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Cytokine Production by Skin-Derived Mast Cells: Endogenous Proteases Are Responsible for Degradation of Cytokines

Wei Zhao,* Carole A. Oskeritzian,† Andrea L. Pozez,† and Lawrence B. Schwartz†‡

The current study characterizes the cytokine protein (ELISA) and mRNA (gene array and RT-PCR) profiles of skin-derived mast cells cultured under serum-free conditions when activated by cross-linking of FcεRI. Prior to mast cell activation, mRNA only for TNF-α was detected, while after activation mRNA for IL-5, IL-6, IL-13, TNF-α, and GM-CSF substantially increased, and for IL-4 it minimally increased. However, at the protein level, certain recombinant cytokines, as measured by ELISAs, were degraded by proteases released by these skin-derived mast cells. IL-6 and IL-13 were most susceptible, followed by IL-5 and TNF-α; GM-CSF was completely resistant. These observations also held for the endogenous cytokines produced by activated mast cells. By using protease inhibitors, chymase and cathepsin G, not tryptase, were identified in the mast cell releasates as the likely culprits that digest these cytokines. Their cytokine-degrading capabilities were confirmed with purified chymase and cathepsin G. Soybean trypsin inhibitor, when added to mast cell releasates, prevented the degradation of exogenously added cytokines and, when added to mast cells prior to their activation, prevented degradation of susceptible endogenous cytokines without affecting either degranulation or GM-CSF production. Consequently, substantial levels of IL-5, IL-6, IL-13, TNF-α, and GM-CSF were detected 24–48 h after mast cells had been activated, while none were detected 15 min after activation, by which time preformed granule mediators had been released. IL-4 was not detected at any time point. Thus, unless cytokines are protected from degradation by endogenous proteases, cytokine production by human mast cells with chymase and cathepsin G cells may be grossly underestimated.

Mast cells are key effector cells in IgE-dependent immediate hypersensitivity reactions (1). Animal studies have revealed their involvement in innate and acquired immune defense against microbes and in several autoimmune hypersensitivity diseases (2). In response to FcεRI-mediated stimulation, mast cells secrete mediators such as histamine, PGD₂, leukotriene C₄, and cytokines that produce clinical symptoms. Based on the protease content of human mast cells, they can be divided into two major types (3–5). Human mast cells with αβ-tryptases and chymase (MCTC)⁴ also express carboxypeptidase A3 and cathepsin G. MC₄ cells, however, produce αβ-tryptases, but not the other proteases. MCTC cells are the predominant type of mast cell in normal skin, perivascular tissue, bronchial smooth muscle, and small bowel submucosa, whereas mast cells that produce αβ-tryptases but not the other proteases of MCTC cells (MCT) are the predominant mast cell type in the alveolar wall and the mucosa of the small intestine. Elevated numbers of MCTC cells in the bronchial smooth muscle of asthmatics correlate with their increased bronchial hyperreactivity (6). The current study will examine cytokine production in FcεRI-activated, skin-derived MCTC cells.

Mature mast cells dispersed from human skin proliferate under serum-free culture conditions with stem cell factor (SCF) (7, 8). They express high levels of Kit and FcεRI on their surface; degranulate in response to FcεRI cross-linking, Substance P, and compound 48/80; and maintain the typical MCTC protease phenotype. However, the cytokine profile of this relatively homogenous population of MCTC cells has not been characterized. Studies of skin-derived mast cells by in situ staining or after they were freshly dispersed are sometimes contradictory. By immunohistochemistry, mast cells of the MCTC phenotype in skin as well as ocular and respiratory tissues appeared to contain IL-4 and IL-13, but little if any IL-5 and IL-6, whereas MC₄ cells contained IL-5 and IL-6 and lesser levels of IL-4 (9, 10). In contrast, another study of human skin-derived mast cells showed IL-6 mRNA and protein production after stimulation (11). Skin-derived mast cells also produce IL-8 (12) and TNF-α (13); however, in this latter study, no IL-4, IL-5, or IL-13 was detected.

Activated lung-derived mast cells have been shown to produce IL-13 (14), IL-16 (15), IL-10 (16), and IL-3 (17), but these studies did not distinguish between MC₄ and MCT cells. Intestinal mast cells produce IL-1β, IL-3, IL-5, IL-6, IL-8, IL-9, IL-13, IL-16, IL-18, and TNF-α (18). Numerous studies have also examined cytokine production by the human mast cell leukemia cell line-1 (HMC-1) and by mast cells derived in vitro from cord blood or bone marrow progenitors (8, 19–29). These types of mast cells differ from tissue-derived mast cells in terms of their maturity and functional phenotypes. Of relevance to the current study, levels of mast cell proteases are far lower in HMC-1 cells and in vitro-derived mast cells than in those that develop in vivo. For example, TNF-α and IL-4 produced by stimulated lung-derived mast cells were each degraded after their release, and a mixture of protease

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4 Abbreviations used in this paper: MCTC, mast cells that produce αβ-tryptases, chymase, carboxypeptidase A3, and cathepsin G; MC₄, mast cells that produce αβ-tryptases but not the other proteases of MCTC; HMC-1, human mast cell leukemia cell line-1; SCF, stem cell factor; SBTI, soybean trypsin inhibitor; ZRETF, diphenyl N-[benzoylcarbonyl-l-Arg-Glu-Thr-Phe-phosphonate.

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inhibitors was not successful at preventing degradation of exogenous IL-4 added to a mast cell extract (30). The current study shows that FcεRI-stimulated skin-derived MCs up-regulate cytokine mRNA and protein production for IL-5, IL-6, IL-13, TNF-α, and GM-CSF, but not IL-4. However, under in vitro culture conditions, secreted cytokines are degraded to varying degrees by endogenous chymase and cathepsin G that are released by degranulation. The presence of soybean trypsin inhibitor (SBTI) prevents this degradation without affecting the activation process, enabling the magnitude of cytokine release to be correctly measured.

Materials and Methods

Reagents

The IgG mAb anti-FcεRIIa 22E7, generously provided by J. P. Kochan (Hoffman-LaRoche, Nutley, NJ) (31), and the IgG anti-tryptase mAb B12 (32) were obtained and used as described. Leupeptin, SBTI, heparin glycosaminoglycan, and aprotinin (Sigma-Aldrich), the chymase inhibitor diphenyl N-benzoxycarbonyl-L-Arg-Glu-Thr-Phε-phosphonate (ZRETFT (33) (Enzyme Systems Products), and purified human neutrophil cathepsin G (0.2 U/mg) and human skin chymase (30 U/mg) (Calbiochem) were obtained as indicated.

Purification and culture of human-derived mast cells

Fresh samples of skin were obtained after breast reduction, mastectomy for breast cancer, or abdominoplasty at the Virginia Commonwealth University Medical Center (Cooperative Human Tissue Network of the National Cancer Institute or the National Disease Research Interchange, as approved by the Human Studies Internal Review Board at Virginia Commonwealth University). Mast cells were obtained from human skin essentially as described (7). After removing s.c. fat by blunt dissection, residual tissue was cut into 1- to 2-mm fragments and digested with type 2 collagenase (1.5 mg/ml), hyaluronidase (0.7 mg/ml), and type 1 DNase (0.3 mg/ml) in HBSS for 2 h at 37°C. The dispersed cells were collected by filtering through a no. 80 mesh sieve and resuspended in HBSS containing 1% FCS and 1 mM MgCl2 and 2.5 mM CaCl2 (TGD buffer (pH 7.4) containing 10 mM HEPES, 0.1% gelatin, and 100 ng/ml DNase (Tyrode’s buffer (7.4) containing 10 mM HEPES, 0.1% gelatin, and 100 ng/ml DNase (7.4)). After removing s.c. fat by blunt dissection, residual tissue was minced to varying degrees by endogenous chymase and cathepsin G that are released by degranulation. The presence of soybean trypsin inhibitor (SBTI) prevents this degradation without affecting the activation process, enabling the magnitude of cytokine release to be correctly measured.

Cytokine ELISAs

Purified and biotinylated mouse or rat mAbs specific for each cytokine and standard recombinant cytokines were purchased from BD Biosciences as follows: IL-4, mouse 8D4-8/rat MP4-25D2; IL-5, rat JES1-39D10/JES1-5A10; IL-6, rat M2Q-13AS/M2Q-39C3; IL-13, rat JES10-5A2/mouse B69-2; GM-CSF, rat BVDV-2B6/BVDV-21C11; and TNF-α, mouse MAB1 (34). Assays were performed according to the manufacturer’s recommendations but were scaled down to 384-well, flat-bottom microtiter plates. Wells were coated overnight at 4°C with capture Abs, blocked with PBS containing 1% BSA, washed in PBS/0.05% Tween 20, and incubated overnight at 4°C with test samples. Standard titration curves were established using serial dilutions of known concentrations of recombinant human cytokines. The next day at room temperature, wells were washed, incubated with biotin detection mAb for 1 h, washed, incubated with avidin-peroxidase for 1 h, washed, and incubated with the peroxidase substrate 2,2’-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid. Absorbance values were determined at 405 nm using a SpectraMax 384 Plus UV-VIS plate reader (Molecular Devices). Under these conditions, the lower limit of detection for each of the cytokines under consideration was 16 pg/ml.

Cytokine degradation by releasate of activated skin mast cells and by purified proteases

Skin-derived human mast cells were stimulated with 22E7 mAb for 15 min in AIM-V medium containing 100 ng/ml SCF at 37°C. Releasates were collected by centrifugation at 1200 rpm for 10 min. Recombinant cytokines were incubated with mast cell releasates at 37°C for up to 24 h in the presence or absence of protease inhibitors. Similar incubations were performed with recombinant cytokines and purified chymase and cathepsin G. Commercial chymase (1 nM N-succinyl-Ala-Ala-Pro-Phe-nitroanilide substrate in 0.45 M Tris (pH 8.0) containing 1.8 M NaCl) and cathepsin G (1 mM MeOSuc-Ala-Ala-Pro-Phe-nitroanilide substrate in 0.1 M HEPES (pH 7.5)) containing 0.3 mg/ml B22E7 mAb, with or without SBTI, were used with synthetic substrates; 1 U of activity cleaves 1 mmol of substrate/min. Similar measurements were made with mast cell releasates. Of note, although chymase failed to hydrolyze the Met-containing peptide, cathepsin G hydrolyzed the Phe-containing chymase substrate. Under the conditions used, 1 μM of commercial cathepsin G with the Met-containing peptide (0.1 M HEPES (pH 7.5), 0.5 μM NaCl, 1 mM substrate) had 0.22 mU/ml of activity with the Phe-containing chymase substrate (0.45 M Tris (pH 8.0), 1.8 M NaCl, 1 mM substrate). Consequently, assuming chymase and cathepsin G were the dominant chymotryptic proteases present in the mast cell releasates, quantitation of chymatic activity in mast cell releasates with the Phe-containing substrate may have been overestimated by up to 8%. Enzymatic activities of purified cathepsin G (2 nM/ml) and chymase (5.5 nM/ml) comparable with those measured in releasates were then incubated with individual cytokines at 37°C for 24 h. To test the effect of serum on the cytokine-degrading activity of mast cell proteases, releasates from activated mast cells were incubated with different concentrations of normal human serum for 1 h prior and then with recombinant cytokines for 24-h incubation at 37°C. Residual cytokine levels in all cases were measured by ELISA.

To assess degradation of IL-6 and IL-13 by Western blotting, heparin (50 ng)/stabilized β-tryptase (5 ng) with or without B12 mAb (100 ng), and commercial chymase (15 ng) and cathepsin G (50 ng) with or without SBTI (1.5 μg), were incubated with IL-6 (150 ng) or IL-13 (200 ng) in 15 μl for 24 h at 37°C. B12 served as an inhibitor of β-tryptase (38) and SBTI as an inhibitor of chymase and cathepsin G. Recovered cytokines were subjected to electrophoresis in a 16% polyacrylamide gel under denaturing and reducing conditions with SDS. Samples were transferred onto nitrocellulose membranes that were blocked and labeled with rat anti-IL-6 or rat anti-IL-13 mAbs. The membranes were then incubated with alkaline phosphatase-conjugated goat anti-rat IgG and developed with 5-bromo-4-chloro-3-indolyl phosphate/nitro-blue tetrazolium for 5 min.

RNA preparation and measurement of specific mRNA

Total RNA was isolated from mast cells using the RNaseasy Mini kit (Qiagen). For standard RT-PCR, 200 ng of total RNA was treated with 10 U RNasin and 2 μg of DNAse (Promega) for 15 min at 37°C. cDNA was synthesized by incubating the RNA with murine Moloney leukemia virus reverse transcriptase and 20 pmol of oligo(T) primer for 1 h at 37°C (Pharmacia). A
portion of the cDNA (typically 1/10 volume) was used for standard PCR. Thirty-five cycles of PCR were performed with 2.5 U of TaqDNA polymerase and 20 pmol of gene-specific sense and antisense primers (R&D Systems). A portion of each PCR product was separated on an agarose gel, stained with ethidium bromide (500 ng/ml), and photographed.

GEArray Q series human common cytokine gene array (SuperArray) was used according to the instructions of the manufacturer to assess levels of specific mRNA molecules in mast cells. The GEArray AmpQ labeling system was used to amplify and label specific mRNAs. Total RNA (3 μg) was converted to cDNA using random primer sets and reverse transcriptase. Cytokine-specific cDNA was then linearly amplified through 30 cycles while biotin-16-dUTP was incorporated into the product. Labeled single-strand probes corresponding to cytokines and housekeeping genes were hybridized to cDNA-coated membranes. Membranes were washed, blocked, and incubated with alkaline phosphatase-conjugated avidin. After washing again, CDP-Star substrate was incubated and chemiluminescent product was detected using the Alphalmager 2000 system (Salph Innotech) equipped with a charge-coupled device camera. Data was acquired using FluroChem software and analyzed with ScanAlyze software (www.superarray.com)). Fluorescent ratios associated with specific genes provided a semiquantitative measure of mRNA levels.

Statistical analysis

For parametric data, one-way ANOVA was used to compare cytokine values among different treatment groups. If significant differences were detected, a Dunnett test was then used to compare all treatment groups vs the control group or a pair-wise comparison was made. For pair-wise comparisons, a two-tailed t test was used. For nonparametric data, the Kruskal-Wallis ANOVA on ranks was performed, followed by Dunn’s test for comparisons against a control group. All p values <0.05 were considered to be significant.

Results

Chymase and cathepsin G in the releasates of skin-derived mast cells degrade certain cytokines

To examine whether cytokines potentially produced by activated skin-derived mast cells could be degraded by released mast cell proteases, these cells were stimulated with mouse anti-FceR1α mAb (22E7) for 15 min and releasates were collected. β-Hexosaminidase levels were measured to assess degranulation, which varied from 34 to 69% in these experiments, whereas spontaneous release was always <7%. Known amounts of cytokines (TNF-α, GM-CSF, and IL-4, 5, 6, and 13) were added to these releasates or to culture medium alone, incubated for up to 24 h at 37°C, and measured by ELISA. As shown in Fig. 1, exogenous IL-5, IL-6, IL-13, GM-CSF, and TNF-α were stable in AIM-V medium (bar 2). Endogenous cytokines were undetectable in incubated releasates (bar 3). When exogenous cytokines were incubated with mast cell releasates (bar 5), exogenous IL-6 and IL-13 were almost completely lost, IL-5 and TNF-α were partially lost, and GM-CSF was preserved. Comparable results were obtained with purified chymase and cathepsin G as indicated in Fig. 1C with IL-6 and IL-13. IL-6 appeared to be more sensitive to cathepsin G than to chymase, whereas IL-13 was quite sensitive to both proteases. Time course studies demonstrated that degradation of both IL-6 and IL-13 was significant by 4 h and nearly complete by 24 h (Fig. 2A). Western blot assessments of protease-treated IL-6 and IL-13 showed that heparin-stabilized β-trypetase failed to alter the electrophoretic mobility of either cytokine. In contrast, degradation of each of these cytokines occurred with cathepsin G and chymase and was prevented by SBTI (Fig. 2B).

Whether protease inhibitors might prevent the loss of cytokines noted above was then examined. Several agents were chosen based on their protease inhibition specificity. Leupeptin can inhibit both serine and cysteine proteases, such as β-trypetase and cathepsin B. SBTI, in contrast, inhibits serine proteases other than β-trypetase, such as chymase and cathepsin G. β-Tryptase-specific neutralizing mAb B12 was also tested. As shown in Fig. 1, SBTI (100 μg/ml) failed to reveal any cytokine in the mast cell releasates (bar 4), as expected during the relatively brief period of mast cell activation (15 min) used to produce the releasates. However, each exog-

FIGURE 1. Certain cytokines are degraded by proteases released from skin-derived mast cells and are protected by protease inhibitors. A, Cytokine degradation and protection. Skin-derived human mast cells (10^7/ml) were stimulated with 22E7 mAb (1 μg/ml) in AIM-V medium for 15 min at 37°C. Releasates (9 × 10^5 mast cell equivalents/ml) were collected and incubated with each one of the indicated cytokines (800 pg/ml) in the presence or absence of aprotinin to inhibit cathepsin G, ZRETF to inhibit chymase, and SBTI to inhibit both proteases. AIM-V medium was used as a negative control (bar 1). Cytokine levels were determined after a 24-h incubation at 37°C. Values are mean ± SE from four independent experiments, each performed in triplicate. ANOVA showed p < 0.001. Cytokine levels in buffer (bar 2) were then compared to each of the other values by Dunnett tests. *, p < 0.05; +, indicates what was included in the incubation mixture. B, Effect of SBTI on mast cell activation. Skin-derived mast cells were stimulated in the absence and presence of SBTI (100 μg/ml) with 22E7 for 15 min as in A. β-Hexosaminidase was measured as a marker of degranulation. A two-sided t test showed no significant difference in percent β-hexosaminidase release between the SBTI+ and SBTI- releasates. C, Purified chymase and cathepsin G degrade IL-6 and IL-13. IL-6 and IL-13 were incubated in buffer or with cathepsin G (2 μM; light gray) or chymase (5.5 μM; dark gray) at 37°C for 24 h and then were measured by ELISA. *, p < 0.05 compared to cytokines in medium alone by ANOVA.
sometimes provided modest protection against the degradation of exogenously added cytokines, together (bar 8) they were as effective as SBTI. These cytokines exhibited similar sensitivities to purified preparations of chymase and cathepsin G. IL-6 and IL-13 were the most sensitive to each protease by itself, TNF-α and IL-5 were less sensitive, and neither degraded GM-CSF. These results suggest that chymase and cathepsin G were responsible for most of the activity in the mast cell releasates that degraded exogenously added IL-6, IL-13, IL-5, and TNF-α. They also suggest that endogenous cytokines might show a similar susceptibility.

To measure cytokine production, skin-derived mast cells (10^6/ml) were incubated in AIM-V medium and stimulated with 22E7 (1 μg/ml) for 24 h in the presence and absence of SBTI. Culture supernatants were collected and subjected to cytokine ELISA. Mean ± SE; n = 3 experiments; * p < 0.05 when comparing each +SBTI group to its corresponding −SBTI group by a two-sided t test.
463 pg/ml, and IL-13 from 124 to 2241 pg/ml. IL-5 levels were negligible at 24 h in the presence and absence of SBTI. For GM-CSF, which was previously shown to be resistant to mast cell protease degradation, mean levels measured in the absence (686 pg/ml) and presence (715 pg/ml) of SBTI were not significantly different. In the absence of 22E7, none of these cytokines was detected at this 24-h time point (data not shown).

The dose response to 22E7 at 6, 24, and 48 h was then examined for both cytokine production and degranulation. Skin-derived human mast cells were incubated with 22E7 (0.001–1 µg/ml) at 10^6 mast cells/ml in the presence of SBTI (100 µg/ml). Negative controls included incubation with nonimmune mouse IgG at 1 µg/ml or medium alone. At 6 h, only TNF-α reached detectable levels (data not shown). Fig. 5 shows levels of cytokines (IL-5, 6, and 13; GM-CSF; and TNF-α) and percent release of β-hexosaminidase at the 24- and 48-h time points. Spontaneous release of β-hexosaminidase was <5% under each condition. Among the cytokines examined, only IL-6 was spontaneously released at detectable levels: 57 pg/10^6 cells at 24 h and 111 pg/10^6 cells at 48 h. In response to anti-FceRI mAb, levels of each cytokine increased in a dose-dependent manner, as did percent release of β-hexosaminidase. Maximal levels of these cytokines produced by activated mast cells were comparable with the levels tested above using recombinant cytokines. At 24 h and 48 h, enhanced production of IL-6, IL-13, GM-CSF, and TNF-α occurred with 0.001 µg/ml 22E7, whereas degranulation, as measured by β-hexosaminidase secretion, was not apparent. Thus, production of certain cytokines may be a more sensitive marker of FceRI-dependent activation than is degranulation. Another feature of note is that levels of β-hexosaminidase and all cytokines except IL-5 were comparable after 24 and 48 h of activation, suggesting that production had been completed by 24 h and that released cytokines and β-hexosaminidase were stable under the experimental conditions used. In contrast, IL-5 secretion was far more prominent at 48 h than at 24 h, and its production required a 22E7 concentration of ≥0.01 µg/ml.

Cytokine mRNA expression by activated skin mast cells

RNA was extracted from skin-derived mast cells that had been stimulated with 22E7 or isotype-matched mouse IgG for 3 h. A cytokine gene array was used to determine expression of the cytokine mRNAs of interest as described in Materials and Methods. As shown in Fig. 6A, IL-5, IL-6, IL-13, GM-CSF, and TNF-α mRNAs were not detected at baseline. By 3 h after stimulation, IL-6 and GM-CSF mRNA levels increased dramatically, whereas those of IL-5, IL-13, and TNF-α did not. Data from three separate
gene array experiments were quantified as shown in Fig. 6B, which revealed significantly increased levels of GM-CSF and IL-6 mRNAs. However, when IL-5, IL-13, and TNF-α mRNAs were examined by conventional RT-PCR, TNF-α mRNA was detected at baseline, and all three of these mRNAs increased 3 h after stimulation with 22E7. These data indicate that increased secretion of these cytokines is due at least in part to increased gene expression.

**IL-4 mRNA but not protein is detected in 22E7-stimulated skin mast cells**

IL-4 is considered separately from the other cytokines because of its distinct behavior. When human rIL-4 is added to AIM-V buffer or to mast cell releasate (Fig. 7A), <5% is recovered by ELISA. To stabilize rIL-4, biotin-conjugated rat IgG anti-human IL-4 mAb (BD Pharmingen; 10 μg/ml) and SBTI (100 μg/ml) were added to the medium or releasate. The concentrations of SBTI and anti-IL-4 were optimal based upon preliminary data. The addition of this biotinylated mAb appears to be protective against the spontaneous loss of IL-4 immunoreactivity in both medium alone and after addition of mast cell releasate and also had no apparent effect on the mast cell degranulation response to 22E7 (data not shown). SBTI offered no significant protection by itself and negligible additional protection when included with anti-IL-4. This is the same mAb used for detection in the IL-4 ELISA. Because it is normally added in excess during the immunosassay, its additional presence prior to capture does not affect the ultimate ELISA dose response. To rule out the possibility that AIM-V medium was responsible for rIL-4 instability, the experiment was repeated using PBS/1% BSA and produced a similar result (data not shown). To evaluate IL-4 production by skin-derived mast cells, SBTI and anti-IL-4 Ab were added to the culture system prior to the addition of 22E7 mAb, with the intention of stabilizing any secreted IL-4. Cell supernatants were collected from 6 to 48 h after stimulation and were subjected to the IL-4 ELISA; however, no IL-4 could be detected (<16 pg/10^6 mast cells), in spite of β-hexosaminidase release values of 45–65%.

To assess IL-4 gene transcription, gene array and RT-PCR were performed as for the other cytokines. Gene array experiments did not detect IL-4; the IL-4 to G3PDH ratio was <0.2 for both unstimulated and stimulated mast cells. RT-PCR, as shown in Fig. 7B, failed to detect IL-4 mRNA in unstimulated mast cells. However, after 22E7 stimulation, low levels of an IL-4 mRNA-derived RT-PCR product were observed. Thus, under the experimental conditions used, IL-4 production by skin-derived mast cells is negligible.

**Discussion**

Proteases released by human skin-derived mast cells were shown to degrade endogenous as well as exogenous recombinant cytokines. IL-6 and IL-13 were the most susceptible, IL-5 and TNF-α were less susceptible, and GM-CSF was totally resistant. B12, a β-tryptase-neutralizing Ab (38), and leupeptin, an inhibitor of certain serine/cysteine proteases such as β-tryptase, failed to affect the loss of cytokines mediated by the mast cell releasate, indicating that proteases other than β-tryptase were involved. Even by Western blotting, no degradation of IL-6 and IL-13 by human β-tryptase was detected, in contrast with the murine tryptase called mouse mast cell protease 6, which degrades murine IL-6 (39). However, another serine protease inhibitor, SBTI, fully protected IL-5, IL-6, IL-13, and TNF-α from degradation. By using more selective proteases inhibitors (aprotinin for cathepsin G and ZRETIF for chymase), by Western blotting of IL-6 and IL-13 that had been exposed to purified preparations of these two proteases, and based on the known abundance of these two proteases in skin MC\(_{TC}\) cells (5, 40), each of these proteases were shown to be responsible for the digestion of susceptible cytokines. Nevertheless, some contributions by other proteases cannot be ruled out by this analysis.

Mast cell chymase has multiple targets of potential biologic import. These include formation of angiotensin II from angiotensin I (41–43), collagen fibrils from type I procollagen (42, 44), bioactive endothelin from big endothelin (42), TGF-β1 from latent TGF-β1 (45, 46), and IL-1β from its inactive precursor (47). Chymase also cleaves membrane-bound SCF to yield a bioactive, cell-free product (48). In contrast, cathepsin G stored in neutrophil granules may facilitate the destruction of engulfed cell debris or microorganisms. Also, cathepsin G can rapidly degrade inflammatory cytokines such as TNF-α (49–51) and IL-6 (52–54).

The cytokine-degrading activities of chymase and cathepsin G are relevant to the MC\(_{TC}\) type of mast cell that predominates in skin, perivascular sites, airway smooth muscle, conjunctiva, and bowel submucosa (1). In contrast, MC\(_{T}\) cells, which lack chymase and cathepsin G and are the predominant mast cell type in alveolar wall and small bowel mucosa, are less likely to be responsible for degrading cytokines. The finding of cytokine degradation by MC\(_{TC}\) cell proteases has several practical as well as clinical implications. In vitro, released proteases cannot diffuse far from their cell source, and natural inhibitors of these proteases are typically absent. Consequently, these proteases can remain in the extracellular fluid as active enzymes for the time it takes the cells to produce and secrete cytokines, resulting in cytokine degradation and an underestimation of the magnitude of cytokines secreted. This might explain why little if any IL-5, IL-6, and IL-13 production by
skin mast cells was detected in several previous studies (9, 10, 13). In vivo, chymase and cathepsin G are likely to be inhibited by natural protease inhibitors and to diffuse away from their tissue sites of release by the time cytokines are produced by these cells. Consequently, these proteases might degrade cytokines already present in the extracellular space that were most likely deposited by other cells, but they are not likely to degrade cytokines produced by their own cell of origin.

With the addition of SBTI, dramatically higher production levels of IL-5, IL-6, IL-13, and TNF-α were detected after skin-derived mast cells had been stimulated with anti-FcεRI Ab. High levels of GM-CSF, which is not destroyed by chymase and cathepsin G, were detected regardless of whether SBTI had been added. All cytokines except IL-5 reached peak levels by 24 h; IL-5 production became detectable at 48 h. Whether IL-5 production directly follows FcεRI cross-linking or is up-regulated in an autocrine fashion by mediators produced during the first 24 h remains to be determined. For example, IL-4 priming enhances IL-5 production by human skin-derived mast cells (55); however, IL-4 production was not detected in the current study. TNF-α is another possible candidate: it activates NF-κB in lung-derived mast cells, thereby leading to the production of cytokines such as GM-CSF and IL-8 (56). Whether TNF-α has a feedback role for skin mast cells remains to be determined.

The relationship between degranulation and cytokine secretion was considered. As shown in Fig. 5, anti-FcεRI Ab at a concentration of 0.001 μg/ml led to the production of IL-5, IL-6, IL-13, and TNF-α, whereas no β-hexosaminidase release was detected. This indicates that cytokine production is a more sensitive measure of mast cell activation than degranulation and also suggests that preformed mediators released by degranulation are not required to stimulate cytokine production. This concept that cytokines could be released from mast cells in the absence of degranulation was previously noted for human cord blood-derived mast cells, which were able to produce IL-6 without degranulation in response to IL-1 (57) and to produce GM-CSF and IL-1β (23) or IL-5, IL-10, and IL-13 (58) without degranulation in response to stimulation of Toll-like receptors.

To examine cytokine gene expression, levels of IL-4, IL-5, IL-6, IL-13, TNF-α, and GM-CSF mRNAs were assessed. Using gene arrays, none of these cytokine mRNAs were detected in unstimulated cells. After cross-linking FcεRI, IL-6, and GM-CSF, mRNA levels were dramatically up-regulated at 3 h, consistent with the later production of IL-6 and GM-CSF protein. IL-5, IL-13, and TNF-α mRNAs were not detected by the gene array in these stimulated cells; however, conventional RT-PCR clearly showed up-regulation of these mRNAs, this technique being more sensitive than gene array. A low level of TNF-α mRNA expression in unstimulated cells was found by RT-PCR. This suggests the possibility of constitutive expression of TNF-α. However, no TNF-α was detected in the tissue culture medium of unstimulated cells and no TNF-α was detected with degranulation 30 min after FcεRI cross-linking. This contrasts with previous studies on enriched, freshly dispersed skin mast cells in which both TNF-α protein and mRNA levels were detected in nonstimulated cells (13, 59, 60). This difference in TNF-α protein expression between cultured and freshly dispersed skin-derived mast cells has not been precisely assessed, but could reflect the influence in vivo of neighboring cells, connective tissue, and interstitial fluid. For example, the cytokine profile produced by cord blood-derived mast cells is influenced by pre-exposure to IL-4 or IL-5, which prime cord blood-derived mast cells to release Th2-like cytokines when stimulated with anti-IgE, including IL-5, IL-13, and GM-CSF (8).

The ability of human mast cells to produce IL-4 has been inconsistently demonstrated, though it is typically far below that of human basophils. The current study found that rIL-4 was unstable at 37°C in culture medium in the presence and absence of mast cell releasate. It was stable in culture medium at 4°C, the temperature at which the capture of IL-4 typically occurs in our standard IL-4 ELISA. Minimal improvement in stability was provided by SBTI alone, suggesting that a serine protease was not responsible. The combination of the bioinactivated anti-IL-4 mAb used in the ELISA dramatically improved rIL-4 stability and did not interfere with the ELISA. However, when anti-IL-4 mAb and SBTI were added to mast cells just prior to when they were stimulated with anti-FcεRI, no IL-4 could be detected in the releasate. Furthermore, only trace amounts of IL-4 mRNA could be detected by RT-PCR. This result is similar to that reported for stimulated cord blood-derived mast cells, which produced GM-CSF, IL-5, and TNF-α, but not IL-4 (61). In contrast, cord blood-derived mast cells in another study were able to express low levels of IL-4 when stimulated with phorbol myristic acid and A23187, but not with FcεRI cross-linking (28).

In summary, human skin-derived MC_TCs produce substantial amounts of IL-5, IL-6, IL-13, TNF-α, and GM-CSF, but not IL-4, in response to FcεRI cross-linking, even at a dose of anti-FcεRI too low to trigger a degranulation response. However, to fully detect most of these cytokines in vitro, released chymase and cathepsin G must be inhibited.

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**References**

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