CXCR3 chemokine receptor-induced chemotaxis in human airway epithelial cells: role of p38 MAPK and PI3K signaling pathways

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G protein-coupled receptor; mitogen-activated protein kinase; phosphatidylinositol 3-kinase; cytoskeleton

THE AIRWAY EPITHELIAL CELLS, which line the tracheobronchial tree, serve as a barrier that protects the body from the environment. Airway diseases such as asthma and chronic obstructive pulmonary disease (COPD) cause loss of the epithelial lining layer and denudation of the mucosal surface (3, 7, 18). Reconstitution of the mucosal surface in these diseases requires epithelial cell migration, spreading, and proliferation (14, 37). Work in our laboratory (20) recently demonstrated that human airway epithelial cells constitutively express a functional CXC chemokine receptor, CXCR3, the cognate receptor for the interferon-γ (IFN-γ)-inducible CXCR3 agonists IP-10/CXCL10 (IFN-γ-inducible protein of 10 kDa), Mig/CXCL9 (monokine induced by IFN-γ) and IP-10/CXCL10 (IFN-γ-inducible T-cell chemoattractant) (10, 34). Activation of CXCR3 induces chemotactic responses to I-TAC and reorganization of the actin cytoskeleton in human airway epithelial cells (20).

In this study, we examined the potency and efficacy of several CXCR3 ligands as chemoattractants for human airway epithelial cells. First, we compared the dose-response relationships of I-TAC, Mig, and IP-10 as chemoattractants in this cell type. Second, we examined the signaling pathways mediating the resultant chemotactic response. Specifically, we assessed the role of a pertussis toxin (PTX)-blockable G protein, intracellular calcium, the MAPks (ERK, p38, and JNK), and the phosphatidylinositol 3-kinase (PI3K) as signaling pathway components in the chemotactic responses to CXCR3 activation.

Our data indicate that I-TAC, Mig, and IP-10 induce chemotaxis in dose-dependent manner with similar potency but with a rank order of efficacy of I-TAC = IP-10 > Mig. The efficacy of the CXCR3 ligands is ~50% that of epidermal growth factor (EGF), a highly potent chemoattractant for airway epithelial cells (9, 27, 37). Finally, CXCR3-induced chemotaxis is G protein dependent and requires signaling by the p38 and PI3K pathways but is not dependent on those activated by changes in intracellular calcium (Ca2+) or ERK.

MATERIALS AND METHODS

Reagents and antibodies. Anti-human CXCR3 mouse IgG1 monoclonal antibody (clone 498011.11) was purchased from R&D Systems (Minneapolis, MN). Phospho- and total MAPK (anti-p38, anti-ERK1/2, and anti-JNK) and PI3K (anti-Akt) antibodies were obtained from Cell Signaling (Beverly, MA). The MEK1/2 inhibitor U0126, p38 inhibitor SB20358, and PI3K inhibitor wortmannin were purchased from Calbiochem (San Diego, CA). Fura-2 AM was obtained from Molecular Probes (Eugene, OR). Fibronectin, collagen VI, fatty acid-free BSA, bradykinin (BK), EGF, and PTX were obtained from Sigma-Aldrich (St. Louis, MO). Human recombinant I-TAC, IP-10, and Mig were purchased from R&D Systems.

Cell culture. Normal human bronchial epithelial cells (NHBEc), obtained from Clonetics-BioWhittaker (Walkersville, MD), were grown on collagen type VI-coated plates (25 μg/ml) in serum-free defined growth medium (BEGM; Clonetics) in 5% CO2 at 37°C. Medium was changed every 2–3 days until cells were 80–100% confluent. Cells from passage 2 or 3 were used as previously described (20). Transformed human airway epithelial cells (16-HBE cell line) were cultured in DMEM plus 4 mM glutamine and 10% FBS (20).

Chemotaxis assay. Chemotaxis in NHBEc and 16-HBE cells was assessed using a commercially available 96-well modified Boyden chamber chemotaxis system (Chemotx; Neuroprobe, Gaithersburg, MD) as previously described (9, 27). In this system, the upper surface

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of each well was separated from a lower chamber containing the chemoattractant ligands by a polycarbonate membrane. A circular area of the membrane in the region that covers each lower well was enclosed by a hydrophobic mask to retain the cell suspension within this area. To facilitate epithelial cell movement, we coated the membrane (pore size 8 μm for 16-HBE and 12 μm for NHBE) with human fibronectin (10 μg/ml) as previously described (9, 27). The system was prepared by loading the bottom wells with 30-μl aliquots of chemotaxis medium (serum-free RPMI, 0.1% BSA), with or without Mig, IP-10, or I-TAC in a range of concentrations. The membrane was then placed over the lower chamber, and a suspension of epithelial cells (0.5–1×10^6 cells in 50 μl) was delivered onto each of the hydrophobically limited regions of the upper surface of the membrane. Chemotaxis was then allowed to proceed at 37°C in 5% CO2 for 6 h. After this period, the membrane was removed and its topside was carefully wiped to eliminate nonmigrated cells. The membrane was then fixed with methanol and stained with Hema 3 (Fisher Scientific). Chemotaxis was assessed by counting the number of cells that entered a pore or passed through to the underside of the membrane. Migrated cells in the entire cross-sectional area of each well were counted under a microscope (×40 magnification). The number of migrated cells was compared in CXCR3 ligand-containing wells and in wells containing buffer only (i.e., serum-free RPMI medium, 0.1% BSA), which served as a negative control. EGF (1 ng/ml), a potent chemoattractant for epithelial cells, was used as a positive control (9, 27, 37). Triplicate wells were used for each condition, and results were averaged.

To examine the effect of CXCR3 blocking antibody, PTX, or inhibitors of signaling pathways, we incubated cells with the appropriate agent for 30 min before and during the chemotaxis assay. Neither the anti-CXCR3 antibody (10 μg/ml; clone 49801.11, R&D Systems) nor PTX (1 μg/ml) affected epithelial cell viability over a 6-h incubation period (n = 2 experiments).

The nonspecific effects of the MEK1/2 inhibitor U0126, the p38 inhibitor SB 20358, and the PI3K inhibitor wortmannin on epithelial cell chemotaxis were assessed using vehicle-treated medium (n = 4 experiments). U0126 had no effect on chemotaxis in the concentration range used (0.1 to 10 μM). SB 20358 and wortmannin inhibited chemotaxis slightly (~25–35%) at the highest inhibitor concentrations used (3 μM and 100 nM, respectively).

**Calcium mobilization.** Intracellular Ca2+ mobilization ([Ca2+]i) was measured as described previously (6). Briefly, NHBE were grown to 50–60% confluence on 12-mm coverslips (Fisherbrand, PA) and loaded with the fluorescent Ca2+ indicator fura-2 AM (5 μM). Cells were incubated in Hanks’ buffered saline solution (HBSS) supplemented with fura-2 AM for 45 min and in HBSS alone for a further 15–60 min to allow deestereification of the dye. Coverslips were placed in a custom-designed bath and transferred to the stage of an inverted epifluorescence microscope equipped with a C&L Instruments fluorimeter system. Fifty microliters of a chemokine, BK, ATP, or HBSS were added to the cells, and fura-2 fluorescence (excitation wavelengths, 340 and 380 nm; emission wavelength, 520 nm) of single cells was acquired at a frequency of 1 Hz. The excitation ratio (340 nm/380 nm) of the fluorescence signals obtained was converted to Ca2+ concentration according to the method of Grynkiewicz et al. (17).

**Western blot analysis.** Cells were grown in six-well plates in full medium until subconfluent and then placed in depleted-medium 24 h before experiments (i.e., BEGM without EGF and bovine pituitary extract for NHBE, or serum-free DMEM for 16-HBE cells). Cells were then treated with 100 ng/ml I-TAC for 1, 5, 10, or 30 min,
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RESULTS

CXCR3-induced airway epithelial cell chemotaxis. I-TAC stimulated chemotaxis of NHBEC and 16-HBE cells in a concentration-dependent manner ($P < 0.03$ for both cell types by ANOVA; $n = 8–12$ experiments) (Fig. 1, A and B). The maximum response to I-TAC equaled 349 ± 88% of the control response (109 ± 27 cells/well in medium alone) in NHBEC and 210 ± 32% of the control response (173 ± 52 cells/well in medium alone) in 16-HBE cells.

The dose-response relationship of the several CXCR3 ligands was compared in both NHBEC and 16-HBE cells (Fig. 1, A and B). All three ligands induced chemotaxis ($P < 0.05$ by ANOVA; $n = 8–12$) with similar potency. However, the rank order of efficacy differed, with I-TAC = IP-10 > Mig.

In 16-HBE cells, CXCR3 blocking antibody (10 $\mu$g/ml) eliminated I-TAC-induced chemotaxis (185 ± 52 and 91 ± 4% of control for I-TAC alone and I-TAC plus anti-CXCR3 antibody, respectively; $n = 3$) (Fig. 2A). These data indicate that cell migration was receptor mediated.

In both NHBEC and 16-HBE cells, chemotactic responses to EGF (1 ng/ml) were significantly greater (789 ± 163% of control for NHBEC, $n = 5$; 797 ± 154% of control for 16-HBE cells, $n = 7$) than maximal responses to I-TAC ($P < 0.05$ for both comparisons with I-TAC).

I-TAC-mediated chemotaxis is blocked by pertussis toxin. I-TAC-induced (10 ng/ml) chemotaxis in NHBEC was completely blocked by PTX (1$\mu$g/ml) pretreatment (101 ± 54% of control; $n = 4$) (Fig. 2B). These data indicate that the CXCR3-mediated chemotaxis involves a PTX-blockable GTP-binding protein.

I-TAC, IP-10, and Mig do not alter intracellular calcium. Neither I-TAC, IP-10, nor Mig induced a change in $[Ca^{2+}]_i$ in NHBEC (Fig. 3; $n = 3$). In contrast, BK or ATP markedly increased $[Ca^{2+}]_i$ (Fig. 3). Similar results were obtained in 16-HBE cells (data not shown).

I-TAC activates ERK1/2 and p38-MAPK but not JNK. I-TAC induced rapid phosphorylation of ERK1/2 and p38 in NHBEC (Fig. 4; $n = 6$). Phospho-ERK and phospho-p38 peaked at 5–10 min and then declined toward baseline levels by 30 min. In contrast, I-TAC did not induce SAPK/JNK phosphorylation in NHBEC (data not shown). A similar effect of I-TAC was observed in 16-HBE cells (data not shown).

I-TAC activates PI3K/Akt pathway. In NHBEC, I-TAC phosphorylated Akt, an activator of the PI3K pathway (24). Phospho-Akt (serine 473) peaked at 5 min and then declined to baseline levels by 30 min (Fig. 4).

p38 but not MEK/ERK inhibition affects I-TAC-stimulated chemotaxis. The MEK inhibitor U0126 (0.01–10 $\mu$M) (15) had no significant effect on I-TAC (10 ng/ml)-induced chemotaxis in NHBEC (Fig. 5A). In contrast, the p38 inhibitor SB 20358 (36) completely abrogated I-TAC-induced chemotaxis in a concentration-dependent manner (0.03–3 $\mu$M) ($P < 0.05$ by ANOVA) (Fig. 5B).

Statistical analysis. Results are given as means ± SE. The statistical significance of differences in group mean data was assessed using one-way and two-way ANOVA and Student’s $t$-tests. The level of significance was set at $P < 0.05$. Curve fitting of chemotactic responses was performed using linear regression with a second-order polynomial equation.

Fig. 3. CXCR3 agonists do not induce a $Ca^{2+}$ flux in NHBEC. NHBEC were loaded with fura-2 AM, and intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) was recorded over time. Cells were stimulated with 1 $\mu$M I-TAC (A and B), IP-10 (C), or Mig (D). To ensure responsiveness, we stimulated cells several minutes later with bradykinin (BK; 10 $\mu$M) (A) or ATP (10 $\mu$M) (B–D), which served as positive controls. Arrows indicate the points at which the stimulus was given. Results shown are from 1 experiment representative of 3.

Fig. 4. I-TAC induces ERK, p38, and phosphatidylinositol 3-kinase (PI3K) phosphorylation in NHBEC. Western blots are shown for total and phospho-ERK, total and phospho-p38, and total and phospho-Akt. Cells were treated with I-TAC for 1–30 min. Results are from 1 experiment representative of 4.
PI3K inhibition affects I-TAC-stimulated chemotaxis. Wortmannin (1–100 nM), a PI3K inhibitor (1), inhibited NHBEC chemotaxis in a concentration-dependent fashion ($P = 0.05$ by ANOVA) (Fig. 5C). Inhibition was not complete, however (~75%).

**DISCUSSION**

Expression of the chemokine receptor CXCR3 has previously been described in a variety of immune (Th1 lymphocytes, natural killer cells, hematopoietic progenitor cells) and structural cells (endothelial cells, microglia, hepatic and renal pericytes, and other) (5, 11, 19, 22, 28, 33). In cells that express CXCR3, activation of this receptor regulates cell movement (5, 8, 10, 22, 33, 34). However, the effects of CXCR3 activation on cell movement are cell type dependent and include both stimulation (e.g., lymphocytes, renal mesangial cells) (5, 33) and inhibition of cell migration (e.g., microvascular endothelial cells) (22). Different chemotactic responses to CXCR3 activation by the same ligands appear to be explained by different signaling pathways. For example, positive chemotactic responses induced by CXCR3 activation are mediated by activation of phospholipase C (PLC) in Th1 lymphocytes and by both PI3K and the ERK MAPK in hepatic stellate cells (5, 33). In contrast, negative chemotactic responses are mediated by G$_i$-independent, cAMP-dependent pathways in microvascular endothelial cells (22).

Work in our laboratory (20) recently demonstrated that human airway epithelial cells constitutively express the chemokine receptor CXCR3. The present study indicates that both normal airway epithelial cells (NHBEC) and the 16-HBE cell line demonstrate robust chemotactic responses to activators of CXCR3. In fact, in normal airway epithelial cells, the efficacy of the CXCR3 ligand I-TAC was approximately one-half the response to EGF, a highly potent chemotactic agent for airway epithelial cells (9, 27, 37).

In NHBEC, the several CXCR3 ligands demonstrated similar potency but different maximal responses (efficacy). I-TAC and IP-10 were equally efficacious and approximately two times more efficacious than Mig.

Initial experiments designed to elucidate the signaling pathways involved in CXCR3-induced chemotaxis examined possible involvement of a PTX-blockable, GTP-binding protein such as G$_{o/1}$. Pretreatment of human airway endothelial cells with PTX completely eliminated chemotactic responses to I-TAC, supporting the role of G$_{o/1}$ in this process (25, 26).

In most but not all cells, activation of chemokine receptors also induces an increase in cytosolic calcium, [Ca$^{2+}$]. However, in our study, the several CXCR3 ligands did not change [Ca$^{2+}$]. These results differ from studies in T cells and renal mesangial cells in which CXCR3 agonists increased [Ca$^{2+}$], (10, 28). Of interest, the absence of a [Ca$^{2+}$] response has been reported with other chemokine receptors and their ligands. For example, RANTES/CCL5 induces CCR5-mediated T-lymphocyte chemotaxis without changing [Ca$^{2+}$] (35). Furthermore, IL-8/CXCL2 induces CXCR2-mediated neutrophil migration without an increase in [Ca$^{2+}$], in cells from PLC-$\beta_2$/PLC-$\beta_3$ knockout mice (23). Together, our data and those of others (23, 35) suggest that changes in [Ca$^{2+}$], are not always necessary for directional sensing and cell shape change.

Our data indicate that I-TAC induces phosphorylation of p38, ERK1/2, and PI3K, strongly suggesting that these pathways are activated by CXCR3 in HAEC. The role of these several pathways in mediating chemotaxis was assessed using specific inhibitors.

**Fig. 5.** p38 MAPK and PI3K inhibitors block I-TAC-induced chemotaxis in NHBEC. The inhibitory effects of U0126, an ERK1/2 inhibitor (A), SB 20358, a p38 inhibitor (B), or wortmannin, a PI3K inhibitor (C), on I-TAC-induced (10 ng/ml) chemotaxis are shown. Values are means ± SE of 4–8 experiments.

**Fig. 6.** A model of CXCR3 signaling in human airway epithelial cells. ERK1/2 and p38 MAPK and PI3K/Akt are all activated by I-TAC. However, only p38 and PI3K/Akt pathways appear to regulate chemotaxis. The p38 pathway is viewed as being essential to chemotaxis in this cell type and can be activated by both PI3K-dependent and -independent pathways. Although the PI3K-independent pathway components are unclear, the small GTPases, Cdc42, and Rho (not shown for clarity) appear to be prime candidates (2, 12, 21, 39).
The role of p38 signaling pathways in CXCR3-induced chemotaxis was assessed using the selective p38α/β isotype inhibitor SB 20358 (36). SB 20358 dose-dependently and completely blocked I-TAC-induced chemotaxis. These data indicate that the p38α/β pathways are necessary for CXCR3-induced airway epithelial cell movement.

To our knowledge, the role of p38 MAPK in CXCR3-induced chemotaxis has not been studied previously in any cell type. However, a role for p38 has been demonstrated in chemotaxis induced by EGF in human bronchial epithelial cells (the BEAS-2B line) (9) and by hepatocyte growth factor in corneal epithelial cells (32). These latter studies, along with our own, support a role for p38 in epithelial cell migration induced by both G protein-coupled receptors (chemokine receptors) and tyrosine kinase receptors (EGF receptor). In fact, p38 may act by regulating the activity of proteins (heat shock protein-27) that control F-actin polymerization, an essential step in generating lamellipodia, which are required for cell movement (16).

In this study, the role of PI3K was assessed using the selective PI3K inhibitor wortmannin (1). Wortmannin dose-dependently but incompletely inhibited I-TAC-induced chemotaxis with maximum inhibition (∼75%) at 1 nM. At this concentration, wortmannin selectively inhibits PI3Kγ without affecting PI3Kα, β, or δ. Of interest, our results on PI3K inhibition are in agreement with results in hepatic stellate cells in which IP-10-induced chemotaxis was only partially inhibited (∼60%) by wortmannin at 100 nM (5). In contrast, I-TAC-induced chemotaxis in T cells was not blocked by wortmannin at concentrations specific for PI3Kγ (<100 nM), strongly suggesting that this kinase does not play a role in CXCR3-induced T-cell movement (33).

Complete inhibition of I-TAC-induced chemotaxis by SB 20358 coupled with the wortmannin results indicating only partial inhibition of chemotaxis suggests that PI3K is one of several mechanisms that regulate p38 (30) and that p38 can be activated in PI3K-independent fashion (2, 12, 21, 39).

The ERK1/2 pathway has been shown to mediate CXCR3-induced chemotaxis in some cell types, e.g., hepatic stellate cells (5). However, inhibition of ERK1/2 in the present study with the use of U0126 had no effect on I-TAC-induced chemotaxis, suggesting that this pathway is not involved in CXCR3-induced chemotaxis in HAEC. ERK1/2 activation may nonetheless be important in other CXCR3-mediated responses of HAEC, e.g., cell proliferation.

To our knowledge, no prior studies have examined the effects of CXCR3 activation on JNK. I-TAC treatment did not phosphorylate JNK MAPK, suggesting that this pathway does not mediate CXCR3-induced chemotaxis in HAEC. However, JNK activation in HAEC by other CXC chemokine receptors, i.e., CXCR4, has been described (13).

In summary, our data indicate that in HAEC, CXCR3-induced chemotaxis is G protein mediated and is critically dependent on signaling by the p38 and PI3K/Akt pathways. CXCR3 agonists do not alter [Ca²⁺], indicating that [Ca²⁺] elevation is not necessary for chemotaxis in this cell type. In contrast, the complete elimination of chemotaxis by inhibition of p38 suggests that the p38 pathway is necessary for chemotaxis induced by CXCR3. Partial inhibition of chemotaxis by inhibiting PI3K suggests that the PI3K pathway is not essential for chemotaxis but, rather, acts as one of several mechanisms by which p38 is regulated. A model depicting our thinking is shown in Fig. 6.

HAEC migration is believed to play a key role in airway morphogenesis and wound repair of denuded airway mucosal surfaces such as occurs in subjects with obstructive airway diseases such as COPD and asthma (14, 18, 27, 37, 38). Of interest, HAEC produce the CXCR3 ligands, i.e., IP-10, Mig, and I-TAC (31). Moreover, epithelial cells in the small intrapulmonary airways demonstrate heightened expression of IP-10 in COPD (29). Therefore, our findings suggest the possibility that an autoregulatory loop involving the IFN-γ-inducible CXC chemokines I-TAC, Mig, and IP-10 may modulate several aspects of epithelial function vital for airway mucosal repair in subjects with lung disease by activating their cognate receptor, CXCR3.

GRANTS

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REFERENCES

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