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Liver-Expressed Chemokine/CC Chemokine Ligand 16 Attracts Eosinophils by Interacting with Histamine H4 Receptor

Takashi Nakayama,* Yoshiko Kato, † Kunio Hieshima,* Daisuke Nagakubo,* Yuichi Kunori, † Takao Fujisawa,* and Osamu Yoshie* ‡ §

Liver-expressed chemokine (LEC)/CCL16 is a human CC chemokine that is constitutively expressed by the liver parenchymal cells and present in the normal plasma at high concentrations. Previous studies have shown that CCL16 is a low-affinity ligand for CCR1, CCR2, CCR5, and CCR8 and attracts monocytes and T cells. Recently, a novel histamine receptor termed type 4 (H4) has been identified and shown to be selectively expressed by eosinophils and mast cells. In this study, we demonstrated that CCL16 induced pertussis toxin-sensitive calcium mobilization and chemotaxis in murine L1.2 cells expressing H4 but not those expressing histamine receptor type 1 (H1) or type 2 (H2). CCL16 bound to H4 with a \( K_d \) of 17 nM. By RT-PCR, human and mouse eosinophils express H4 but not H3. Accordingly, CCL16 induced efficient migratory responses in human and mouse eosinophils. Furthermore, the responses of human and mouse eosinophils to CCL16 were effectively suppressed by thioperamide, an antagonist for H3 and H4. Intravenous injection of CCL16 into mice induced a rapid mobilization of eosinophils from bone marrow to peripheral blood, which was also suppressed by thioperamide. Collectively, CCL16 is a novel functional ligand for H4 and may have a role in trafficking of eosinophils.


Histamine receptor type 4 (H4) is the most recently identified subtype of histamine receptors (9, 10). H4 is selectively expressed by cells such as eosinophils and mast cells and mediates histamine-induced chemotaxis and calcium mobilization in these types of cells (11, 12). Chemoattractant receptors including those for chemokines often interact with quite diverse molecules. For example, CCR6, the receptor for CCL20 (1), is also a receptor for \( \beta \)-defensins (13). HIV-1 Tat protein attracts monocytes via CCR2 and CCR3 (14). Histidyl-tRNA synthetase and asparaginyl-tRNA synthetase, the autoantigens in myositis, attract T cells, IL-2-activated monocytes, and immature dendritic cells via CCR5 and CCR3, respectively (15). Formyl peptide receptor-like 1, the low-affinity receptor for the bacterial chemotactic peptide fMLF, is also a receptor for lipoxin A4 and acute phase protein serum amyloid A (16, 17). Given such versatility in the chemoattractant receptors in terms of ligand-specificity, we tested a large panel of human chemokines on histamine receptors H1, H2, and H4 (18). In this study, we report that CCL16 is a functional ligand for H4 and induces an efficient migration of human and mouse eosinophils via H4.

Materials and Methods

Materials

All recombinant human chemokines were purchased from R&D Systems (Minneapolis, MN). Histamine and thioperamide were purchased from Sigma-Aldrich (St. Louis, MO). Pertussis toxin (PTX) was purchased from Invitrogen (Carlsbad, CA). A murine L1.2 pre-B cell line was kindly provided by E. Butcher (Stanford University School of Medicine, Stanford, CA).

Stable expression of histamine receptors

The coding regions of human histamine receptors H1, H2, and H4 were amplified from a cDNA library generated from PHA-stimulated PBMC by PCR. The cDNAs were cloned into a retroviral vector pMX-IRES/EGFP (19) and recombinant retroviruses were obtained. L1.2 cells were infected with the recombinant viruses and stable transfectants expressing green fluorescence were selected by FACs.
**Calcium mobilization assay**

Cells were suspended at 10^6 cells/ml in HBSS containing 1 mg/ml BSA and 10 mM HEPES (pH 7.4), and loaded with 3 μM Fura 2-AM fluorescence dye (Molecular Probes, Eugene, OR). After washing, cells were placed in F2000 fluorescence spectrophotomer (Hitachi, Tokyo, Japan) and stimulated with recombinant human chemokines or histamine. Eosinophils were further purified by negative selection using anti-CD16-bound micromagnet (Miltenyi Biotec). The eosinophils were then centrifuged at 250 × g for 20 min. Eosinophils were layered onto a discontinuous Percoll density gradient consisting of 1.06, 1.07, and 1.09 g/ml and were centrifuged at 250 × g for 20 min. Eosinophils were layered between 1.07 and 1.09 g/ml. Eosinophils were further purified by two times negative selection using anti-CD90 (Thy 1.2), anti-CD45R (B220), and anti-CD8α (Ly-2)-bound micromagnetic beads and iMagnet (BD Biosciences).

After negative selections, the purity of eosinophils was consistently >90%, and their viability was consistently >95%.

**Eosinophil mobilization in mice**

Female BALB/c mice, 6 wk old, were purchased from CLEA and kept in specific pathogen-free conditions for at least 1 wk before experiments. Mice were injected i.v. with 100 μl of PBS without or with 0.5 nmol of CCL16. At various time points, mice were given anesthesia by inhalation of diethyl ether and peripheral blood was collected. After blood collection, mice were sacrificed by cervical dislocation and femurs were obtained. The ends of femurs were removed and bone marrow cells were recovered by flushing with 1 ml of PBS. Sperms were stained with May–Grünewald and Giemsa solutions. Differential cell counts were performed for at least 500 cells/slide. All animal experiments were performed in accordance with the guideline of the Center of Animal Experiments, Kinki University School of Medicine.

**Results**

**CCL16 induces calcium mobilization via H4**

Histamine receptors H1, H2, and H4 are variably expressed by hemopoietic cells, while H3 is selectively expressed by neurons in the CNS (10–12, 18). To examine whether any chemokines act as an agonist for the histamine receptors, we performed calcium mobilization assays using murine L1.2 pre-B cells stably expressing H1, H2, or H4 (L1.2-H1, L1.2-H2, and L1.2-H4, respectively). Among 32 recombinant human chemokines tested at 100 nM (CC chemokines: CCL1, 2, 3, 4, 5, 7, 8, 11, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 24, 25, 26, 27, and 28; CXC chemokines: CXCL4, 8, 9, 10, 12, 13, and 16; CXCL1; CX3CL1), CCL16 was found to induce vigorous calcium mobilization in L1.2-H4. Fig. 1A shows that, while histamine-induced calcium mobilization via H1, H2, and H4, CCL16 dose-dependently induced calcium mobilization via H4 but not via H1 or H2. Furthermore, H4-mediated calcium mobilization induced by CCL16 was completely suppressed by PTX, supporting that H4 couples with the Gαi class of G proteins (9, 10). Exposition of histamine receptors H1, H2, and H4 to 100 nM CCL16 with an IC50 of 50 nM for H3 and H4 (10, 22), completely suppressed CCL16- and histamine-induced calcium mobilization via H4. Conversely, histamine desensitized CCL16-induced calcium mobilization via H4. Furthermore, thiopemide, an antagonist for H3 and H4 (10, 22), completely suppressed CCL16- and histamine-induced calcium mobilization via H4. Collectively, CCL16 is a novel agonist for H4.

**CCL16 induces chemotactic responses via H4**

We next examined chemotactic responses of L1.2-H1, L1.2-H2, and L1.2-H4 to CCL16. As shown in Fig. 2A, both CCL16 and histamine induced vigorous cell migration in L1.2-H4 with a typical bell-shaped dose-response curve. In contrast, neither CCL16 nor histamine induced any significant migration in L1.2-H1 or L1.2-H2. Thus, these histamine receptors are not coupled with the Gαi class of G proteins in L1.2 cells. As shown in Fig. 2B, thiopemide, an antagonist for H3 and H4 (10, 22), dose-dependently suppressed migratory responses of L1.2-H4 to 100 nM CCL16 with an IC50 of 50 μM. As shown in Fig. 2C, CCL16 and histamine were additive in inducing migration of L1.2-H4 cells. As shown in Fig. 2D, a checkerboard-type analysis confirmed that CCL16 induced mostly chemotaxis, not chemokinesis, in L1.2-H4. As shown in Fig. 2E, PTX, the inhibitor of the Gαi class of G proteins, suppressed CCL16-induced migratory responses of L1.2-H4.

**Specific binding of CCL16 to H4**

We next examined binding of CCL16 to H4. As shown in Fig. 3A, 125I-labeled CCL16 specifically bound to L1.2-H4. Scatchard analysis revealed a single class of binding sites with a Kd of 17 nM and 41,000 sites/cell (Fig. 3B). Furthermore, binding of 125I-labeled CCL16 at 10 nM was completely inhibited by unlabeled CCL16, histamine, and thiopemide, with an IC50 of 8.8, 4.1, and 57 nM, respectively (Fig. 3C).
Eosinophil responses to CCL16

H4 is selectively expressed by eosinophils and mediates eosinophil migration to histamine (10, 11). By RT-PCR, we confirmed that human and mouse eosinophils expressed H4 but not H3 (Fig. 4A).

We, therefore, examined induction of eosinophil migratory responses by CCL16. As shown in Fig. 4B (left), CCL16 dose-dependently induced a highly efficient migration of human eosinophils. In fact, many more eosinophils migrated to CCL16 at 100 or 1000 nM than to eotaxin/CCL11 at its optimal concentration of 10 nM. CCL16 also induced less efficient but still vigorous migration in mouse eosinophils (Fig. 4B, right). Furthermore, thioperamide, an antagonist for H3 and H4 (10, 22), dose-dependently suppressed migratory responses of human and mouse eosinophils to CCL16 (Fig. 4C). Fig. 4D further shows induction of calcium mobilization in mouse eosinophils by CCL16 and histamine as well as their mutual desensitization. Again, thioperamide completely suppressed CCL16-induced calcium mobilization in mouse eosinophils.

Eosinophil mobilization from mouse bone marrow by CCL16

Expression of H4 was consistently seen in the human, rat, mouse, and guinea pig bone marrow (Fig. 4A) (10, 22). Since CCL16 is selectively expressed in the liver and present in the normal plasma
Histamine is an important biogenic amine and one of the major products of mast cells. Histamine exerts diverse physiological functions via four subtypes of histamine receptors, namely, H1, H2, H3, and H4, which are all GPCRs (18). These receptors are differentially expressed in various types of cells and mediate diverse effects by coupling with different classes of G proteins (10–12, 18). For example, H1 triggers responses such as smooth muscle contraction and vascular permeability and plays an important role in allergy, while H2 mediates gastric acid secretion. H3 is expressed in the CNS and controls release of histamine and neurotransmitters by neurons (18). Notably, H1, H2, and H3 share less protein sequence identity with each other than with other biogenic amine receptor family members, suggesting that they may have evolved from different ancestral genes (Fig. 6). H4 is the most recently identified subtype of histamine receptor with an amino acid identity of ~38% to H3 (9, 10). H4 has been shown to be primarily expressed by eosinophils and responsible for histamine-induced migration of eosinophils (10, 11). In the present study, we have demonstrated that CCL16, which is a low-affinity ligand for CCR1, CCR2, CCR5, and CCR8 (6, 8), is also a novel agonist for H4. CCL16 induced vigorous calcium mobilization and chemotaxis through H4 (Figs. 1 and 2) and specifically bound to H4 with a $K_d$ of 17 nM (Fig. 3). At the molar basis, CCL16 and histamine are almost equivalent in potency as an agonist for H4 (Figs. 2 and 3). Fig. 6 shows the phylogenic relationships of the chemokine receptors (for the sake of simplicity, only four members are
shown) and the histamine receptors. Chemokine receptors are closely related to GPCRs for peptide ligands, while histamine receptors H1 and H2 are the members of GPCRs for biogenic amines. However, H3 and H4, which are most closely related to each other, are only remotely related to other biogenic amine receptors including H1 and H2 and have a closer phylogenetic relationship with GPCRs for peptide ligands than other biogenic amine receptors. At any rate, this is the first time of a demonstration of an interaction of a chemokine with a GPCR quite different from the chemokine receptors. Given the versatility of the GPCR system, however, such unexpected interactions may be more frequent than anticipated.

In consistence with the selective expression of H4 in eosinophils (Fig. 4A) (10, 11), CCL16 induced vigorous migration in human and mouse eosinophils in vitro (Fig. 4) and rapid mobilization of bone marrow eosinophils into peripheral blood in mice (Fig. 5). Furthermore, thioperamide, an antagonist for H3 and H4 (10, 22), effectively suppressed all of those responses of eosinophils to CCL16 (Figs. 4 and 5). H3 is known to be selectively expressed in the CNS and primarily by neurons (18). By using RT-PCR, we confirmed that human and mouse eosinophils express H4 but not H3 (Fig. 4A). Furthermore, human eosinophils do not express CCR1, CCR2, CCR5, or CCR8 (23). Thus, it can be safely concluded that H4 mediates eosinophil responses to CCL16. Since CCL16 is constitutively expressed by the liver parenchymal cells and present in the normal plasma at relatively high concentrations (average, 50 ng/ml) (6), CCL16 may have a role in mobilization of eosinophils from the bone marrow (Fig. 5). In addition, CCL16 is known to be induced in monocytes by IL-10 (3). Therefore, CCL16 may be produced by monocytes and tissue macrophages in Th2-dominant inflammatory conditions and attract eosinophils into inflammatory sites. Furthermore, CCL16 and histamine, whose effects on H4 are additive (Fig. 2), may cooperatively promote eosinophil migration in allergic conditions. CCL16 may also play a significant role in certain liver diseases such as drug-induced liver damages that are known to be associated with liver infiltration of eosinophils and/or blood eosinophilia (24–27). Furthermore, CCL16 may also play a role in migration of other hemopoietic cells such as dendritic cells and mast cells that also express H4 (12, 28). Even though CCL16 is effective only at relatively high concentrations, the versatility of CCL16 as a ligand for CCR1, CCR2, CCR5, CCR8, and even H4 may allow it to have a substantial role in trafficking of various types of hemopoietic cells. These possibilities remain to be seen.

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