INVESTIGATIVE REPORT

Decrease in Chymase Activity is Associated with Increase in IL-6 Expression in Mast Cells in Atopic Dermatitis

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Mast cell chymase and interleukin (IL)-6 can be involved in atopic dermatitis and chymase can degrade IL-6. Our aim was therefore to study the expression of mast cell chymase and IL-6 in atopic skin using enzyme- and immunohistochemistry and to analyse their interaction in vitro. Chymase activity was significantly reduced in mast cells in skin lesions whereas the percentage of IL-6 mast cells was increased. Low recombinant human (rh)-chymase concentration (10–100 ng/ml) stimulated and higher concentration (1,000 ng/ml) inhibited the proliferation of T cells and peripheral blood mononuclear cells. Rh-IL-6 inhibited T-cell proliferation, and even inhibited the proliferation induced by rh-chymase. Pretreatment of rh-IL-6 with a high rh-chymase concentration prevented the IL-6-induced inhibition in T-cell proliferation. The results suggest that reduction in chymase activity can give rise to increased cellular effects of mast cell IL-6 and that chymase and IL-6 can modify each other’s effects. Key words: mast cells; chymase; IL-6; atopic dermatitis.

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Atopic dermatitis (AD) is a common inflammatory skin disease in which an epidermal barrier dysfunction leads to enhanced skin penetration of antigens. Mast cells (MCs) are the first-line responders to allergen stimulation (1) and cell injury (2). The numbers of MCs are increased both in non-lesional and lesional AD skin (3, 4). Chymase, a major protein in the secretory granules of MCs, is a chymotrypsin-like enzyme that belongs to serine proteinases. Chymase gene CMA1 variants have been found to be associated with AD (5, 6), although some other reports have not confirmed the results (7). There are some reports that point to an important role for chymase in the pathogenesis of allergic diseases and AD. An intradermal injection of chymase has been shown to increase the accumulation of MCs, eosinophils, neutrophils and mononuclear cells in mouse skin (8, 9). Additionally, chymase has stimulated cutaneous angiogenesis and contributed to cell damage in AD by increasing the expression of metalloproteinases (10). In mouse models, a chymase inhibitor has been shown to ameliorate the symptoms of AD (11).

Another way by which MCs can regulate immune responses is through interleukin (IL)-6. IL-6 is a multifunctional cytokine produced by many different cell types, e.g. endothelial cells, macrophages, dendritic cells, epithelial cells, MCs, T and B cells. The production of IL-6 is stimulated by several factors such as trauma or the cytokines IL-1, IL-33, tumour necrosis factor and platelet-derived growth factor (2, 12–17). Interest towards the role of IL-6 in allergic diseases dates back 20 years. An IL-6 receptor genetic variant 358Ala was recently found to be associated with a persistent form of AD (18). Increased production of IL-6 by peripheral blood T cells has been observed in AD patients (19). Furthermore, house dust mites and skin colonising yeast Malassezia sympodialis, factors known to exacerbate AD, have been reported to increase IL-6 expression (20, 21). Both bone marrow- and skin-derived MCs produce IL-6 after stimulation of the FcεRI-receptor by IgE-antigen complexes (22, 23). On the other hand, the IL-6 produced by FcεRI-stimulated skin MCs can be degraded by MC chymase (23).

Both chymase and IL-6 can be involved in AD pathogenesis and chymase may degrade and hence regulate the activity of IL-6. Therefore, the relationship between chymase and IL-6 in MCs in AD was investigated in this study.

PATIENTS, MATERIAL AND METHODS

Study subjects and skin biopsies

The description of study subjects has been presented in our previous article (24). Briefly, 17 patients with moderate-to-severe AD (11 women and 6 men, mean age 35 years, range 19–69) volunteered to take part in the study after providing written informed consent. The severity of AD was defined according to the Rajka-Langeland Severity Index (score range 3–9; score 3–4 denotes mild, 4.5–7.5 moderate and 8–9 severe disease) (25). Additionally, the Eczema Area and Severity Index score (EASI, score range 0–72), the Dermatology Life Quality Index score (DLQI, score range 0–30) (26), the Visual Analogue Scale score for the mean level of itching over the past week (VAS, 0–10 cm, where 0 = no itching and 10 = worst itching ever experienced), and the mean total serum IgE level were measured.

Four-mm punch biopsies were collected from lesional skin under local anaesthesia. A control biopsy was taken from non-lesional skin of each subject at least 2 cm from the site of lesional skin biopsy. The samples were immediately embedded...
in OCT compound (Sakura Finetek, Torrance, CA, USA) and frozen prior to preparing 5-µm cryosections.

The Ethics Committee of the Kuopio University Hospital, Kuopio, Finland, approved the protocol.

Enzyme-histochemical and immunohistochemical stainings and analyses of skin biopsies

The enzyme-histochemical techniques used were developed by the research group (27). Prior to the enzyme-histochemical staining of tryptase+ and chymase+ MCs, cryosections were fixed in a mixture of 0.6% formaldehyde, 0.5% acetic acid and 10 mM sodium phosphate buffer for 8 min. For the staining of tryptase, the sections were incubated for 10 min in a solution containing 1 mM Z-Gly-Pro-Arg-4-methoxy-2-naphthylamide (-MNA) as the substrate (Bachem, Bubendorf, Switzerland), 0.5 mg/ml Fast Garnet GBC salt as the chromogen (Sigma-Aldrich, Schnelldorf, Germany), 0.5 mg/ml α1-proteinase inhibitor (α1-PI, Sigma-Aldrich) and 100 mM Tris-HCl buffer (pH 7.5). The enzyme activity of MC chymase was demonstrated by using the mixture of 1 mM Suc-Val-Pro-Phe-MNA (Bachem), 0.5 mg/ml Fast Black K salt (Sigma-Aldrich), 0.5 mg/ml α1-proteinase inhibitor and 100 mM Tris-HCl buffer (pH 7.5). Incubation time was 30 min. α1-PI and aprotinin were added to the substrate solutions to reduce any possible background staining.

In the immunohistochemical staining of MC chymase, the cryosections were first fixed in cold acetone for 10 min. MC chymase was identified using commercially available monoclonal mouse anti-human antibodies (5950-4906, AbD Serotec, Martinsried, Germany). The bound antibodies were visualised with the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA) and 0.05% 3,3′-diaminobenzidine, 0.04% nickel chloride, and 0.03% hydrogen peroxide, as described previously (28). Unrelated mouse IgG was used as the control at the same concentration as the specific antibodies. Sequential double-staining method for IL-6 in mast cells

The techniques for the double-staining method and staining of IL-6 in MCs have been described elsewhere (12, 29). After enzyme-histochemical staining of tryptase, at least 10 random sites of upper dermis per skin sample were photographed (objective × 20). The total number of tryptase+ cells was counted from each picture. The dye was then removed by 15% Tween 20 incubation overnight, and immunohistochemical staining of IL-6 was performed using anti-IL-6 mouse monoclonal antibody (MAB2061, 25 µg/ml, R&D Systems, Minneapolis, MN, USA) and the Vectastain Elite ABC kit. Unrelated mouse IgG controlled the staining. The previously photographed sites were re-photographed at the identical skin area using visual landmarks in the skin sections. Tryptase+ cells with IL-6 immunoreactivity were identified and counted by comparing the photographs side by side. The mean percentage of tryptase+ MCs showing IL-6 immunoreactivity was calculated.

Cultivation of T cells and peripheral blood mononuclear cells with rh-chymase, rh-IL-6 and α1-proteinase inhibitor

A heparinised peripheral blood sample was obtained from atopic patients. Peripheral blood mononuclear cells were separated on Ficoll-Paque™ PLUS (GE Healthcare Bio-Sciences, Uppsala, Sweden) by centrifugation at 1000 × g for 10 min as described previously (30). The total T-cell pool was isolated by filtering PBMCs through nylon wool fibre columns (Polysciences, Warrington, PA, USA) for 1 h according to the instructions of the manufacturer.

T-cell proliferation experiments were performed using cells separated from peripheral blood mononuclear cells (PBMCs). In these experiments, rh-IL-6 (0.1 and 0.5 µg/ml, 206-IL, R&D Systems) or diluent control (0.005% BSA in PBS) was preincubated with rh-chymase (0.1, 1.0 and 10 µg/ml, C8118, Sigma-Aldrich) or diluent control (stock 20 mM Tris-HCl, 0.8 M NaCl, 25% glycerol, pH 7.6) at room temperature for 2 h. Before the preincubation, 1% heat-inactivated bovine serum albumin (BSA) was used for blocking possible binding sites for rh-IL-6 on the plastic surface of test tubes. After blocking, the tubes were washed with PBS. The concentration of BSA was kept as low as possible (5 µg/ml) during the preincubation to prevent any possible competitive inhibition of rh-chymase (31). After preincubation, samples from the incubation mixtures were collected, and then 10 mg/ml α1-proteinase inhibitor (α1-PI; producing the concentration of 0.8 µg/ml) was added to the remaining solutions to inactivate rh-chymase. A sample from each incubation solution was added to T cells (1 × 10^6 cells/ml of RPMI-1640 without serum) in the wells of a 96-well plate as described in the Results. After incubation for 48–72 h, 5 µCi/ml 3H-thymidine in RPMI-1640 was added to wells for 24 h (the final concentration 0.63 µCi/ml). The T-cell proliferation response was measured by analysing 3H-thymidine incorporation with a liquid scintillation counter using ULTIMA Gold™ fluid. Additionally, the same experiments were performed with PBMCs.

Statistical analysis

Statistical analyses were performed using SPSS for Macintosh (SPSS, Chicago, Illinois). The differences in tryptase+ and IL-6+ MCs between lesional and non-lesional skin were evaluated with the paired Student’s t-test. The Wilcoxon test was performed to compare the difference in the percentage of IL-6+ MCs in the ratios of MCs with chymase activity to MCs with chymase protein between lesional and non-lesional dermis. Linear associations between these parameters and the parameters of clinical severity of AD were tested using the Spearman correlation coefficients. The results from experiments with rh-chymase, rh-IL-6 and T cells or PBMCs were analysed using the Mixed model method.

RESULTS

Chymase activity is decreased in mast cells in AD lesions

The number of MCs with chymase immunoreactivity was greater in lesional dermis than in non-lesional dermis (154 ± 72 cells/mm² vs 111 ± 46 cells/mm², p = 0.035). In contrast, relatively reduced enzyme activity of chymase was seen in lesional dermis: 47 ± 19% of MCs with chymase immunoreactivity in non-lesional skin but only 30 ± 13% of the corresponding MCs in lesional skin displayed chymase enzyme activity (p<0.001).

The percentage of mast cells expressing IL-6 is higher in lesional than in non-lesional dermis

IL-6 expression was detected in infiltrating and endothelial cells in upper dermis and in keratinocytes in epidermis (Fig. 1). As demonstrated by sequential double-staining micrographs (Fig. 1), many IL-6+ cells...
in dermis were tryptase⁺ MCs. The mean number of tryptase⁺ MCs did not differ significantly between lesional and non-lesional skin (148 ± 67 cells/mm² vs 131 ± 58 cells/mm², p = 0.34). Similarly, the mean number of IL-6⁺ MCs was non-significantly greater in lesional than in non-lesional dermis (98 ± 63 cells/mm² vs 77 ± 45 cells/mm², p = 0.14). However, the percentage of IL-6⁺ MCs was statistically significantly greater in lesional dermis (62 ± 15%) than in its non-lesional counterpart (56 ± 12%, p = 0.047).

The association between IL-6 expression and chymase activity was further studied, because chymase can cause proteolytic degradation of IL-6 and at the same time be inactivated by protease inhibitors. A non-significant weak negative correlation was detected between the percentage of IL-6⁺ MCs and the ratio of MCs with chymase activity to MCs with chymase protein (Chy activity/Chy protein; rₛ = –0.25, p = 0.3).

No correlations between clinical severity of AD and IL-6⁺ mast cells or chymase activity

The number and the percentage of IL-6⁺ MCs were compared to clinical parameters: the Rajka-Langeland Severity Index (mean 7 ± 1), the EASI score (mean 12.3 ± 6.6), the DLQI score (mean 12.7 ± 6.8), the levels of itching (mean 5.4 ± 2), and the total IgE serum levels of study subjects (1,862.3 ± 1,873.6 kU/l) (24). No correlations were detected between these parameters and the number or percentage of IL-6⁺ MCs.

Clinical parameters did not correlate with Chy activity/Chy protein ratio or with the number of MCs with chymase immunoreactivity or chymase enzyme activity.

Low concentrations of rh-chymase stimulate and rh-IL-6 inhibits T-cell proliferation

Additionally, we wanted to clarify if MC chymase and IL-6 are able to modify each other’s effects in vitro.
Table I. Proliferation response of T cells in co-culture experiments with T cells and rh-IL-6 and/or rh-chymase before and – in parentheses – after adding α1-proteinase inhibitor (α1-PI)

<table>
<thead>
<tr>
<th>Incubation mixture</th>
<th>Patient 1</th>
<th>Patient 2</th>
<th>Patient 3</th>
<th>Patient 4</th>
<th>Patient 5</th>
<th>Patient 6</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diluent control + diluent control</td>
<td>100 (112)</td>
<td>100 (24)</td>
<td>100 (112)</td>
<td>100 (90)</td>
<td>100 (112)</td>
<td>100 (88)</td>
<td>100 (90%NS)</td>
</tr>
<tr>
<td>Diluent control + rh-IL-6 10 ng/ml</td>
<td>Not done</td>
<td>91 (27)</td>
<td>80 (112)</td>
<td>69 (120)</td>
<td>88 (123)</td>
<td>58 (69)</td>
<td>77% (90%NS)</td>
</tr>
<tr>
<td>Diluent control + rh-IL-6 50 ng/ml</td>
<td>59 (116)</td>
<td>59 (28)</td>
<td>67 (118)</td>
<td>61 (110)</td>
<td>73 (129)</td>
<td>30 (72)</td>
<td>58% (96%NS)</td>
</tr>
<tr>
<td>Rh-chymase 10 ng/ml + diluent control</td>
<td>197 (114)</td>
<td>116 (27)</td>
<td>354 (100)</td>
<td>382 (93)</td>
<td>210 (114)</td>
<td>243 (91)</td>
<td>250* (90%)</td>
</tr>
<tr>
<td>Rh-chymase 100 ng/ml + diluent control</td>
<td>138 (123)</td>
<td>43 (30)</td>
<td>97 (110)</td>
<td>103 (95)</td>
<td>222 (131)</td>
<td>184 (87)</td>
<td>131** (96%NS)</td>
</tr>
<tr>
<td>Rh-chymase 1,000 ng/ml + diluent control</td>
<td>107 (147)</td>
<td>21 (25)</td>
<td>113 (118)</td>
<td>115 (99)</td>
<td>145 (148)</td>
<td>66 (93)</td>
<td>95% (105%NS)</td>
</tr>
<tr>
<td>Rh-chymase 10 ng/ml + rh-IL-6 50 ng/ml</td>
<td>75 (94)</td>
<td>82 (69)</td>
<td>97 (108)</td>
<td>79 (104)</td>
<td>89 (125)</td>
<td>45 (77)</td>
<td>78% (96%NS)</td>
</tr>
<tr>
<td>Rh-chymase 100 ng/ml + rh-IL-6 50 ng/ml</td>
<td>88 (120)</td>
<td>32 (35)</td>
<td>95 (104)</td>
<td>98 (99)</td>
<td>120 (118)</td>
<td>58 (76)</td>
<td>82% (92%NS)</td>
</tr>
<tr>
<td>Rh-chymase 1,000 ng/ml + rh-IL-6 50 ng/ml</td>
<td>120 (141)</td>
<td>22 (28)</td>
<td>102 (110)</td>
<td>114 (112)</td>
<td>132 (141)</td>
<td>55 (90)</td>
<td>91% (104%NS)</td>
</tr>
</tbody>
</table>

* Patients with atopic dermatitis.
† Patients with mucosal atopic symptoms.

Results are expressed as relative T-cell proliferation response expressed in percentages (in parenthesis the results after adding α1-PI).

*p-values of the differences between T-cell responses were determined using the Mixed model analysis: *, † p < 0.001, **, †† p < 0.05.

‡, ‡‡ incubation mixtures without α1-PI or rh-chymase and α1-PI or rh-IL-6 vs rh-chymase at the same concentration.

NS: not significant.

alone stimulated significantly the PBMC proliferation response in all experiments at 100 ng/ml (Table SI1). The concentration of rh-chymase giving maximum stimulation differed somewhat between the study subjects. However, at 1,000 ng/ml rh-chymase, the stimulation of the PBMC proliferation rate was decreased in 3 of 4 experiments. The pattern of stimulation and then inhibition by rh-chymase alone in these 3 subjects was similar to that induced by the combination of rh-chymase and rh-IL-6 (Table SI1). As in the experiments with T cells, the combination of rh-IL-6 and rh-chymase stimulated the proliferation of PBMC more than what could be achieved with rh-IL-6 alone, although the difference was statistically significant only at the rh-chymase concentration of 100 ng/ml (p < 0.001).

α1-PI significantly reduced the proliferation of PBMCs in wells treated with only diluent controls (Table SI1). Therefore, the significant inhibitions observed in experiments with rh-IL-6 and α1-PI or rh-chymase and α1-PI were dependent on the effect of α1-PI. The combination of α1-PI and 10–100 ng/ml rh-chymase reduced significantly the PBMC proliferation response. At the rh-chymase concentration of 1,000 ng/ml, the change in the proliferation rate was not as clear. This indicates that α1-PI added to the incubation mixture was not able to inactivate rh-chymase sufficiently at the highest concentration. Similarly, in experiments with both rh-IL-6 and rh-chymase, the decrease in the PBMC proliferation response was seen at rh-chymase concentrations of 10 and 100 ng/ml but not at 1,000 ng/ml.

DISCUSSION

The present study shows that the percentage of MCs expressing IL-6 is slightly increased, but simultaneously the activity of MC chymase is relatively decreased, in lesional AD skin compared to non-lesional skin. The result on IL-6 differs from the finding in psoriatic skin where no such increase was found between lesional and non-lesional skin (12). Moreover, the percentage of IL-6+ MCs in non-lesional psoriatic skin appears to be clearly lower than in non-lesional AD skin seen in the current study (36 ± 14% and 56 ± 12%, respectively) (12), suggesting a possible importance of IL-6 in AD.

The result of relatively decreased chymase activity in AD lesions is similar to the findings in lesional psoriatic skin and other chronic skin inflammations (32). Analogous to the situation with IL-6 in AD and psoriasis, the percentage of TNF-α+ MCs has previously been reported to be higher in lesional than in non-lesional AD skin, but not so in psoriatic skin (29).

Previously, two studies concerning the expression of chymase in AD with somewhat conflicting results have been published. Järvikallio et al. (4) studied the chymase protein immunohistochemically in biopsies from 7 AD patients and 8 healthy controls. They found that the percentage of MCs containing the chymase protein did not differ significantly between the lesional and non-lesional skin of AD patients but was decreased compared with the skin of healthy subjects. In a more recent report, an elevated number of chymase+ MCs was shown in 7 AD lesions compared to non-lesional AD skin (n = 11) and psoriatic skin (n = 8) (33). The present study found that although the expression of chymase protein was greater in lesional than in non-lesional dermis, the level of chymase enzyme activity was relatively decreased in MCs in AD lesions. In agreement with the present work, a reduced percentage of MCs exhibiting chymase activity in AD lesions was demonstrated in the study by Järvikallio et al. (4). The reduced chymase activity in lesional skin is possibly due to the action of protease inhibitors localised within MCs (34).
The in vitro experiments revealed that the relative decrease in chymase enzyme activity seen in the lesional skin may not be associated with a reduction in the proinflammatory effect of chymase, but may rather increase its actions. Low concentrations of rh-chymase stimulated significantly both PBMCs and T-cell proliferation, while at the highest concentration of rh-chymase, the stimulatory effect decreased in most of experiments. One possible explanation is that the partial inactivation of chymase can lead to reduced degradation of IL-4, a cytokine that is expressed at increased levels in MCs in AD lesions (35, 36). Another substrate of chymase is IL-6 (23). Indeed, the pretreatment of rh-IL-6 with the highest concentrations of rh-chymase prevented the inhibition in T-cell proliferation caused by rh-IL-6 alone. Similarly, the pretreatment of rh-IL-6 with rh-chymase resulted in a higher proliferation rate of PBMCs than treatment with rh-IL-6 alone. On the other hand, the stimulatory effect of low rh-chymase concentrations was prevented by rh-IL-6 in experiments with T cells. Thus, it can be concluded that only the highest rh-chymase concentration used during preincubation was able to cause sufficient degradation and inactivation of rh-IL-6 in current experimental setting with T cells.

Since IL-6 production is increased after stimulation by IgE-antigen complexes (23), it may be an important proinflammatory mediator of MCs in allergic inflammation and AD. Furthermore, IL-6+ MCs in AD lesional skin were slightly increased. Unexpectedly, in the T-cell proliferation experiments of the current study, rh-IL-6 significantly inhibited the proliferation rate. No such inhibition was seen in experiments with PBMCs, where monocytes and other lymphocytes than T cells can modify the response. Previously, IL-6 has been shown to prevent apoptosis of T cells, promote Th2 and Th17 differentiation and suppress the function of regulatory T cells (37). In a mouse model of airway hyperreactivity, IL-6-stimulated MCs prevented the inhibition of effector T-cell proliferation by regulatory T cells (22). Blockade of IL-6 receptor led to increase in the number of regulatory T cells and to decrease in Th2 cell number in a mouse model of allergic asthma (38). Similar results were presented in another study, where anti-IL-6 receptor antibody suppressed partially splenic CD4+ T-cell activation and IL-2 production but increased the frequency of regulatory T cells among the cultured spleen cells (39). The results may indicate a different influence of IL-6 on T-cell proliferation depending on cell type. The role of dendritic cell-derived IL-6 in preventing the inhibition of effector T cells by regulatory T cells has been demonstrated in psoriasis (14). However, there are no studies investigating the IL-6 effect on T cells isolated from AD patients. As rh-IL-6–induced inhibition of the T-cell proliferation rate was prevented by α-PI, the effect of rh-IL-6 may be dependent on some unknown proteolytic mechanism.

The levels of neither IL-6 nor chymase correlated to clinical severity of AD as assessed by several parameters. However, it is noteworthy that the pathogenesis of AD is very complex and probably no single factor is sufficiently dominating to show a clear correlation with the clinical severity of the disease.

One limitation of the study is the use of nylon wool fibre columns for the purification of T cells. It has been shown that this can reduce T-cell proliferation and may lead to altered production of cytokines (40). However, the possible influence of this factor would remain constant throughout the T-cell experiments. In future studies, the different subtypes of T cell, such as effector T cells and regulatory T cells, should be isolated and used in the experiments.

In summary, the present study reveals that the expression of IL-6 is slightly but significantly increased, whereas chymase activity is relatively decreased, in MCs in AD lesions, possibly due to the action of protease inhibitors. Unexpectedly, rh-IL-6 inhibited T-cell proliferation as well as even prevented the stimulation of T-cell proliferation induced by low concentrations of rh-chymase. On the other hand, pretreatment of rh-IL-6 with higher rh-chymase concentration abolished the inhibitory effect of IL-6 on T cells. Thus, MC chymase and IL-6 are able to modify each other’s effects depending on their concentrations, and these effects are further regulated by protease inhibitors.

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The authors declare no conflict of interest.

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