Expression of CXCR4 in Eosinophils: Functional Analyses and Cytokine-Mediated Regulation

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Expression of CXCR4 in Eosinophils: Functional Analyses and Cytokine-Mediated Regulation

Hiroyuki Nagase,* Misato Miyamasu,† Masao Yamaguchi,‡ Takao Fujisawa,‡ Ken Ohta,§ Kazuhiko Yamamoto,‡ Yutaka Morita,* and Koichi Hirai†‡

We examined the expression of transcripts of a panel of chemokine receptors in human eosinophils and found intense constitutive expression of CXCR4 mRNA. Although surface CXCR4 protein was hardly detectable in the peripheral blood or freshly isolated eosinophils, surface expression of CXCR4 became gradually apparent during incubation at 37°C. In contrast, the level of CCR3 expression was virtually unchanged during the incubation. Stromal cell-derived factor-1α (SDF-1α), the natural ligand of CXCR4, elicited an apparent Ca2+ influx in these cells and induced a strong migratory response comparable to that by eotaxin. The surface expression of CXCR4 in eosinophils was up-regulated by IFN-γ, TNF-α, and TGF-β while it was down-regulated by IL-4 and eosinophil-directed hemopoietins such as IL-5. The CXCR4 expression did not always parallel the apoptotic changes in cytokine-treated eosinophils. In contrast to IL-4 and IFN-γ, IL-5 potently reduced the level of CXCR4 mRNA. It seems unlikely that CXCR4 is fundamentally involved in the pathogenesis of allergic disorders by inducing the migration of eosinophils toward inflammatory sites, because a Th2-dominant state down-regulates eosinophil CXCR4 expression. However, CXCR4 may affect the size of the mobilizable pool by holding eosinophils at noninflamed tissues. Th2-dominant state may favor the liberation of eosinophils by down-regulating CXCR4 expression. The interplay between CXCR4 and SDF-1α in eosinophils potentially plays an important role in the accumulation of these cells at the allergic inflammatory sites. The Journal of Immunology, 2000, 164: 5935–5943.

Massive accumulation of eosinophils is a characteristic aspect of inflammation associated with allergic diseases. Historically, eosinophils had long been assumed to play an antiinflammatory role in allergic disorders by virtue of their antagonizing effects on mast cell-derived mediators such as histamine. However, it has become apparent that the eosinophils involved in these conditions are highly destructive: eosinophil-derived mediators, especially various cationic proteins, contribute to the tissue damage associated with allergic diseases. Along with the progression of allergic reactions, eosinophils migrate from the blood compartment to inflamed tissues and function as allergic inflammatory cells (1). The processes involved in tissue eosinophilia consist of a complex interplay of various pathways and are not fully understood. However, several chemoattractants generated at inflammatory sites potentially play a pivotal role in the recruitment of eosinophils in humans as well as in animals (2).

Chemotactic cytokines, termed chemokines, are now recognized as essential participants in the sequence of events by which circulating leukocytes migrate toward inflammatory sites. Chemokines are divided into two major subfamilies based on the sequence of arranged cysteine groups: the CXC subfamily and the CC subfamily. Two minor subfamilies, i.e., the CX3C and the C chemokines, are also categorized. To date, ~40 chemokines have been identified, and 15 chemokine receptors, i.e., five CXC chemokine receptors (CXCRs), eight CCRs, one CX3CR, and one XCR, have been cloned. It has been reported that eosinophils express CCR3, and ligands of CCR3 such as eotaxin induce strong migration of eosinophils (3, 4). Expression of CCR1 (5), and under certain circumstances expression of CXCR2 (6), have also been reported in eosinophils. The expression and function of these receptors have been extensively investigated. In contrast, there is little information regarding the expression and function of other chemokine receptors in eosinophils.

In this study, we have examined the expression of transcripts of a panel of chemokine receptors in human eosinophils and found intense constitutive expression of CXCR4 mRNA as well as CCR3 mRNA. Here, we demonstrate that surface expression of CXCR4 is inducible in eosinophils and that stromal cell-derived factor-1α (SDF-1α) elicits strong eosinophil migration comparable to that induced by eotaxin. The effects of cytokines on eosinophil CXCR4 expression have also been investigated.

Materials and Methods

Reagents and mAbs

The following reagents were purchased as indicated: recombinant human SDF-1α, eotaxin, IL-4, and TGF-β1 (PeproTech, London, U.K.); IFN-γ (Shionogi Pharmaceutical, Osaka, Japan); TNF-α (Dainippon Pharmaceutical, Osaka, Japan); C5a, cytochalasin B, and cycloheximide (Sigma, St. Louis, MO); ionomycin (Seikagaku, Tokyo, Japan); pertussis toxin (PTX) (Calbiochem-Behring, La Jolla, CA); anti-Fas mAb (CH-11, subclass IgM)

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and mouse IgM a1 with irrelevant specificity (Medical and Biological Laboratories, Nagoya, Japan and Organon Teknika, West Chester, PA, respectively). Anti-CCR3 mAb (7) was provided by Dr. H. Kawasaki (Institute of Medical Science, University of Tokyo, Tokyo, Japan). IL-3 and IL-5 were kindly donated by Kirin Brewery (Tokyo, Japan) and Suntory (Osaka, Japan and Laboratories, Nagoya, Japan and Organon Teknika, West Chester, PA, respectively).

Eosinophil separation and culture conditions

Eosinophils were separated from normal volunteers who had no history of allergy, as previously described (8). In brief, buffy coat cells were obtained from venous blood by dextran T500 sedimentation. Eosinophils were isolated by Percoll (1.088 g/ml; Pharmacia, Uppsala, Sweden) density centrifugation. Unless Percoll separation achieved purity of 90%, the eosinophils were further purified by negative selection using anti-CD16-bound micromagnetic beads (Miltenyi BioTech, Bergisch-Gladbach, Germany) and a magnetic-activated cell sorter column (Miltenyi BioTech) as the second step (9). After this negative selection, the mean eosinophil purity was consistently >99%, and the viability was consistently >95%. Eosinophils (0.5–1.0 × 106) were cultured in RPMI 1640 (Life Technologies, Minneapolis, MN) and anti-CD16-PE (Coulter) at 105 g/ml for 60 min at 4°C. During flow cytometry procedures, contaminating neutrophils were discriminated on the basis of their different fluorescence properties.

Flow cytometry of isolated eosinophils

Isolated eosinophils were washed in PBS supplemented with 3% FCS and 0.1% NaCl, and then incubated with anti-CCR4 mAb (12G5; Pharmingen, San Diego, CA) at 10 μg/ml for 60 min at 4°C. An isotype-matched mouse IgG2a with irrelevant specificity (UPC 10; Sigma) was used as a negative control. After washing, the cells were stained with FITC-labeled goat F(ab)2 against mouse IgG (Jackson ImmunoResearch, West Grove, PA) at 7 μg/ml for 30 min at 4°C. During flow cytometry procedures, contaminating neutrophils were discriminated on the basis of their different fluorescence properties.

Stained eosinophils were analyzed using EPICS XL System II (Coulter, Miami, FL). At least 3000 eosinophils were assessed to calculate the median value of fluorescence intensity. The median values of fluorescence intensity of human eosinophils were converted to the numbers of the molecules of equivalent soluble fluorochrome units (MESF) using Quantum 25 microbeads (Flow Cytometry Standards, San Juan, Puerto Rico) on each day of an experiment. Surface receptor levels expressed in MESF units were calculated using the following formula: (MESF of eosinophils stained with anti-CCR4 mAb) / (MESF of eosinophils stained with isotype control IgG).

Flow cytometry of eosinophils in whole blood

FACS analysis of eosinophils in whole venous blood was performed as previously described (11). In brief, blood was anti-coagulated with EDTA, and an equal volume of FACS buffer (PBS with 3% FCS and 0.1% NaN3) was added. Cells were stained with anti-CCR4-FITC (12G5; R&D Systems, Minneapolis, MN) and anti-CD16-PE (Coulter) at 10 μg/ml for 45 min at 4°C. An isotype-matched mouse IgG2a-FITC (R&D Systems) and mouse IgM a1 with irrelevant specificity (Medical and Biological Laboratories, Nagoya, Japan and Organon Teknika, West Chester, PA, respectively) were added. Cells were stained with anti-CCR4-FITC (12G5; R&D Systems, Minneapolis, MN) and anti-CD16-PE (Coulter) at 10 μg/ml for 45 min at 4°C. An isotype-matched mouse IgG2a-FITC (R&D Systems) and mouse IgM a1 with irrelevant specificity (Medical and Biological Laboratories, Nagoya, Japan and Organon Teknika, West Chester, PA, respectively).

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* The direct and reverse oligo primers for chemokine receptor sequences and their expected bp size are shown.
mouse IgG1-PE (Immunotech, Marseille, France) with irrelevant specificity were used as negative controls. Contaminating erythrocytes were eliminated with a lysis buffer (Ortho Diagnostic Systems, Tokyo, Japan). Granulocytes were discriminated on the basis of different forward/side scatter properties, and electronic gates were set on CD16-negative cells to identify eosinophils.

**Measurement of intracellular calcium concentration**

Purified eosinophils (purity, >99%) were resuspended in HBSS with Ca\(^{2+}\) and Mg\(^{2+}\) (Life Technologies) and 2% BSA at a cell density of 2.0 × 10\(^6\)/ml. Fura-2 AM (Dojindo, Tokyo, Japan) was added at a final concentration of 2 μM. After incubation for 20 min, excess dye was removed by centrifugation, and the cells were resuspended in a buffer containing 119 mM NaCl, 5 mM KCl, 1 mM CaCl\(_2\), 1 mM MgCl\(_2\), 0.03% human serum albumin, and 25 mM PIPES, pH 7.4, at a concentration of 1.6 × 10\(^6\) cell/ml. Ca\(^{2+}\) influx was measured using excitation at 340 and 380 nm on a Hitachi F-2500 fluorescence spectrometer (Hitachi, Tokyo, Japan). Calibration was performed using 0.1% Triton X-100 for total Ca\(^{2+}\) release and 10 mM EGTA to chelate free Ca\(^{2+}\).

**Chemotaxis assay of eosinophils**

Eosinophil migration was measured using a 96-well multiwell Boyden chamber (Neuroprobe, Bethesda, MD) and a 10-μm-thick polyvinylpyrrolidone-free polycarbonate membrane filter with pores of 5 μm in diameter (Neuroprobe), as previously described (8). Aliquots of 362 μl of triplicate samples were transferred into the lower wells, while 200 μl of a cell suspension that contained 1.5 × 10\(^6\) eosinophils was introduced into each well of the top compartment. After incubation for 90 min, the eosinophil peroxidase (EPO) activity of cells at the bottom of wells was determined with 200 μl of 0.05 M Tris-HCl, pH 8.0, containing 0.1% (v/v) Triton X-100, 0.1 mM o-phenylenediamine dihydrochloride (OPD; Sigma) and 50 mM hydrogen peroxide. The OD was read at 490/570 nm in an ELISA reader (Model 550; Bio-Rad, Hercules, CA). Data were analyzed with the MicroPlate Manager III program (Bio-Rad), and the numbers of migrated eosinophils were calculated based on a standard curve established with varying known numbers of eosinophils.

**Eosinophil degranulation**

Freshly isolated eosinophils (purity, >99%) were cultured for 24 h with or without IFN-γ (10 ng/ml). These cells were pretreated with 5 μg/ml of cytochalasin B for 5 min and then stimulated with SDF-1α (333 ng/ml) or C5a (5 × 10\(^{-9}\) M) for 5 h. The level of eosinophil-derived neurotoxin (EDN) was measured with an EDN ELISA kit (Medical and Biological Laboratories).

**Analysis of apoptotic cells**

Differential analysis of apoptotic and necrotic cells was performed using a MEBCYTO apoptosis kit (Medical and Biological Laboratories). Apoptotic cells were quantitatively determined by their ability to bind annexin V and exclude propidium iodide. Cells stained with propidium iodide were used as negative controls. Contaminating erythrocytes were eliminated with a lysis buffer (Ortho Diagnostic Systems, Tokyo, Japan). Granulocytes were discriminated on the basis of different forward/side scatter properties, and electronic gates were set on CD16-negative cells to identify eosinophils. As shown in Fig. 3, eosinophils in fresh whole blood, eosinophils in whole blood eosinophils, and eosinophils in Percoll-separated eosinophils did not express significant amounts of CXCR4 on their surface. However, as observed with Percoll-separated eosinophils, expression of eosinophil CXCR4 in whole blood eosinophils became evident after incubation for 24 h at 37°C (Fig. 3C). These results indicate that the purification step did not modulate the expression of CXCR4, and we therefore used Percoll-separated eosinophils in the following experiments.

**CXCR4-mediated eosinophil activation**

In the next series of experiments, we investigated whether eosinophil CXCR4 is functionally activated by a specific ligand for CXCR4, SDF-1α. As shown in Fig. 2, freshly isolated eosinophils did not express significant amounts of CXCR4 on their surface, but SDF-1α elicited a small but apparent Ca\(^{2+}\) influx in these cells (Fig. 4A). In cells incubated for 24 h at 37°C, much stronger influx of Ca\(^{2+}\) was observed (Fig. 4C). The magnitude of Ca\(^{2+}\) influx elicited by SDF-1α was comparable to that induced by eotaxin (Fig. 4D). Furthermore, SDF-1α-induced Ca\(^{2+}\) influx was completely abrogated by treatment with PTX (Fig. 4E), indicating the involvement of G proteins of the G\(_i\) class in the signal transduction pathways. To determine whether CXCR4 expressed in eosinophils has functional relevance, we tested the migration-inducing ability of SDF-1α. As shown in Fig. 5, SDF-1α was capable of inducing a migratory response in eosinophils: although SDF-1α did not induce significant migration in freshly isolated cells, eosinophils incubated for 24 h at 37°C exhibited a migratory response toward SDF-1α in a dose-dependent manner. Apparent migration was observed at 33 ng/ml of SDF-1α, and much stronger migration was observed at 333 ng/ml of SDF-1α. It should be noted that SDF-1α induced eosinophil chemotaxis as strongly as eotaxin, which is known as the most potent eosinophil chemoattractant. Furthermore, the migration induced by SDF-1α was chemotactic rather than chemo kinetic: when the same concentration of SDF-1α was added to both the upper and lower wells, the migration was significantly reduced (data not shown). No significant decrease in...
granulation was noted in cells that had migrated in response to SDF-1α compared with freshly isolated or 24-h-cultured eosinophils (Fig. 6). In fact, in vitro experiments showed that CXCR4 was not involved in the process leading to degranulation: SDF-1α failed to induce significant release of EDN from eosinophils cultured for 24 h with or without IFN-γ or from freshly isolated cells (Fig. 7).

Modulation of surface CXCR4 expression in eosinophils by cytokines

In the next series of experiments, we examined the effects of cytokines on CXCR4 expression by eosinophils during 24 h of incubation. As shown in Table II, TGF-β1, IFN-γ, and TNF-α up-regulated the expression of CXCR4. In contrast, eosinophil-directed hemopoietins, i.e., IL-3, IL-5, and GM-CSF, almost completely attenuated the surface expression of CXCR4. In addition, IL-4 also drastically down-regulated the CXCR4 expression (Table II and Fig. 8A). As shown in Fig. 8B, as small as a femtomolar level of IL-5 was sufficient to inhibit the CXCR4 expression. Half-maximal inhibition was observed at a concentration of ~100 fM of IL-5 with dose-dependent inhibition seen between 0.1 fM and 10 pM. For IL-4, half-maximal inhibition was observed at a concentration of ~1 pM with dose-dependent inhibition seen between 10 fM and 10 pM. On a molar basis, IL-5 was 10-fold more potent than IL-4. Furthermore, delayed addition of IL-5 or IL-4 effectively down-regulated CXCR4 expression. When eosinophils were cultured for 24 h without addition of any factors and then treated with IL-5 or IL-4, the level of CXCR4 expression in the

FIGURE 2. Time course of surface CXCR4 expression in purified eosinophils. Eosinophils were purified by means of Percoll density gradient centrifugation followed by negative selection using anti-CD16 microbeads. Purified eosinophils (purity, >99%) were cultured at 4°C (A) or 37°C (B) for the indicated times in culture medium. The cells were then treated with anti-CXCR4 mAb or isotype control mouse IgG (shaded peak) for 60 min at 4°C, stained with FITC goat F(ab’)2 against mouse IgG for 30 min at 4°C and analyzed by flow cytometry. A and B are representatives of three separate experiments, and the other showed similar results. C. The time course of surface CXCR4 expression is shown. All data are expressed as the mean ± SEM (n = 3) of MESF values calculated as described in Materials and Methods. **p < 0.01 vs MESF values of eosinophils cultured at 37°C. D. Expression of CCR3 on freshly isolated (0 h) and 24-h-cultured eosinophils (a representative of three separate experiments) is shown.

FIGURE 3. Expression of CXCR4 on eosinophils in whole blood. Eosinophils in whole blood were double-stained with anti-CXCR4-FITC and anti-CD16-PE and analyzed by flow cytometry. Granulocytes were discriminated on the basis of different forward/side scatter properties (A) and electronic gates on CD16-negative cells were set to eliminate neutrophils (B). C. Surface CXCR4 expression on eosinophils in fresh whole blood and eosinophils in whole blood cultured for 24 h at 37°C. The shaded area shows the fluorescence of cells stained with isotype-matched mouse IgG2a-FITC. The data are representative of three independent experiments, all showing similar results.
eosinophils was decreased at 6 and 24 h after the addition of each cytokine (data not shown).

Substantial evidence has shown that isolated eosinophils rapidly undergo apoptosis. In fact, when apoptotic cells were quantita-

tively determined based on their ability to bind annexin V and exclude propidium iodide, we found an increase in the proportion of apoptotic cells after 24 h of culturing (<5% vs ~20% for freshly isolated and 24-h-cultured eosinophils, respectively). We determined whether the surface expression of CXCR4 is related to apoptotic changes in cytokine-treated eosinophils. IL-4 exerted no significant inhibitory effect on apoptosis, but it almost completely suppressed CXCR4 expression. Conversely, IFN-γ significantly reduced the number of apoptotic

FIGURE 4. SDF-1α caused calcium influx to eosinophils. Calcium influx in highly purified eosinophils (purity, >99%) just after purification (A and B) and eosinophils cultured for 24 h at 37°C without (C and D) or with (E) further treatment with PTX. E. Cultured cells were treated with PTX at 100 nM for 2 h at 37°C, and then stimulated sequentially with SDF-1α and ionomycin. The data shown are representative of two independent analyses from different donors, each showing similar results. The concentration of chemokines and ionomycin were 400 ng/ml and 1 μM, respectively.

FIGURE 5. SDF-1α induced migration of eosinophils. The migration-inducing activity of SDF-1α and eotaxin was analyzed using eosinophils just after purification (purity, 96.3 ± 1.3%) and eosinophils cultured for 24 h at 37°C. The data are expressed as the percentage of total cells introduced into each of the upper wells (mean ± SEM, n = 4). The effects of the concentration of chemokines were analyzed by the two-way ANOVA test. When this test indicated a significant differences between concentrations (indicated as ++, p < 0.01), Fisher’s protected least significant difference (PLSD) test was used to compare individual groups. **, p < 0.01 vs values of eosinophil migration in control buffer at the same time point.

FIGURE 6. Photomicrographs of eosinophils that were freshly isolated (A), 24-h-cultured (B), and migrated in response to SDF-1α (C). The photos are representatives of two different experiments and another showed similar results. Magnification, ×600.
with FITC-conjugated annexin V and propidium iodide. Annexin V-negative and propidium iodide-negative cells are defined as alive. Annexin V-positive and propidium iodide-positive cells are considered to be apoptotic. The number of living cells, whereas it failed to up-regulate CXCR4 expression (Table II). Finally, treatment of eosinophils with anti-Fas mAb slightly but significantly decreased eosinophil CXCR4 expression (Table II). In contrast to those cultured eosinophils, we observed that surface CXCR4 protein was hardly detected in freshly isolated eosinophils (Fig. 2). However, surface CXCR4 expression gradually became apparent during incubation at 37°C. Because surface CXCR4 in cultured eosinophils was widely variable, it is of importance to clarify the control mechanisms of eosinophil CXCR4 expression. In this study, we observed that several cytokines exerted reciprocal and divergent effects on eosinophil CXCR4 expression (Table II). The patterns of cytokine-mediated regulation of CXCR4 expression in eosinophils seem to be distinct from those in other types of leukocytes such as T lymphocytes. In T lymphocytes, IL-4 up-regulates (13–15) and IFN-γ down-regulates (16) CXCR4 expression, while both cytokines exerted completely opposite effects on eosinophils.

Studies of time course and various conditions for CXCR4 expression raised a possibility that the expression of CXCR4 is related to apoptotic changes. IL-5, IL-3, and GM-CSF, all of which are known to rescue eosinophils from apoptosis (17–19), completely attenuated CXCR4 expression in eosinophils. Furthermore, the concentration of IL-5 necessary for the inhibition of CXCR4 induction matched the reported range required for inhibition of eosinophil apoptosis (20) (Fig. 5B). Consequently, it would be reasonable and even attractive to speculate that CXCR4 is preferentially induced in eosinophils undergoing apoptosis, and that these cells are then eliminated from the circulation by the interplay between CXCR4 and ubiquitously expressed SDF-1α. However, the level of eosinophil CXCR4 expression did not always parallel protein expression of CXCR4. In addition, CXCR4 mRNA remained at the baseline level in eosinophils cultured with IFN-γ. In contrast, none of these cytokines had any effect on the level of CCR3 mRNA (Fig. 10).

**Discussion**

In this study, we have demonstrated the functional expression of CXCR4 in human eosinophils. Among the 15 chemokine receptors, intense expression of CXCR4 as well as CCR3 transcripts was always detected in freshly isolated eosinophils (Fig. 1). While we were preparing this manuscript, Chelucci et al. reported the surface expression of CXCR4 in the eosinophil lineage during the culture of purified hemopoietic progenitors (12), although they did not examine the functional relevance of CXCR4 in these cells. In contrast to those cultured eosinophils, we observed that surface CXCR4 protein was hardly detected in freshly isolated eosinophils (Fig. 2). However, surface CXCR4 expression gradually became apparent during incubation at 37°C. Because surface CXCR4 in cultured eosinophils was widely variable, it is of importance to clarify the control mechanisms of eosinophil CXCR4 expression. In this study, we observed that several cytokines exerted reciprocal and divergent effects on eosinophil CXCR4 expression (Table II). The patterns of cytokine-mediated regulation of CXCR4 expression in eosinophils seem to be distinct from those in other types of leukocytes such as T lymphocytes. In T lymphocytes, IL-4 up-regulates (13–15) and IFN-γ down-regulates (16) CXCR4 expression, while both cytokines exerted completely opposite effects on eosinophils.

### Table II. Modulation of CXCR4 expression and eosinophil survival by cytokines or anti-Fas mAb

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<tr>
<td>IL-4</td>
<td>19.9 ± 1.2**</td>
<td>52.2 ± 8.0</td>
<td>21.8 ± 2.2</td>
<td>25.5 ± 9.4</td>
</tr>
<tr>
<td>IL-5</td>
<td>20.3 ± 4.0**</td>
<td>85.9 ± 4.2*</td>
<td>2.7 ± 0.3*</td>
<td>11.1 ± 3.8</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>7.1 ± 0.9**</td>
<td>83.8 ± 2.0**</td>
<td>3.1 ± 0.3*</td>
<td>12.7 ± 1.8</td>
</tr>
<tr>
<td>TGF-βI</td>
<td>133.3 ± 6.0*</td>
<td>54.2 ± 2.3</td>
<td>22.3 ± 5.1</td>
<td>23.0 ± 5.0</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>139.0 ± 11.9*</td>
<td>76.7 ± 4.8**</td>
<td>3.6 ± 0.6**</td>
<td>18.9 ± 4.4</td>
</tr>
<tr>
<td>TNF-α</td>
<td>196.7 ± 31.9*</td>
<td>63.4 ± 3.1*</td>
<td>12.0 ± 2.8**</td>
<td>24.2 ± 1.5</td>
</tr>
<tr>
<td>Control IgM</td>
<td>100</td>
<td>45.5 ± 7.5</td>
<td>26.9 ± 6.6</td>
<td>26.9 ± 5.1</td>
</tr>
<tr>
<td>anti-Fas mAb</td>
<td>76.1 ± 8.8</td>
<td>33.1 ± 6.0*</td>
<td>29.0 ± 6.8</td>
<td>37.5 ± 4.8</td>
</tr>
</tbody>
</table>

Notes: Eosinophils (purity, >99%) were incubated at 37°C for 24 h with the indicated cytokines, and CXCR4 expression was analyzed by flow cytometry. The concentration of each cytokine was 10 ng/ml, except for TNF-α, which was used at 100 ng/ml. In separate experiments, eosinophils were incubated with 1 μg/ml of anti-Fas mAb or the same concentration of control IgM for 24 h and then analyzed by flow cytometry. The data are expressed as the percentage of the calculated MESF of control cells, which were incubated in culture medium alone or incubated with control IgM (mean ± SEM, n = 4). * p < 0.05; ** p < 0.01 vs each control (nil = 100%). Eosinophils were also double-stained with FITC-conjugated annexin V and propidium iodide. Annexin V-negative and propidium iodide-negative cells are defined as alive. Annexin V-positive and propidium iodide-negative cells are defined as apoptotic, and propidium iodide-positive cells are defined as necrotic. Data are expressed as the mean ± SEM of the percentage of total cell number (n = 4).

* p < 0.05; ** p < 0.01 vs each control.
the apoptotic changes (Table II). IL-4, which is devoid of anti-apoptotic effects, almost completely attenuated CXCR4 expression (Table II). On the contrary, IFN-γ, which is known to block eosinophil apoptosis (Ref. 20 and Table II), efficiently up-regulated CXCR4 expression. It might be possible that each cytokine may regulate eosinophil CXCR4 expression via different pathways or mechanisms. However, these discrepancies observed between apoptotic changes and CXCR4 expression in cytokine-treated eosinophils, together with the direct determination of CXCR4 expression in nonapoptotic populations (Fig. 9), strongly suggest that eosinophil CXCR4 expression cannot be explained merely as a consequence of apoptotic changes in these cells.

Spontaneous sustained increase in CXCR4 expression similar to our present findings has also been reported for monocytes and lymphocytes (21, 22). In these cells, surface expression of CXCR4 seems not to be regulated at the mRNA level but mainly at the protein level, such as receptor trafficking between the intracellular space and cell surface (23). In the present study, despite the constitutive expression of CXCR4 mRNA (Fig. 1), freshly isolated eosinophils did not express significant amounts of CXCR4 protein on their surface (Fig. 2). Furthermore, spontaneous sustained increase in CXCR4 expression was reversibly down-regulated by delayed addition of IL-4 and IL-5. These findings strongly suggest that surface CXCR4 expression in eosinophils is regulated, at least in part, at the level of receptor trafficking. However, the expression of surface CXCR4 dose not seem to be regulated merely at the level of posttranscription. The process leading to the expression of CXCR4 in eosinophils was partly inhibited by cycloheximide, indicating that the sustained induction of surface CXCR4 expression involves a cycloheximide-sensitive component. Although a direct relationship between the level of mRNA and the amount of surface protein was not always observed in cytokine-mediated alteration of CXCR4 expression, IL-5 apparently down-regulated the level of both mRNA and surface protein of CXCR4 (Table II and Fig. 10). Taken together, it seems plausible that surface CXCR4 expression in eosinophils is modulated not only at the level of posttranscription but also at least in part at the level of transcription.

The most striking findings of our study are that surface expression of CXCR4 in eosinophils has functional relevance, and that SDF-1α induced strong eosinophil migration comparable to that
of evidence indicates that CXCR4 regulates myelopoiesis and lymphopoiesis in a stage-specific fashion: CXCR4 is preferentially expressed in immature progenitors in the bone marrow or thymus during the maturational stage (31–35), and SDF-1α retains these cells via CXCR4 to prevent their liberation from these tissues into the circulation. Thus, CXCR4 in immature leukocytes potentially serves as an anchor for these cells by inhibiting their movement rather than inducing their migration. A similar situation can be imagined for CXCR4 in mature eosinophils: eosinophil CXCR4 may affect the size of the mobilizable pool, and anchorage of mature eosinophils by SDF-1α may be crucial for retaining eosinophils in noninflamed tissues. Th2-dominant state may favor the liberation of eosinophils by down-regulating CXCR4 expression, which in turn would permit enhanced accumulation of eosinophils at allergic inflammatory sites by eosinophil-active chemokines such as eotaxin. The presence of IL-5 in the serum, which is often observed in patients with allergic and helminthic disorders, may increase the distribution of eosinophils to inflamed tissues.

In summary, we have demonstrated that functional expression of CXCR4 is inducible in eosinophils, and that SDF-1α elicits strong migration comparable to that induced by eotaxin. Th2 cytokines such as IL-4 and IL-5 drastically inhibited the expression of CXCR4. The interplay between CXCR4 and SDF-1α may affect the distribution and migration of eosinophils, thus indicating that CXCR4 in eosinophils might represent an important mechanism in diseases in which eosinophils play pathogenic roles.

Acknowledgments

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