Mouse Mast Cell Protease-4 Recruits Leukocytes in the Inflammatory Phase of Surgically Wounded Skin

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Objective: Mouse mast cell protease-4 (mMCP-4, also known as chymase) has both pro- and anti-inflammatory roles depending on the disease model. However, its effects have not been studied in surgically wounded skin. Given the significant clinical applications of modulating the inflammatory response in wound healing, we examined the role of mMCP-4 and the effect of its inhibitor chymostatin on leukocyte and polymorphonuclear cell (PMN) recruitment in our skin model.

Approach: Recruitment was assessed on day-1 postwounding of three groups of mice ($n = 10$ each): mMCP-4 null mice, wild-type (WT) mice treated with the mMCP-4 inhibitor chymostatin, and WT with no other intervention. Leukocytes were stained with CD-45 cell marker, and PMN cells were stained with chloroacetate esterase.

Results: The WT mice had 27 ± 9 leukocytes per field compared with 11 ± 6 for the mMCP-4 nulls, a decrease of 60% ($p = 0.03$), whereas the chymostatin-injected group had a count comparable with the uninjected WT controls at 24 ± 9 . The WT group had a PMN count of 96 ± 12 cells, compared with just 24 ± 8 in the mMCP-4 null group, a decrease of 75% ($p = 0.001$), whereas the chymostatin-treated group had 60 ± 18 cells, a decrease of 38% compared with the WT group ($p = 0.03$).

Innovation: We showed that the inflammatory process can be influenced by impeding the arrival of PMNs into the surgically injured site using the mMCP-4 inhibitor chymostatin.

Conclusion: Chymase contributes to the recruitment of white blood cells in surgically wounded skin.

Keywords: wound healing, inflammation, mouse mast cell protease-4, leukocytes recruitment, polymorphonuclear cells, chymase

INTRODUCTION

MAST CELLS ARE ubiquitous immune cells that originate in the bone marrow, yet they only complete their maturation and differentiation once

they reach their target organ.^{1,2} Each mast cell has on average 75 granules that can contain a wide variety of different mediators. Once the immature mast cells reach their destination,

their receptors interact with the organ's milieu, leading to gene transcription and dictation of which mediators are formed in the granules.^{1,3,4} The diversity of these mediators accounts for the contribution of mast cells to a variety of biological processes, including inflammation, immunity, tumorigenesis, allergies, angiogenesis, and wound healing.⁵

In humans, chymase (CMA1) is one of the mediators found in the granules of mast cells. The murine counterpart of human chymase is the mouse mast cell protease 4 (mMCP-4),⁶ a chymase that is stored as active protease in the mast cell granules. An interesting feature of chymase is that it has been found to have both anti-inflammatory and proinflammatory properties.⁷ Depending on the context, mMCP-4 has been found to have anti-inflammatory properties, such as in models of allergic airway inflammation,⁸ renal fibrosis,⁹ post-traumatic brain injury,¹⁰ and sepsis,⁶ and yet, in other contexts exhibiting proinflammatory behavior such as in bleomycin-induced lung inflammation,¹¹ chronic testicular inflammation,¹² and inflammatory bowel disease.¹³

Inflammation is the early phase of wound healing, beginning immediately after injury. It is characterized with the recruitment of white blood cells (WBCs) into the wound site, particularly neutrophils and monocytes.¹⁴ This cellular response forms within the first 24 h of injury.¹⁵ In a model of skin injury, mice deficient in chymase were found to have significantly reduced inflammation after second degree burn injury,¹⁶ and chymase was also implicated in other cutaneous inflammatory process such

as in rosacea¹⁷ and atopic dermatitis.¹⁸ The role of mast cells in inflammation is well known, particularly in the inflammatory phase of wound healing,¹⁹ and dysregulation of the inflammatory response has also been shown to cause pathologic wound healing.

In burn wounds there is generalized increase in capillary permeability. There is also coagulative necrosis at the center of the burn, as well as a peripheral zone of stasis with sluggish circulation, and an outermost zone of hyperemia that is a result of the extensive vasodilation.²⁰ These changes are not seen in surgical wounds, where the inflammation remains local. Surgical wound complications are a substantial source of morbidity, particularly in patients with comorbid conditions.²¹ As attenuating the inflammatory response in skin injury has significant potential clinical applications, we investigated the role of mMCP-4 in surgical wound healing. We hypothesized that chymase influences the inflammatory process by modulating the recruitment of WBCs into the site at the time of injury, and thus investigated this process using both mMCP-4 null mice and the chymase inhibitor chymostatin.

CLINICAL PROBLEM ADDRESSED

A physiological inflammatory response highlighted by appropriate recruitment of WBCs is critical to physiological wound healing. Under- or overactivation can disrupt the healing process, and can lead to clinical symptoms such as infection, wound dehiscence, or hypertrophic scarring.²² Having the ability to modulate this phase can

Figure 1. Macroscopic view of the wound model. **(A)** The dashed lines/arrows indicate the intradermal injections of chymostatin. **(B)** Appearance of the wound on postoperative day 1. Scale bar = 1 cm.

become a clinical option to treat or prevent such conditions.

MATERIALS AND METHODS

Animals and surgical procedures

Ten mMCP-4-deficient mice (line 003410), and 20 wild-type (WT) mice (line C57BL/6 B6) were obtained from the Taconic Farms, Inc. (Germantown, NY). Animals were housed in an accredited facility, and all procedures were carried out according to an approved protocol and to our institutional IACUC policies. After surgical preparation of the wound site, under inhalation anesthesia (isoflurane 1.3%) a 1 cm² area of dorsal skin was excised using forceps and scissors in all animals and covered with a semiocclusive dressing (3MTM TegadermTM). Ten WT mice received an intradermal injection of chymostatin along each of the four borders of the wound (Fig. 1A). A chymostatin solution was prepared by diluting 5 mg of powdered chymostatin in 250 μ L of dimethyl sulfoxide to give a 20 mg/mL stock solution. Forty microliters of the stock solution was further diluted in 4 mL of phosphate-buffered saline to obtain the standard working concentration of 300 μ M chymostatin. Mice were injected with 0.1 mL of this working solution at each wound border (arrows, Fig. 1A). The mice were followed-up for 24 h after wounding (Fig. 1B), and tissue specimens were obtained and fixed in 10% neutral-buffered formalin solution for 24 h, then placed in 70% ethanol at 4°C.

Tissue processing, histology, and analysis

Fixed tissue specimens were embedded in paraffin, and 6 μ m cross-sectional cuts were obtained and stained with anti-CD-45 to identify leukocytes, and for chloroacetate esterase (CAE) activity to identify the polymorphonuclear cells (PMNs): neutrophils, eosinophils, basophils, and mast cells. Mast cells can be differentiated from the other myeloid cells by their metachromatic staining and larger size.

Digital photographs of the two edges of the wounds were taken using an optical microscope at 40 $\times$ magnification for leukocyte counting, and at 10 $\times$ magnification for PMNs counting. One section was taken per mouse, and two fields per section. Leukocyte and PMN (including mast cell) counts were quantified by number of cells per field. All measurements were analyzed and quantified by three blinded observers using digital planimetry (ImageJ; NIH, Bethesda, MD).

Statistical analysis was carried out using analysis of variance and Bonferroni *post hoc* correction.

RESULTS

mMCP-4 increases recruitment of leukocytes in the inflammatory phase of wound healing

Leukocyte recruitment was assessed on day 1 postwounding using CD-45 staining to quantify infiltrates (Fig. 2). The WT mice had 27 ± 9 CD45⁺ leukocytes per field in their wound site compared with 11 ± 6 leukocytes for the mMCP-4 nulls, a significant decrease of 60% ($p = 0.03$), whereas the chymostatin-injected group had a count comparable with the not-injected WT controls at 24 ± 9 (Fig. 3A). Day 1 postwounding CAE staining for PMNs showed that the WT group had 96 ± 12 positive cells per field, compared with just 24 ± 8 positive cells in the mMCP-4 null group (Fig. 4), a

Figure 2. CD-45 staining for leukocytes on wound samples. The CD 45 pan-leukocyte marker staining marks leukocytes in a brown-copper hue. mMCP-4 nulls show significantly less leukocyte infiltration compared with wild type. The chymostatin-treated group shows a leukocyte infiltrate comparable with the one observed in the wild-type group. Scale bar = 50 μ m. mMCP-4, mouse mast cell protease-4.

Figure 3. Analysis from measurement of stained histological sections. **(A, B)** mMCP-4 nulls ($n=10$) had 60% less leukocytes and 75% less PMNs compared with wild-type controls ($n=10$), and 60% less PMNs compared with the chymostatin-treated group ($n=10$). **(C)** No significant differences among groups were observed with regard to the number of mast cells in the inflammatory infiltrate. * $p<0.05$. PMNs, polymorphonuclear cells.

significant decrease of 75% ($p=0.001$), and compared with 60 ± 18 in the chymostatin-treated group, a decrease of 38% compared with the WT group ($p=0.07$). The mMCP-4 group also had 60% less PMNs compared with the chymostatin group ($p=0.03$) (Fig. 3B). In addition, there was no difference in the mast cell count between the three groups, with 8 ± 2 mast cells per field in the WT mice, 9 ± 4 mast cells in the mMCP-4 nulls group, and 6 ± 5 mast cells in the chymostatin group (Fig. 3C).

DISCUSSION

Our results showed that the mMCP-4 nulls and the mice treated with the chymase inhibitor chymostatin had significantly fewer PMNs compared with WT mice, with a 75% and 38% decrease of PMNs, respectively, compared with control mice. That the chymostatin-treated group showed a decrease in PMN recruitment, although its magnitude was not as high as the mMCP-4 null group, substantiates the role of chymase in this process.

The observed decrease in total leukocytes in the mMCP-4 null group was not replicated in the chymostatin-treated group, which showed counts comparable with the WT group (27 ± 9 and 24 ± 9 , respectively). One potential explanation for the attenuated decrease in the chymostatin group compared with the mMCP-4 nulls is that mast cells release their mediators immediately after skin injury,²³ and the injection of the chymostatin after wounding would not have been timely enough to oppose the near-instantaneous release of chymase.

The mechanism of action of chymase in inflammation is complex and can be the result of either direct or indirect action. The reason why chymase has an anti-inflammatory behavior in one environment while having the opposite effect in another is because of the chymase's dependence on what stimulates the mast cell (mechanical, chemical, thermal, IgE mediated, etc.), and the presence of local mediators that are cleavable by the chymase.⁷

In models of skin injury, one mechanism by which chymase influences neutrophil recruitment is by activating the potent chemoattractant and

Figure 4. Chloroacetate esterase staining for PMNs and mast cells on wound samples. Scale bar=200 μ m.

activator of neutrophils: neutrophil-activating-peptide II (NAP-2)²⁴ from its inactive precursor connective tissue-activating peptide III (CTAP-III). Furthermore, chymase's ability to activate CTAP-III into the active NAP-2 is a thousand-fold higher than neutrophils.²⁵ In addition to modulating neutrophil recruitment, chymase has a direct effect on inflammation by modulating the activity of inflammatory cytokines such as IL-6, IL-13, and TNF α .²⁶ For example, Zhao *et al.* showed that the mast cell proteases degraded cytokines such as IL-6, whereas Ilves and Harvima showed that low concentration of chymase stimulated the proliferation of mononuclear cells and T cells, and that this effect was mediated by chymase's degradation of IL-6, which is an inhibitor in T cell proliferation.¹⁸

Chymase can also influence leukocyte recruitment by facilitating the extravasation of leukocytes into wounds, as it potentiates the vasodilatory effect of histamine.²⁷ The mechanism by which chymase is thought to contribute to this process is by its action on the surrounding tissue and its extracellular matrix elements, whereby their degradation by the chymase results in fewer obstacles to the flow of fluid,²⁷ and consequently the entry of leukocytes to the injury site. Another study showed that inhibition of the chymase leads to the elimination of the vascular permeability, and this effect is thought to be mediated by the degradation of the fibrin clot by the chymase.²⁸ In addition, chymase activates TGF- β , proendothelins, promatrix metalloproteinases, and degrades thrombin and plasmin, all of which underline chymase's role in tissue remodeling during the inflammatory phase of injury and subsequent fluid extravasation.²⁹

In addition to direct effect on tissue components, chymase is able to degranulate nearby mast cells that have not been activated by the injury.³⁰ As such the chymase would contribute to inflammation by leading to the release of the other proinflammatory mediators present in mast cells, such as TNF, IL-6, and IL-1.

The inflammatory process involves the immediate action of mediators in addition to the later recruitment of WBCs to the injury site, and the clinical advantages of controlling inflammation are diverse and valuable. WBCs recruitment might be an important mechanism for defense against infection, particularly in patients with depressed immunity such as diabetes. Conversely, reducing recruitment might be important in control hypertrophic scarring. This study showed that chymase has an important role in the recruitment of WBCs, specifically PMNs, in surgically wounded skin. We showed that it is possible to limit the inflammatory process by delaying or impeding the arrival of PMNs into the surgically injured site with chymostatin. This suggests that clinical modulation of the inflammatory response by controlling the effects of chymase warrants further exploration, such as investigating the expression of cytokines and other inflammatory mediators, as well as its potential effect on the healing of the surgical wound.

INNOVATION

Healing skin injuries remains a central clinical concern, particularly in the presence of comorbid conditions. Furthermore, current treatment options for pathological scarring are lacking.²² PMNs

and their mediators have an integral role in the wound healing process, and many pathological conditions stem for either their under- or overactivation. We have shown that PMNs recruitment can be modulated through the manipulation of mMCP-4, and these pathways can be considered for targeting in clinical scenarios.

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AUTHOR DISCLOSURE AND GHOSTWRITING

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. No ghostwriting was used in this article.

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KEY FINDINGS

- mMCP-4 contributes to the recruitment of PMNs and leukocytes into surgically wounded skin.
- We were able to inhibit PMN, but not leukocyte recruitment into surgically wounded skin using chymostatin, an mMCP-4 inhibitor.
- The recruitment effects were independent from mast cell count.

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Abbreviations and Acronyms

mMCP-4 = mouse mast cell protease 4
 PMNs = polymorphonuclear cells
 WBCs = white blood cells
 WT = Wild type