Human Conjunctival Mast Cells

Expression of Fc\(\epsilon\)RI, c-kit, ICAM-1, and IgE

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Objective: To characterize the expression and regulation of conjunctival mast cell surface receptors important in allergic inflammation.

Methods: Mast cells were isolated from human conjunctival tissues of cadavers. Mast cell surface markers were identified using flow cytometry with antibodies to IgE, Fc\(\epsilon\)RI, c-kit, and intercellular adhesion molecule-1 (ICAM-1). We evaluated the effect of 24-hour tumor necrosis factor \(\alpha\) (TNF-\(\alpha\)) or interleukin 4 (IL-4) incubation on the expression of mast cell c-kit, ICAM-1, and surface-bound IgE.

Results: Staining of mast cells (c-kit and/or tryptase positive) yielded positive results for all of the variables measured. The intensity of mast cell c-kit staining increased with TNF-\(\alpha\) incubation, but decreased below that of unstimulated mast cells when incubated with IL-4. Anti–ICAM-1 and anti–IgE staining were increased over that of unstimulated cells when incubated with TNF-\(\alpha\) or IL-4.

Conclusions: In this model, TNF-\(\alpha\) up-regulates mast cell surface receptors and cell-bound IgE. Interleukin 4 up-regulates mast cell ICAM-1 and cell-bound IgE, but down-regulates c-kit.

Clinical Relevance: Conjunctival mast cells play a critical role in the pathogenesis of atopic ocular disease. Characterization of the expression and regulation of mast cell surface receptors is important to the development of potential novel treatments for ocular inflammation.


CONJUNCTIVAL MAST cells and their mediators are implicated, at least in part, in all types of atopic ocular disease. Human studies of these ocular processes, however, primarily have consisted of pathological studies of biopsy specimens (demonstrating increased numbers of mast cells, eosinophils, and other inflammatory cells) and evaluation of tear film for mediators and cellular infiltration.1-5 A method to isolate and purify human conjunctival mast cells from cadaveric donor tissue has been reported previously, and these mast cells have been shown not only to express message for tumor necrosis factor \(\alpha\) (TNF-\(\alpha\)), but also to release TNF-\(\alpha\) protein. Release of TNF-\(\alpha\) from challenged mast cells stimulates up-regulation of intercellular adhesion molecule-1 (ICAM-1) (CD54) on epithelial cells.7 We herein expand on these findings by demonstrating that human conjunctival mast cell surface receptors (c-kit, ICAM-1, and Fc\(\epsilon\)RI) and surface-bound IgE can be detected using flow cytometry and manipulated using incubation with the cytokines interleukin 4 (IL-4) and TNF-\(\alpha\). This work gives us the potential to examine further the mechanisms of mast cell activation and survival and the role of the mast cell in the progression of ocular allergic inflammation to sight-threatening diseases such as atopic keratoconjunctivitis (AKC) and vernal keratoconjunctivitis (VKC).

RESULTS

MAST CELL PURIFICATION

The cell preparations consisted of 5% to 30% mast cells and 0.7 \(\times\) 10\(^6\) to 1.0 \(\times\) 10\(^6\) total cells per gram of conjunctival tissue digested. Contaminating cells were erythrocytes, mononuclear cells, and epithelial cells. The viability of the cell preparations was routinely greater than 85%.

FLOW CYTOMETRIC ANALYSIS OF MAST CELL SURFACE MARKERS

In the representative contour plots of unstimulated cells shown in Figure 1, the percentage of total cells staining positive is indicated in each quadrant. Figure 1, A, shows that cells staining positive for intracellular tryptase also stained positive for Fc\(\epsilon\)RI (6.9% of total cells). Cells staining positive for Fc\(\epsilon\)RI also stained positive for...
MATERIALS AND METHODS

REAGENTS AND SOLUTIONS

Collagenase (type I), hyaluronidase (type 1-S), HEPES, trypan blue, Percoll (silica particles coated with polyvinyl pyrrolidone used for cell separation), fetal calf serum, RPMI 1640, gentamicin, penicillin, streptomycin, amphotericin B, Hank's balanced salt solution (without calcium, magnesium, or phenol red) (HBSS), bovine serum albumin, sodium azide, and phenylmethyl sulfonyl fluoride were obtained from Sigma Chemical Company (St Louis, Mo). Wright stains were performed using a commercially available staining kit (Diff-Quik; Baxter Scientific Products, McGaw Park, Ill). Rabbit anti–chicken IgY-fluorescein isothiocyanate (FITC) polyclonal antibody was obtained from Serotec (Raleigh, NC). Mouse anti–human c-kit–PE monoclonal antibody and mouse anti–human ICAM-1–FITC polyclonal antibody were obtained from Pharmingen (San Diego, Calif). Isotype controls for flow cytometry were purchased from the same manufacturer as the respective specific antibodies. The chicken anti–human tryptase IgY polyclonal antibody was donated by Promega (Madison, Wis), and the mouse anti–human high-affinity IgE receptor chain (FcεRI) monoclonal antibody was donated by Jarema Kochan, PhD, Hoffman LaRoche, Inc (Nutley, NJ).

The Tyrode physiological salt solution plus gelatin (TG) used in these studies consisted of 137-mmol/L sodium chloride, 2.6-mmol/L potassium chloride, 0.35-mmol/L sodium phosphate monobasic, 11.9-mmol/L sodium bicarbonate, 5.3-mmol/L glucose, and 1-g/L gelatin (adjusted to pH of 7.4 with hydrochloric acid). Calcium chloride, 2 mmol/L, and 1-mmol/L magnesium chloride were added to TG to make TGCM. Percoll stock solution was prepared by mixing the commercial Percoll solution and 10-fold HEPES buffer plus deionized water to obtain an osmolality of 285 mOsm/kg water. The desired densities of Percoll were prepared by mixing the Percoll stock solution with TG to a density of 1.041 g/mL. Flow cytometry staining buffer (HBSS-BAP) consisted of HBSS, 1-g/L bovine serum albumin, 0.5-g/L sodium azide, and 18-mg/mL phenylmethyl sulfonyl fluoride in 2 mL of ethanol. Mast cell culture medium consisted of RPMI 1640 supplemented with 20% fetal calf serum, 2.5-mmol/L l-glutamate, 400-μg/mL streptomycin, 20-μg/mL gentamicin, and 5-μg/mL amphotericin B.

MAST CELL ISOLATION, PURIFICATION, AND CULTURE

Human conjunctival mast cells were obtained as previously described. Briefly, upper and lower bulbar conjunctivae were aseptically collected with permission within 6 hours (average time, 4.5 hours) of death from organ and tissue donors and transported in corneal preservation medium (Dexsol; Chiron Ophthalmics, Irvine, Calif). Tissue acquisition protocols were approved by the University of Wisconsin Human Subjects Committee, Madison. On arrival, tissues were resuspended in mast cell culture medium, stored at 4°C, and equilibrated at 37°C overnight before isolation. A series of digestions (30 minutes at 37°C) with hyaluronidase and collagenase (2000 U/mL in TGCM) were used to disrupt tissue. Freed cells were collected, layered over single-density Percoll gradients (density, 1.041 g/mL), and centrifuged for 20 minutes at 400g. The resulting cell pellet was resuspended in mast cell culture medium, transferred to a 24-well plate (0.5 mL per well), and equilibrated at 37°C until the cells were resuspended in HBSS-BAP for surface receptor and/or intracellular tryptase staining. Mast cells were identified using flow cytometry with a mast cell–specific chicken surface-bound IgE (9.0% of total cells) (Figure 1, B). Whereas other tryptase-negative cells in the preparation also stained positive for IgE (21.5% of total cells) (Figure 1, B), these cells were negative for FcεRI. Figure 1, C, shows staining of ICAM-1 on the surface of c-kit–positive, unstimulated mast cells (8.2% of total cells). Figure 1, D, shows that c-kit–positive, unstimulated mast cells stained positive for IgE (9.4% of total cells) as well as some c-kit–negative cells (7.1% of total cells). Figure 2 depicts the data in Figures 1, C, and 1, D, as overlay histograms (antibody staining over isotype control). Staining of cells with anti–c-kit antibody is shown in Figure 2, A. The staining of c-kit–positive cells that also stained positive for ICAM-1 and IgE are shown in Figures 2, B, and 2, C, respectively.

The results of TNF-α and IL-4 stimulation of human conjunctival mast cells expressed as mean fluorescence units over unstimulated cells (mean ± SEM [n = 3]) are shown in Figure 3. Twenty-four-hour incubation with TNF-α resulted in up-regulation (compared with unstimulated control cells) of c-kit (27.5 ± 6.4 U) and ICAM-1 (13.3 ± 6.6 U). Incubation with IL-4 resulted in a similar increase in ICAM-1 expression (13.5 ± 10.5 U) but decreased expression of c-kit below the level detected on unstimulated cells (−7.0 ± 8.0 U). Surface-bound IgE was increased by incubation with TNF-α (62.5 ± 22.5 U) and IL-4 (56.0 ± 21.0 U).

COMMENT

We have previously reported that isolated conjunctival mast cells release histamine on challenge with anti-IgE antibody, suggesting the presence of IgE bound to functional FcεRI receptors on these cells. In the present study, we have identified the FcεRI receptor on mast cells (tryptase-positive cells) using immunostaining combined with flow cytometry. Mast cells that stained positive for FcεRI also stained positive for IgE. Some tryptase-negative cells (nonmast cells) in the preparation also stained positive for IgE, but were negative for FcεRI. These cells are likely conjunctival epithelial cells or mononuclear cells with IgE bound to their surface. We have previously reported that purified conjunctival epithelial cells stain positive for IgE and the low-affinity IgE receptor CD23.
anti–human tryptase IgY polyclonal antibody or mouse anti–human c-kit–PE monoclonal antibody. Differentiation of cells was determined on cytospins using Wright stain. Viability of cells was determined by trypan blue exclusion.

FLOW CYTOMETRIC ANALYSIS OF MAST CELLS

A 2-color immunostaining technique (FITC- vs PE-conjugated antibodies) was used to double-label mast cells and identify surface receptors. In the immunostaining procedure, antibodies to 3 different mast cell–specific markers (intracellular tryptase, surface-expressed FcεRI, and c-kit) along with antibodies to surface-expressed ICAM-1 and surface-bound IgE, were used. Cells were initially fixed and made permeable using a modification of the method published by Schmid et al,6 to allow for intracellular staining of tryptase and mast cell identification. A chicken anti–human tryptase polyclonal antibody was used to label tryptase, and a rabbit anti–chicken Ig1-FITC polyclonal second antibody was used to detect anti–tryptase-labeled cells. When mast cells were identified with anti–c-kit staining, intracellular staining for tryptase was not required (accordingly, cells were not fixed and made permeable), and propidium iodide was added to allow analysis of viable cells.

When anti-IgE staining was combined with FcεRI, a goat anti–IgE polyclonal antibody was combined with a donkey anti–goat IgG-PE polyclonal second antibody. In other combinations, a sheep anti–human IgG-FITC polyclonal antibody was used. A mouse anti–human FcεRI monoclonal antibody combined with a goat anti–mouse PE polyclonal second antibody was used to detect FcεRI. Mouse anti–human monoclonal antibodies against c-kit (PE-conjugated) and ICAM-1 (FITC-conjugated) were used to detect these receptors. Species- and chromagen-appropriate isotype antibody controls were included for each antibody used.

When a second antibody was used, cells were washed and resuspended in HBSS-BAP to a concentration of 1 × 10^6 cells per milliliter (50 µL per tube). One of the following first antibodies was then added to these cells at a volume of 10 µL per tube: chicken anti–human tryptase polyclonal antibody (1:50 dilution in HBSS-BAP), 10-µg/mL mouse anti–human FcεRI monoclonal antibody, or 10-µg/mL goat anti–human IgE polyclonal antibody. Rabbit IgG, donkey IgG, goat IgG, or mouse IgG, all 10 µL per tube (1:10 dilution of each in HBSS-BAP), was also added to block nonspecific binding of antibody. After incubation of the mixture for 30 minutes (shaking ice bath), the cells were washed twice in 2 mL of HBSS-BAP and then resuspended in rabbit anti–chicken Ig1-FITC polyclonal second antibody (1:50 dilution in HBSS-BAP) and PE-conjugated goat anti–mouse IgG-PE polyclonal antibody or donkey anti–goat IgG-PE polyclonal antibody (100 µL per tube). After a second 30-minute incubation, the cells were washed and resuspended in HBSS-BAP (300 µL per tube) for analysis using a commercially available flow cytometer (FACScan; Becton Dickinson, San Jose, Calif). When directly conjugated antibodies were used, the cells were resuspended after isolation, incubated with 10 µL per tube of anti–surface receptor antibodies (PE- and FITC-conjugated), and prepared for flow cytometry. The cells were analyzed via dot-plots for PE vs FITC staining.

CYTOKINE REGULATION OF MAST CELL c-kit, ICAM-1, AND IgE

To study the effect of cytokines on mast cell surface receptors, semipurified conjunctival mast cell preparations were incubated overnight (37°C) with recombinant TNF-α or IL-4 (200 U/mL) or alone in mast cell medium. The cells were then resuspended for flow cytometry and double-stained with a mouse anti–human c-kit–PE polyclonal antibody vs a mouse anti–human ICAM-1–FITC monoclonal antibody or a sheep anti–human IgE–FITC polyclonal antibody. The cells were analyzed using histograms to evaluate c-kit–positive cells.

Our findings of ICAM-1 and c-kit expression on conjunctival mast cells and their changes in expression in response to cytokine stimulation are consistent with what others have described using mast cell lines and mast cells from other tissues.11-14 Intercellular adhesion molecule-1 is important for migration of inflammatory cells and antigen presentation, and it has been proposed that contact between mast cells and lymphocytes via ICAM-1 may result in mast cell degranulation.13 Coculture of mast cells with murine T-cell hybridoma cells (activated by anti–CD3 antibody) has been shown to induce histamine release.15 Mast cells cocultured with these activated T cells also had enhanced FcεRI aggregation–induced degranulation, and this enhancement was inhibited by anti–ICAM-1 antibody. Frandji et al16 demonstrated the importance of IL-4 in antigen presentation by mast cells with the observation that it could be enhanced by granulocyte-macrophage colony-stimulating factor–cerebral spinal fluid only in combination with IL-4. As T cells appear to be a significant contributor to the pathological characteristics of AKC and VKC, greater understanding of mast cell–T-cell interactions would be beneficial in discerning the mechanisms of these diseases.

Incubation of conjunctival mast cells with IL-4 or TNF-α resulted in up-regulation of ICAM-1. Although TNF-α and IL-4 have been found in tear films of patients with allergic conjunctivitis, AKC, and VKC, the actual effect of these cytokines on the ocular surface is not known. Up-regulation of ICAM-1 by IL-4 has been described on cells of a human leukemic mast cell line, HMC-1.14 It has been reported that IL-4 promotes the expression of leukocyte function–associated antigen-1 (CD11a) and ICAM-1 on cultured human cord blood mast cells, and that it induces homotypic aggregation of mast cells, which was completely blocked by anti–leukocyte function–associated antigen-1 (CD11a) or ICAM-1 antibodies.13 This suggests that IL-4 has an important role in interactions of mast cells with other inflammatory cells by up-regulating mast cell adhesion molecules.

Recently it has been demonstrated that human conjunctival mast cells produce TNF-α and that conjunctival epithelial cell cultures incubated with supernatants from calcium ionophore (A23187)–stimulated mast cells.
Figure 1. Representative contour plots of double staining for tryptase vs FcεRI (A), IgE vs FcεRI (B), and anti-c-kit vs intercellular adhesion molecule-1 (ICAM-1) (C) and IgE (D) in terms of fluorescence intensity. The quadrants were set by gating the appropriate isotype control into the lower left quadrant (ie, negative cells were contained in the lower left quadrant). The percentage of total cells staining positive is indicated in each quadrant. Double positive-stained mast cells are in upper right quadrants. Cells appearing in the upper left quadrants stained positive for only tryptase (A), IgE (B), or c-kit (C and D). Cells appearing in the lower right quadrants stained positive for only FcεRI (A and B), ICAM-1 (C), or IgE (D). The IgE-positive tryptase and c-kit–negative cells (lower right quadrant) in plots A and D represent epithelial cells that express the low-affinity IgE receptor FcεRII (data shown in Cook et al9).

Figure 2. Representative overlay histograms of unstimulated human conjunctival mast cells. The black histograms represent antibody (positive) events; the gray histograms, isotype control (negative) events. The y-axis scales are number of events; the x-axis scales, fluorescence intensity. Staining of cells with anti–c-kit antibody is shown in panel A. The gate used to define the population of c-kit–positive cells that was used in panel B showing anti–intercellular adhesion molecule-1 (ICAM-1) staining and in panel C showing anti-IgE staining is represented by the rectangle in panel A.
had increased levels of ICAM-1 on their surface.7 Tumor necrosis factor α up-regulates ICAM-1.17,26 and conjunctival epithelial cells have been shown to release TNF-α.19 This TNF-α release from conjunctival mast cells and/or epithelial cells may be an important mechanism of autocrine regulation of conjunctival adhesion receptors. In our study, TNF-α and IL-4 were added to a mixed culture of cells (primarily mast cells and epithelial cells) containing an undefined combination of cell mediators from all cell types present (but presumably similar to the environment from which they were isolated). Accordingly, the responses observed may or may not be due to a direct effect of the cytokine added.

Interestingly, our research showed down-regulation of c-kit following incubation with IL-4, which is in agreement with the finding of down-regulation of c-kit messenger RNA expression by IL-4 in leukemic myeloid cells and HMC-1 cells.12 This may be part of a negative feedback mechanism and prominent in the resolution of the acute allergic response in the conjunctiva. Mast cell c-kit receptor ligation to stem cell factor is known to be critical to mast cell survival, but the in vivo results of this ligand are not well understood. Murine fibroblasts have been shown in vitro to aggregate mast cells via membrane-bound stem cell factor, presumably facilitating mast cell differentiation and survival.13 If these interactions between mast cells and fibroblasts happen in vivo and collagen release is a result, this could be a critical aspect of the conjunctival scarring in AKC, which is due to collagen release from fibroblasts.2

The basis for the increase in surface-bound IgE levels following IL-4 or TNF-α incubation is not fully understood. Two possible explanations are increased availability of IgE and/or an increase in the absolute number of FcεRI receptors. It has been reported that IL-4 induces FcεRI receptors on human mast cells.20

The array of surface receptors expressed on mast cells has not been thoroughly characterized. We believe the ability to measure and manipulate these receptors on freshly isolated cells gives us the unique opportunity to study mast cell interactions with other isolated cells in vitro as a model of ocular inflammation. This model could be a useful tool to examine the effects of mast cell-stabilizing drugs on cytokine-induced expression and mechanism of sight-threatening disease.

Accepted for publication October 26, 1998.

This work was supported in part by Alcon Laboratories, Fort Worth, Tex, and in part by the Research Division of Prevent Blindness America, Fight for Sight, Inc, New York, NY, in memory of Alexander P. and Mary E. Hirsch.

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