cardiovascular diseases, but the components that influence these processes remain unclear. One bioactive peptide that may mediate some of the ascribed features is the octapeptide, angiotensin II (AngII), derived from the decapetide, angiotensin I (AngI). Several in vitro studies show that MC chymase can produce AngII, and there are some in vivo data implicating chymase in this process (Takai et al., 1999; Caughey, Raymond & Wolters, 2000; Muramatsu et al., 2000). To determine the involvement of MC proteases in the generation of AngII from AngI, we utilized a mouse strain that lacks an enzyme essential for heparin proteoglycan biosynthesis, NDST-2. The loss of the enzyme causes a granular storage defect, rendering the CTMCs virtually devoid of neutral serine proteases. We also used a strain lacking the chymase, mMCP-4, to define its contribution to the AngII formation.

We employed an ex vivo system, where AngI is added to peritoneal cells from wild type and knock-out mice, and the conditioned media was analyzed for presence of AngI-derived products. Initial studies using ELISA-based detection of AngII revealed that degranulation of MC drastically increased AngII formation. Comparison of wild type and NDST-2−/− cell cultures showed that the reduction in proteases in the heparin-deficient cells caused an impaired ability to generate AngII from AngI, a result corroborated by high performance liquid chromatography (HPLC)-analysis of the conditioned media. HPLC analysis gave further insights into the generation of other angiotensin-related peptides. Besides AngII, Ang(1-9), Ang(1-7) and Ang(5-10) could be detected and quantified, and their formation was also influenced to the same extent as AngII following degranulation. Inhibition of chymotrypsin-like activity and removal of mMCP-4 did not affect the rate of AngI proteolysis. It did, however, influence the levels of peptides derived from AngI, as all peptides except Ang(5-10), which could not be detected, reached higher levels in mMCP-4−/− cultures compared to wild type.
Additional inhibitory studies showed that a carboxypeptidase A inhibitor (CPI) decreased the rate of AngI processing in wild type cultures. This effect was more pronounced when combined with mMCP-4 deficiency, where AngI processing was drastically decreased and formation of AngII essentially blocked. Addition of CPI to wild type or mMCP-4−/− cell cultures completely prevented the formation of Ang(1-9) and Ang(1-7), which clearly showed that these bioactive peptides were generated by CPA or a carboxypeptidase-like enzyme. In a physiological context, this implies a regulatory, homeostatic role for MCs, where the MC proteases, CPA and chymase, generate the vasoconstrictor, AngII, but limit its effects by producing its antagonists, Ang(1-9) and Ang(1-7). Furthermore, it may be of clinical interest to inhibit both chymase and CPA, or selectively inhibit either of them, e.g. following ischemia to limit the adverse effects of angiotensin-derived peptides.

**Paper III: A Key Role for Mast Cell Chymase in the Activation of Pro-matrix Metalloprotease-9 and Pro-matrix Metalloprotease-2**

In this paper, we explore how MCs participate in the regulation of the extracellular matrix (ECM). Previous studies show that chymase can act directly on ECM-components, such as fibronectin (Tchougounova et al., 2000; Tchougounova, Pejler & Åbrink, 2003). Furthermore, *in vitro* studies revealed that chymase can activate proMMP-9 (Fang et al., 1996), an enzyme capable of degrading parts of the ECM as well as activating growth factors that influence the synthesis of ECM-components. MMP-9 belongs to a family comprised of 25 known members with the common feature that they are all metalloproteases synthesized as zymogens, which require proteolytic processing to become active. Gelatinases, the sub-group to which MMP-9 belongs, has another member called MMP-2 that is associated with tumor progression and angiogenesis (Itoh et al., 1998), processes also associated with MCs.

We investigated the relevance of MC-dependent activation of MMP-9 by culturing mouse peritoneal cells, in either the presence or absence of MCs. In the absence of MCs, endogenous proMMP-9 was not activated, whereas addition of MCs increased the level of active MMP-9 in a dose-dependent manner. We used the heparin-deficient mouse strain, NDST-2−/−, to elucidate the involvement of heparin-dependent MC proteases in the generation of active MMP-9. Zymographic analysis did not reveal any active MMP-9 in NDST-2−/− peritoneal cell cultures, thus implying a role for MC proteases in the proteolytic activation of proMMP-9. Analysis of peritoneal cell cultures lacking the chymase, mMCP-4, showed that proMMP-9 accumulated in these cultures, while active MMP-9 was absent, indicating that mMCP-4 is involved in MMP-9 formation. This result gained additional support upon analysis of tissue homogenates, where the deficit in mMCP-4 caused a complete abolition of active MMP-9 in the studied tissues, whilst homogenates of tissues from wild type mice contained both proMMP-9 and its active form. Further comparison of wild type and mMCP-4−/− tissue homogenates suggested that chymase might activate proMMP-2, as mMCP-4 deficiency caused a partial reduction in active MMP-2 levels.
To substantiate the discoveries, we carried out in situ zymographies on selected tissues. This analysis revealed a strong reduction in gelatinolytic activities in the dermal region of the ear, as well as in the surroundings of blood vessels and bronchioli of the lungs, in mMCP-4−/− compared to wild type. Histochemical and amino acid analysis was carried out to investigate whether the reduction in MMP-activity and lack of mMCP-4 caused alterations in ECM-composition. These studies showed increased collagen-deposition in mMCP-4−/−-skin. Moreover, the intensity following immunohistochemical detection of fibronectin was higher in lungs and ears from mMCP-4-knock out mice, implying increased fibronectin levels in these animals.

Taken together, the results presented in this study show that chymase contributes to the formation of active MMP-2 and MMP-9, and may be a key-player in this process. Furthermore, we suggest that chymase influences tissue homeostasis, partly through activation of MMPs and partly by acting on the ECM directly. The present study thus clearly establishes a potential link between MC proteases and the development of pathologies associated with changes in ECM, e.g. rheumatoid arthritis.


In paper III, we detected a decreased activation of MMP-2 in tissues collected from mice lacking the chymase, mMCP-4. We conducted this study to determine if this chymase, or another MC protease, directly acts on proMMP-2 to generate active MMP-2.

The rate of MMP-2 formation from exogenously added proMMP-2 increased in peritoneal cell cultures after induction of MC degranulation, which indicated an involvement of MC proteases in proMMP-2 activation. In agreement with the degranulation study, elimination of MCs from peritoneal cell cultures caused a decreased formation of active MMP-2, but addition of MCs restored proMMP-2 activation. The MC-dependent proMMP-2 activation was susceptible to inhibitors of serine proteases, but not to inhibitors of other enzyme-classes. It appears that mMCP-4 was not the major proMMP-2 activator, as MMP-2 formation was similar in cultures lacking this chymase. However, the activation involved a MC protease requiring the proteoglycan, serglycin, for its storage, as determined by analysis of cultures of serglycin-deficient peritoneal cells.

The results in this paper indicate that an MC serine protease, dependent on serglycin proteoglycan for its storage, can generate active MMP-2. This provides an alternative route to the commonly proposed mechanism, which involves a ternary complex between proMMP-2, TIMP-2 and MMP-14.
Concluding remarks and future perspectives

MCs are intricate entities, capable of providing excellent protection against invading pathogens, but also capable of causing serious harm to their host. Owing to their wide array of mediators, they can influence the progression of several pathologies with which they are associated. Depending on the stimuli, their effect can be detrimental or beneficial. The present investigations reveal that MC proteases influence the integrity of the surrounding ECM, and that some of this effect is mediated through activation of matrix degrading enzymes. However, MC are implicated in both excessive ECM break-down as well as excessive ECM deposition, and the different pathways which are affected need further investigation. In order to understand the mechanisms behind these opposing effects, identification of the affected mediators in each process is needed. One tool for this identification is proteomics, which enables simultaneous detection of proteins affected by a certain process. The use of this tool may reveal novel targets for MC proteases and other MC components, and hence explanations for how MC participate in different physiological conditions.

In addition to analysis of MC proteins, it is of interest to investigate the composition of the proteoglycans, and especially their GAG-chains. The characterization of sugars, referred to as glycomics, has attained increased attention as mounting evidence show that the modifications of the sugar residues, and the composition of polysaccharides, generate sequences that regulate protein- and cell-interactions. An example of this is a penta-saccharide sequence in heparin, which is required for the inhibition of thrombin by anti-thrombin. Interaction-based technology, e.g. surface plasmon resonance and affinity chromatography, may reveal additional sequences required for specific interactions and thereby provide explanations for the involvement of MCs in different processes.

The construction of gene targeted mice, and the use of MC “knock-ins” are of great interest for unveiling the physiological effects of MCs. In depth analysis, using e.g. proteomics and glycomics, to elucidate what the gene-deficiency affects in various disease models will hopefully suggest what mediators should be targeted in each disease.

An intriguing prospect would be to govern the MC response in different pathologies, making use of its beneficial effects and minimizing its deleterious potential. For example, it would be very useful if the angiogenic-related mediators could be blocked, whilst not compromising the mediators capable of inducing apoptosis when treating tumors. Based on indications from the studies conducted in this thesis, inhibitors of chymase and CPA could be of therapeutic interest.
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