Molecular Cloning of a Novel Human CC Chemokine (Eotaxin-3) That Is a Functional Ligand of CC Chemokine Receptor 3*

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Previously, we mapped the novel CC chemokine myeloid progenitor inhibitory factor 2 (MPIF-2)/eotaxin-2 to chromosome 7q11.23 (Nomiyama, H., Osborne, L. R., Imai, T., Kusuda, J., Miura, R., Tsui, L.-C., and Yoshie, O. (1998) *Genomics* 49, 339–340). Since chemokine genes tend to be clustered, unknown chemokines may be present in the vicinity of those mapped to new chromosomal loci. Prompted by this hypothesis, we analyzed the genomic region containing the gene for MPIF-2/eotaxin-2 (SCYA24) and have identified a novel CC chemokine termed eotaxin-3. The genes for MPIF-2/eotaxin-2 (SCYA24) and eotaxin-3 (SCYA26) are localized within a region of ~40 kilobases. By Northern blot analysis, eotaxin-3 mRNA was constitutively expressed in the heart and ovary. We have generated recombinant eotaxin-3 in a baculovirus expression system. Eotaxin-3 induced transient calcium mobilization specifically in CC chemokine receptor 3 (CCR3)-expressing L1.2 cells with an EC50 of 3 nM. Eotaxin-3 competed the binding of 125I-eotaxin to CCR3-expressing L1.2 cells with an IC50 of 13 nM. Eotaxin-3 was chemotactic for normal peripheral blood eosinophils and basophils at high concentrations. Collectively, eotaxin-3 is yet another functional ligand for CCR3. The potency of eotaxin-3 as a CCR3 ligand seems, however, to be ~10-fold less than that of eotaxin. Identification of eotaxin-3 will further promote our understanding of the control of eosinophil trafficking and other CCR3-mediated biological phenomena. The strategy used in this study may also be applicable to identification of other unknown chemokine genes.

Chemokines are a group of structurally related, mostly basic, heparin-binding cytokines that play important roles in inflammatory and immunological processes by their capacity to recruit selective subsets of leukocytes (1–4). Chemokines are divided into two major subfamilies, CXC and CC, based on the arrangement of the first two of the four conserved cysteine residues; the two cysteines are separated by a single amino acid in CXC chemokines and are adjacent in CC chemokines. Furthermore, lymphotactin/single C motif 1, which retains only the second and fourth of the four cysteine residues conserved in other chemokines, and fractalkine/neurotactin, a transmembrane molecule with an N-terminal chemokine domain with a unique CX3C motif, represent the new C and CX3C subfamilies (1–4). Classically, CXC chemokines generally attract neutrophils, and their genes are clustered on chromosome 4q12-q21, whereas CC chemokines mainly attract monocytes, and their genes are clustered on chromosome 17q11.2. It is now known, however, that CXC chemokines with an ELR motif just prior to the first cysteine residue are potent chemotactants for neutrophils, and their genes are clustered on chromosome 4q12-q21, whereas those without the ELR motif act mainly on lymphocytes, and their genes are localized to different chromosomal loci. Similarly, a number of novel CC chemokines, which are highly specific for lymphocytes and dendritic cells, have their genes mapped to loci different from the major CC chemokine gene cluster at chromosome 17q11.2 (3). Furthermore, there are several mini-clusters of chemokine genes outside the major chemokine gene clusters (3). Thus, unknown chemokine genes may be present in the vicinity of chemokine genes mapped to new chromosomal loci.

Accumulation of eosinophils in the blood and affected tissues is a classical sign of inflammatory responses to helminths and allergens. Even though eosinophils are important effector cells against helminths, they are also implicated in tissue damages in allergic diseases. Because of the pathologic potential of eosinophils, identification of specific factors that regulate their accumulation in tissues is important. Using an experimental model of asthma in guinea pigs, Jose et al. (5) have identified a CC chemokine (eotaxin) that is a potent and selective chemoattractant for eosinophils. Subsequently, we (6) and others (7, 8) have identified the human homologue of eotaxin and its specific receptor, now termed CCR3.1 CCR3 has been shown to be expressed selectively on eosinophils at high levels and also on basophils and some Th2-type T cells (9–11). Furthermore, besides eotaxin, many other CC chemokines have been listed as functional ligands for CCR3 (7–9). Recently, the novel CC chemokine myeloid progenitor inhibitory factor 2 (MPIF-2)/eotaxin-2 was reported as a new ligand for CCR3 (12, 13). We have mapped the gene for MPIF-2/eotaxin-2 (SCYA24) to human chromosome 7q11.23 (14). Human chromosome 7 is one of the main sequencing targets of the Genome Sequencing Center.

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The nucleotide sequence reported in this paper has been submitted to the DDBJ/GenBank™/EBI Data Bank with accession number AB010447.

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1 The abbreviations used are: CCR3, CC chemokine receptor 3; MPIF-2, myeloid progenitor inhibitory factor 2; RACE, rapid amplification of cDNA ends; PCR, polymerase chain reaction; MES, 4-morpholineethanesulfonic acid; HPLC, high performance liquid chromatography.
experimental procedures

Reagents—MIP-1α (macrophage inflammatory protein 1α), RANTES (regulated on activation normal T expressed and secreted), MCP-4 (monocyte chemoattractant protein 4), and eotaxin-2 were purchased from PeproTech (Rocky Hill, NJ). Eotaxin, MCP-1, TARC (thymus and activation-regulated chemokine), LARC (liver and activation-regulated chemokine), SLC (secondary lymphoid tissue chemokine), and fractalkine were prepared as described previously (16, 17).

Cells—The panel of murine L1.2 pre-B cells stably transfected with the known CC chemokine receptors and orphan receptors was described previously (16). Peripheral blood eosinophils were isolated as follows. Heparinized venous blood was obtained from healthy donors. After sedimentation with dextran, red blood cells were lysed by hypotonic treatment, and mononuclear cells were removed by Ficoll-Paque sedimentation. Neutrophils were depleted by magnetic cell sorting (Miltenyi Biotec, Bergisch, Germany) after labeling cells with anti-CD16 microbeads. The purity of eosinophils thus obtained was determined by standard cytological staining and was routinely >95%. Basophils were separated from heparinized blood by Percoll density gradient centrifugation as described previously (18). The purity of basophils was routinely ~20%.

human genome sequence data base search—The human genome sequence data base of the Genome Sequencing Center at Washington University was searched for the MIPF-2/eotaxin-2 nucleotide sequence using BLAST Search. A sequence (H_RG356E01.seq) was found to contain the genomic sequence of MIPF-2/eotaxin-2. Using the DNA sequence data, the full-length cDNA sequence was obtained by the polymerase chain reaction (PCR) method (19) using human lung Marathon-Ready cDNA (CLONTECH) as a template. Two-step polymerase chain reaction (PCR) was carried out. The cDNA was first amplified with the first gene-specific primer (5′-RACE primer, CTCCCTGAAGACCTGGGACCTGGGTGCGAAGCTA) and the AP2 primer (5′-RACE primer, TTCCCTGGACCTGGGTGCGAAGCTA) and amplified with the second gene-specific primer (5′-RACE primer, CTTCAGAAAAGATTCCGCAGGCTCCCCAGAG; and 3′-RACE primer, CATATCCAAAGACTGCTGTTGCTAATACGACGC) and AP1 primer (CLONTECH), which is complementary to the adapter ligated at both ends of the cDNA. PCR was performed in a 20-μl reaction mixture containing 0.2 μM each dNTP, 4 μM of each primer, 0.625 units of AmpliTaq Gold™ (Perkin-Elmer), and 1× buffer supplied with the polymerase. The reaction was carried out at 94 °C for 45 min; then 40 cycles of 94 °C for 1 min, 68 °C for 2 min, and 72 °C for 1 min; and finally, 72 °C for 5 min. Nested PCR was carried out using the primary PCR products as templates. The cDNA was amplified with the second gene-specific primer (5′-RACE primer, GCTCTAGAAGAGTGGTCTTCGAGGTGTTTTCCAGAAG; and 3′-RACE primer, TCTCCGACGCTGGGACCTGGGTGCGAAGCTA) and the AP2 primer (CLONTECH), which is complementary to the cDNA adapter ligated at both ends of the cDNA. PCR was performed in a 50-μl reaction mixture containing 0.2 mM each dNTP, 10 μM of each primer, 1.25 units of AmpliTaq Gold™, and 1× buffer supplied with the polymerase. The amplification reaction was carried out as follows: 94 °C for 5 min; then 30 cycles of 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min; and finally, 72 °C for 5 min. The amplification products were cloned into the pGEM-T Easy vector (Promega) by T-A ligation and sequenced on both strands.

Northern Blot Analysis—This was carried out as described previously (16). In brief, a multiple-tissue blot filter was purchased from Genosys (Houston, TX). The filter was hybridized with the 32P-labeled eotaxin-3 cDNA probe at 65 °C for 1 h in QuikHyb hybridization solution (Stratagene) containing denatured salmon sperm DNA at 100 μg/ml. After washing at 65 °C for 30 min in 0.1× SSC and 0.1% SDS, filters were exposed to x-ray films at ~80 °C with an intensifying screen for 1 week.

Production of Eotaxin-3 in a Baculovirus Expression System—Recombinant eotaxin-3 was prepared using the Bac-to-Bac baculovirus expression system (Life Technologies, Inc.) following the protocol recommended by the manufacturer. Briefly, the eotaxin-3 cDNA containing the entire coding region was excised with BamHI and XhoI and subcloned into the BamHI-XhoI sites of the pFastBac baculovirus transfer vector downstream of the polyhedrin gene promoter. The resulting recombinant plasmid, pFastBac-eotaxin-3, was transformed into Echerichia coli DH10Bac containing a shuttle vector “bacmid” with a mini-attTn7 target site and the helper plasmid. The mini-attTn7 element on the pFastBac1 plasmid transposes to the mini-attTn7 target site on the bacmid in the presence of transposition protein provided by the helper plasmid. Insertion of the mini-Tn7 element into the mini-attTn7 target site disrupts the lacZ coding sequence, making colonies containing recombinant bacmids white in the presence of 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside. The resulting recombinant bacmid was transfected into Sf9 cells using Cellfectin reagent (Life Technologies, Inc.), and the recombinant virus was obtained. High Five™ cells (Invitrogen) were infected with the recombinant virus at a multiplicity of infection of 10–20. The culture supernatants collected 2 days after infection were cleared by filtration through 0.22-μm membranes and were dialyzed against 50 mM MES (pH 6.5) and 0.1 M NaCl and applied to a 1-ml cation-exchange HiTrap S column (Amersham Pharmacia Biotech) equilibrated with 50 mM MES (pH 6.5) and 0.1 M NaCl. The column in the fast protein liquid chromatography system (Amersham Pharmacia Biotech) was eluted with 45 ml of a linear gradient of 0.1–1.0 M NaCl in 50 ml of MES at a rate of 1 ml/min. The fractions containing recombinant eotaxin-3 were pooled and injected into a reverse-phase high-performance liquid chromatography column (4.6 × 250 mm, YMC Bio, Tokyo, Japan) eluted with 0.1% trifluoroacetic acid. The column was eluted with 60 ml of 0–60% acetonitrile in 0.05% trifluoroacetic acid at a flow rate of 1 ml/min. Fractions containing recombinant eotaxin-3 were pooled and lyophilized. Protein concentrations were determined with a BCA kit (Pierce). The amino-terminal sequence analysis was done in a protein sequence analyzer (Shimazu, Tokyo).

Chemokine Binding Assay—This was carried out as described previously (16). In brief, cells were suspended at 3 × 10^6 cells/ml in buffer A (Hanks’ balanced saline solution supplemented with 1 mg/ml bovine serum albumin and 10 mM HEPES (pH 7.4)) and incubated with 2 μM Fura-2/AM (Molecular Probes, Inc., Eugene, OR) at 37 °C for 1 h in the dark. Cells were washed twice with buffer A and resuspended at 2.5 × 10^6 cells/ml. To measure intracellular calcium, 2 μM of the cell suspension was placed in a quartz cuvette. Emission fluorescence at 530 nm was measured upon excitation at 340 and 380 nm every 0.2 s on an LS 50B spectrofluorometer (Perkin-Elmer) to obtain the ratio of fluorescence intensity (F(340/380)). To determine EC_{50}, a dose-response curve was generated.

Chemokine Binding Assay—The chemotaxis assay was performed using a 48-well microchemotaxis chamber as described previously (20). In brief, 1 × 10^6 cells were incubated for 1 h at 15 °C with 125I-labeled eotaxin (specific activity, 200 Ci/mmol; Amersham Pharmacia Biotech) in the presence of increasing concentrations of unlabeled competitors in 200 μl of 25 mM HEPES (pH 7.6), 1 mM CaCl_2, 5 mM MgCl_2, 120 mM NaCl, and 0.5% bovine serum albumin. After incubation, cells were separated from unbound radiolabeled ligands by centrifugation through a mixture of dibutyl phthalate/olive oil (4:1). All assays were done in duplicate.

Migration Assay—The chemotaxis assay was performed using a 48-well microchemotaxis chamber as described previously (20). In brief, chemotacticants were diluted in RPMI 1640 medium supplemented with 1 mg/ml bovine serum albumin and placed in the lower wells (25 μl/well). Cells were suspended in RPMI 1640 medium, 20 mM HEPES (pH 7.6), and 1% bovine serum albumin at 2 × 10^6 cells/ml and added to the upper wells (50 μl/well), which were separated from the lower wells by a polyvinylpyrrolidone-free polycarbonate filter with 5-μm pores. The chamber was incubated for 1 h at 37 °C in 5% CO₂ and 95% air. Filters were removed, and cells that migrated were stained with Diff-Quik. Basophils that migrated were stained with toluidine blue (18). Cells that migrated were counted in 5–10 randomly selected high-power fields (>400) per well. All assays were done in triplicate.

RESULTS

Search for a Novel Chemokine Gene—Previously, we mapped the gene for the novel CC chemokine MIPF-2/eotaxin-2 (SCF124) to chromosome 7g11.23 (14). By analyzing the human genome DNA sequence data base of the Genome Sequencing Center at Washington University by BLAST Search, we identified the genomic sequence derived from chromosome 7 that contains the gene for MIPF-2/eotaxin-2 (H_RG356E01.seq). In an attempt to identify a novel chemokine gene in the vicinity of the gene for MIPF-2/eotaxin-2, we carried out an exon search...
on this genomic sequence using the DNA sequence analysis tool GRAIL (15). Two exon sequences corresponding to the second and third exons of a chemokine-like protein were found at a site ~40 kilobases apart from the gene for MPIF-2/eotaxin-2. The sequencing of H_RG356E01.seq (107 kilobases) has now been completed and deposited in the GenBank™/EBI Data Bank with accession number AC005102. The physical map of the region is shown in Fig. 1.

Cloning of a Novel CC Chemokine cDNA—To determine the full-length cDNA sequence of a putative novel chemokine, we generated primers based on these genomic sequences and carried out 5’- and 3’-RACE (19) using human lung Marathon-Ready™ cDNA as a template. As shown in Fig. 2, the full-length cDNA of 467 base pairs was obtained. Comparison of the cDNA sequence with the genomic sequence (GenBank™/EBI Data Bank accession number AC005102) defines the exon-intron junctions: exon 1, positions 95,006–95,105; exon 2, positions 95,206–95,320; and exon 3, positions 97,420–97,670. The cDNA contained a long open reading frame starting from the first methionine codon and encoding a highly basic polypeptide of 94 amino acids with a calculated molecular mass of 10,635 Da. The 3’-noncoding region contains a typical AATAAA polyadenylation signal, but no ATTTA motif for rapid mRNA degradation, which is frequently found in the 3’-noncoding regions of cytokines and chemokines (21). The deduced polypeptide sequence contains a highly hydrophobic amino-terminal region characteristic of a signal peptide with a predicted cleavage site between Ala-23 and Thr-24. The predicted amino acid identities of the mature protein to other known human CC chemokines are ranged from 45% with MIP-1β to 22% with LARC. We also constructed a phylogenetic tree using the mature amino acid sequences of all the known human CC chemokines (Fig. 3). Even though the amino acid identity of the novel chemokine (indicated as eotaxin-3) to MPIF-2/eotaxin-2 is only 33%, it has the closest relationship with MPIF-2/eotaxin-2 and is located just at the branching point between the CC chemokines clustered on chromosome 17q11.2 and those 40 kilobases apart from the gene for MPIF-2/eotaxin-2. The polyadenylation signal is double-underlined.

Expression of Eotaxin-3 mRNA in Human Tissues—We examined constitutive expression of eotaxin-3 mRNA in various human tissues by Northern blot analysis. Eotaxin-3 transcripts of ~0.8 kilobases were detected at low levels in the heart and ovary (Fig. 4). By reverse transcription-PCR, however, its transcripts were detected ubiquitously in various tissues (data not shown).

Production of Recombinant Eotaxin-3 Protein—To obtain recombinant eotaxin-3, insect cells were infected with a recombinant baculovirus encoding eotaxin-3 under the control of the polyhedrin promoter. Recombinant eotaxin-3 was purified from pooled culture supernatants by cation-exchange chromatography and reverse-phase high performance liquid chromatography. Eotaxin-3 was eluted from the reverse-phase column as a single major peak at an acetonitrile concentration of 30% (Fig. 5A) and migrated as a single band of ~12 kDa on SDS-polyacrylamide gel (Fig. 5B). Amino acid sequence analysis demonstrated that the amino terminus of mature eotaxin-3 starts at

FIG. 1. Physical map of the region at human chromosome 7q11.23 that contains the genes for MPIF-2/eotaxin-2 and eotaxin-3. The map is depicted based on the bacterial artificial chromosome nucleotide sequence deposited by the Genome Sequence Center at Washington University (GenBank™/EBI Data Bank accession number AC005102). Arrows indicate the transcriptional orientation. kb, kilobases.

FIG. 2. Full-length cDNA of eotaxin-3. The nucleotide and deduced amino acid sequences are shown. The signal sequence is underlined. The polyadenylation signal is double-underlined.

FIG. 3. Phylogenetic tree of the human CC chemokines. Evolutionary distances are estimated using the program phylip (23) based on the protein alignment data generated by the program prrp (23). The chromosome where each chemokine gene is mapped is indicated on the right.

FIG. 4. Northern blot analysis of eotaxin-3 mRNA expression in various human tissues. A filter blotted with 2 μg/lane of poly(A)+ RNA from the indicated human tissues (CLONTECH) was hybridized with 32P-labeled eotaxin-3 cDNA. After washing, a film was exposed to the filter at ~80 °C with an intensifying screen for 1 week. PBL, peripheral blood leukocytes.
Thr-24 as predicted.

Eotaxin-3 Induces Calcium Flux in CCR3-transfected Cells—To examine the interaction of eotaxin-3 with each cloned receptor, we measured eotaxin-3-induced calcium mobilization in a panel of L1.2 cells stably expressing CCR1–7, CXCR1, CXCR5, XCR1, CCR9, GPR1, GPR15/BOB, GPR25, STRL33/BONZO, Mas, and CMKR-L2 (16). As shown in Fig. 6, eotaxin-3 induced calcium flux specifically in CCR3-expressing L1.2 cells. On the other hand, MIP-1α, MCP-1, TARC, RANTES, LARC, and SLC properly induced calcium flux in L1.2 cells expressing their respective receptors even after treatment with eotaxin-3. As shown in Fig. 7, eotaxin and eotaxin-3 induced calcium mobilization in CCR3-expressing L1.2 cells with EC₅₀ values of ~0.4 and ~3 nM, respectively. These results demonstrate that eotaxin-3 is a specific functional ligand for CCR3, but has a potency about one-tenth of that of eotaxin.

Cross-desensitization in CCR3-transfected Cells—Using CCR3-expressing L1.2 cells, we analyzed cross-desensitization between eotaxin-3 and other known CCR3 ligands: eotaxin, eotaxin-2, MCP-4, and RANTES. As shown in Fig. 8, eotaxin-3 desensitized the cells almost completely to eotaxin-3, eotaxin-2, and RANTES and partially to eotaxin and MCP-4. Conversely, eotaxin and MCP-4 almost completely desensitized the cells to eotaxin-3, whereas RANTES and eotaxin-2 only partially desensitized the cells to eotaxin-3. Thus, the rank order of potency for calcium mobilization in CCR3-expressing L1.2 cells between eotaxin-3 and these other CCR3 ligands is as follows: eotaxin, MCP-4 > eotaxin-3 > eotaxin-2, RANTES.

Binding Studies—To compare the binding affinity of eotaxin and eotaxin-3 with that of CCR3, we carried out a displacement-type binding experiment in which binding of [¹²⁵I]eotaxin to CCR3-expressing L1.2 cells was competed by unlabeled competitors. As shown in Fig. 9, both eotaxin and eotaxin-3 showed complete competition with IC₅₀ values of 1.9 and 13 nM, respectively.

Chemotactic Activity—We examined the chemotactic activity of eotaxin-3 in comparison with eotaxin. As shown in Fig. 10, eotaxin induced chemotactic responses in eosinophils with a typical bell-shaped dose-response curve and a maximum effect at 100 nM. On the other hand, eotaxin-3 induced migration of eosinophils only at 1000 nM. Similarly, eotaxin induced migration of basophils at 30–300 nM, whereas eotaxin-3 induced migration of basophils only at 300 nM.

**DISCUSSION**

In the past few years, a number of novel chemokines, particularly CC chemokines, have been discovered by searching the public and private expressed sequence tag data bases. Some of the chemokines are of particular interest because of their constitutive expression in lymphoid tissues, their specific activities on lymphocytes and dendritic cells, and their genes being mapped to chromosomal sites different from the major chemo-
kine gene clusters at chromosome 4 and 17 (3). Even though the expressed sequence tag data bases have been quite useful for identification of many new chemokines, those that are expressed in small amounts in tissues or by a restricted type of cells may be rarely represented in expressed sequence tag data bases. Since chemokine genes tend to be clustered, it may also be possible to discover novel chemokines at chromosomal loci where some chemokines are already mapped. Previously, we mapped the novel CC chemokine MPIF-2/eotaxin-2 to chromosome 7q11.23 (14). In the present study, by analyzing the genomic sequence containing MPIF-2/eotaxin-2 (H_RG356E01.seq/AC005102), we have indeed identified a novel human CC chemokine termed eotaxin-3 (Fig. 2) in the vicinity of MPIF-2/eotaxin-2. The genes for MPIF-2/eotaxin-2 (SCYA24) and eotaxin-3 (SCYA26) are localized within a region of ~40 kilobases and thus represent a new cluster of chemokine genes at chromosome 7q11.23 (Fig. 1). It is thus likely that MPIF-2/eotaxin-2 and eotaxin-3 were generated by gene duplication from a common ancestor. In a phylogenetic tree, they are indeed most closely related to each other even though they also appear to have diversified a long time ago (Fig. 3). Notably, the CC chemokines of the major chemokine cluster at chromosome 17 appear to be mostly generated after diversification of eotaxin-3 from a common ancestor (Fig. 3).

We have shown that eotaxin-3 is capable of inducing transient calcium mobilization specifically in CCR3-expressing L1.2 cells (Fig. 6). Eotaxin and eotaxin-3 induced transient calcium mobilization in CCR3-expressing L1.2 cells with EC50 values of 0.4 and 3 nM, respectively (Fig. 7). Eotaxin-3 partially desensitized CCR3-expressing L1.2 cells to eotaxin and MCP-4 and completely desensitized these cells to eotaxin-2 and RANTES (Fig. 8). Eotaxin and eotaxin-3 competed the binding of 125I-eotaxin to CCR3-expressing L1.2 cells with IC50 values of 1.9 and 13 nM, respectively (Fig. 9). Collectively, eotaxin-3 is yet another functional ligand for CCR3. The potency of eotaxin-3 as a CCR3 ligand seems, however, to be ~10-fold less than that of eotaxin (Fig. 7). Consistently, eotaxin-3 was chemotactic for normal peripheral blood eosinophils and basophils only at high concentrations (Fig. 10). However, eotaxin-3 may have more relevant biological functions such as myeloid progenitor inhibition shown for MPIF-2/eotaxin-2 (12). Eotaxin-3 may also act on an unknown receptor different from CCR3 with a higher potency.

Northern blot analysis has shown that eotaxin-3 mRNA is constitutively expressed in the heart and ovary (Fig. 4). By reverse transcription-PCR, however, its mRNA is detected ubiquitously in various tissues (data not shown). This may indicate that eotaxin-3 is expressed ubiquitously at low levels by cells in various tissues or by only a minor fraction of cells present in various tissues. Previously, MPIF-2/eotaxin-2 mRNA could not be detected in any human tissues by Northern blot analysis (12). The transcripts were detected only by reverse transcription-PCR in monocytes treated with granulocyte/macrophage colony-stimulating factor and in T cells activated by anti-CD3 antibody in the presence of interleukin-2 (12). It remains to be seen which types of cells produce
eotaxin-3 and whether production of eotaxin-3 is up-regulated by some external stimuli. It is also notable that, like eotaxin-3, eotaxin is constitutively expressed in the heart, in addition to the small intestine and colon (6). Eosinophils are known to migrate physiologically into intestinal tissues, but do not usually exist in heart tissues except for rare complications associated with high blood eosinophilia (22). Thus, eotaxin and eotaxin-3 may have some other physiological roles in the heart. By its low potency on CCR3, eotaxin-3 may even negatively regulate migration of eosinophils into the heart.

In conclusion, identification of eotaxin-3 will further promote our understanding of eosinophil trafficking and other CCR3-mediated pathophysiological phenomena. Furthermore, it remains to be seen whether eotaxin-3 has functions other than that of a low potency ligand for CCR3. The strategy of identification of eotaxin-3 used in this study may also be applicable to identification of other unknown chemokines.

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REFERENCES

1. Baggiolini, M., Dewald, B., and Moser, B. (1997) Annu. Rev. Immunol. 15, 675–705
2. Rollins, B. (1997) Blood 90, 909–928
3. Yoshie, O., Imai, T., and Nomiyama, H. (1997) J. Leukocyte Biol. 62, 634–644
4. Zlotnik, A., Morales, J., and Hedrick, J. A. (1999) Crit. Rev. Immunol. 19, 1–47
5. Jose, P. J., Griffiths-Johnson, D. A., Collins, P. D., Walsh, D. T., Moqbel, R., Totty, N. F., Truong, O., Hsuan, J. J., and Williams, T. J. (1994) J. Exp. Med. 179, 881–886
6. Kitaura, M., Nakajima, T., Imai, T., Harada, S., Combadiere, C., Tiffany, H. L., Murphy, P. M., and Yoshie, O. (1996) J. Biol. Chem. 271, 7725–7730
7. Ponath, P. D., Qin, S., Post, T. W., Wang, J., Wu, L., Gerard, N. P., Newman, W., Gerard, C., and Mackay, C. R. (1996) J. Exp. Med. 183, 2437–2448
8. Daugherty, B. L., Siciliano, S. J., DeMartino, J. A., Malkowitz, L., Sirtolina, A., and Springer, M. S. (1996) J. Exp. Med. 183, 2349–2354
9. Heath, H., Qin, S., Rao, P., Wu, L., LaRosa, G., Kassam, N., Ponath, P. D., and Mackay, C. R. (1997) J. Clin. Invest. 99, 178–184
10. Uguccioni, M., Mackay, C. R., Ochensberger, B., Loetscher, P., Rhs, S., LaRosa, G. J., Rao, P., Ponath, P. D., Baggioioli, M., and Dahinden C. A. (1997) J. Clin. Invest. 100, 1137–1141
11. Sallusto, F., Mackay, C. R., and Lanzavecchia, A. (1997) Science 277, 2005–2007
12. Patel, V. P., Kreider, P. L., Li, Y., Li, H., Keung, K., Salleedo, T., Nardelli, B., Pippalla, V., Gentz, S., Thotakura, R., Parmelee, D., Gentz, R., and Garotta, G. (1997) J. Exp. Med. 185, 1163–1172
13. Forssmann, U., Uguccioni, M., Loetscher, P., Dahinden, C. A., Langen, H., Thelem, M., and Baggioioli, M. (1997) J. Exp. Med. 185, 2171–2176
14. Nomiyama, H., Osborne, L. R., Imai, T., Kusuda, J., Miura, R., Teui, L.-C., and Yoshie, O. (1998) Genomics 49, 339–340
15. Uberbacher, E. C., and Mural, R. J. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 11261–11265
16. Yoshida, R., Nagira, M., Kitaura, M., Imagawa, N., Imai, T., and Yoshie, O. (1998) J. Biol. Chem. 273, 7118–7122
17. Imai, T., Hieszima, K., Haskell, C., Baba, M., Nagira, M., Nishimura, M., Kakiuzki, M., Takagi, S., Nomiyama, H., Schall, T. J., and Yoshie, O. (1997) Cell 81, 521–530
18. Yamada, H., Hirai, K., Miyamasu, M., Ikura, M., Misaki, Y., Shoji, S., Takaish, T., Kasahara, T., Morita, Y., and Ito, K. (1997) Biochem. Biophys. Res. Commun. 231, 365–368
19. Frohman, M. A. (1993) Methods Enzymol. 218, 340–356
20. Imai, T., Yoshida, T., Baba, M., Nishimura, M., Kakiuzki, M., and Yoshie, O. (1996) J. Biol. Chem. 271, 21514–21521
21. Shaw, G., and Kamen, R. (1996) Cell 86, 659–667
22. Fauci, A. S., Harley, J. B., Roberts, W. C., Ferrans, V. J., Gralnick, H. R., and Bjornson, B. H. (1982) Ann. Intern. Med. 97, 78–92
23. Gotoh, O. (1995) Comput. Appl. Biosci. 11, 543–551