CXCR3 surface expression in human airway epithelial cells: cell cycle dependence and effect on cell proliferation


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CXCR3, the sole receptor for the interferon-γ-inducible CXC chemokine I-TAC (CXCL11), expresses chemokine receptors, such as CXCR3, are also expressed by structural cells (4, 6, 17–19, 25, 32). In structural cells (e.g., endothelial cells, renal mesangial cells, trophoblastic cells, keratinocytes), CXCR3 induces a number of pleiotropic responses important for organogenesis (25, 28), angiostasis (3, 29), tissue repair and remodeling (9, 28), ion transport (2), and tumor metastasis (17). For example, in endothelial cells, activation of CXCR3 by its cognate ligands induces apoptosis and angiostasis (19, 29). In contrast, CXCR3 activation in renal mesangial cells and T cells induces cell proliferation (19, 29, 35). Differing cellular responses to CXCR3 activation appear to be explained by expression of at least two receptor splice variants termed CXCR3-A and -B (19). These two proteins signal through different pathways (19).

Our group (18) has recently demonstrated that human airway epithelial cells constitutively express both CXCR3 splice variants (i.e., CXCR3-A and -B). In fact, CXCR3 mRNA levels are about one-third of the β-actin value, and ~80,000 receptor binding sites are expressed per cell. Activation of CXCR3 induces robust airway epithelial cell chemotaxis, which is mediated by both MAPK and phosphatidylinositol 3-kinase signaling pathways (33a). Of interest, because airway epithelial cells also release the chemokines IP-10 (MIG), and I-TAC (33), there is the possibility of autocrine and/or paracrine regulation of cell movement.

In other cell types (e.g., T cells, renal mesangial cells), CXCR3-A and -B expression varies by several orders of magnitude (19). Consequently, we hypothesized that the relative expression of CXCR3-A and -B mRNA differs in normal human bronchial epithelial cells (NHBECS). Second, because previous studies in some structural cells (endothelial cells) indicate that CXCR3 surface expression may be cell cycle dependent (29), we hypothesized that CXCR3 expression on the surface of NHBECS would vary with the cell cycle. Third, because CXCR3 activation regulates cell proliferation in other cell types (6, 29), we hypothesized that activation of CXCR3 by its ligand, I-TAC, would affect NHBECS proliferation.

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In the present study, performed in human airway epithelial cells, we quantitated mRNA expression of the CXCR3-A and -B splice variants by real-time RT-PCR. We also examined the relationship of CXCR3 expression on the cell surface to cell cycle. Cell cycle was determined by flow cytometry with the use of the DNA-binding dye 7-AAD and by fluorescence microscopy, which used calcin B1 expression and mitotic spindle formation as cell cycle markers. Finally, we examined the effect of CXCR3 activation by its ligand, I-TAC, on cell growth and thymidine incorporation.

Our experiments indicate that human airway epithelial cells express mRNA for both CXCR3-A and -B splice variants, with CXCR3-B predominating. Furthermore, CXCR3 is present mostly on the cell surface in the late S + G2/M phases of the cell cycle. Finally, activation of CXCR3 by I-TAC induces DNA synthesis and cell proliferation.

METHODS

Cell isolation and culture. NHBEC, passages 1 and 2 (Cambrex, Walkersville, MD), were cultured in collagen-coated 6-well plates at 100,000 cells/well or in 12-well plates at 25,000 cells/well in serum-free, basal growth medium (BEBM; Cambrex) supplemented with the following components to form complete medium (BEGM): epidermal growth factor (EGF), bovine pituitary extract (BPE), insulin, triiodothyronine, transferrin, hydrocortisone, retinoic acid, epinephrine, and GA-1000 antibiotic. Cells were grown until 80–100% confluent. In contrast, transformed human airway epithelial cells (the 16-HBE cell line) were cultured in DMEM plus 4 mM glutamine and 10% FBS until 80–100% confluent.

Real-time RT-PCR. Total RNA was isolated as previously described (18) by guanidinium-phenol extraction (TRI reagent; Sigma-Aldrich, St. Louis, MO) from NHBEC and 16-HBE cells. RNA purity and yield were determined by absorbance at 260 and 280 nm (Beckman DU640 spectrophotometer). This protocol routinely yields 260 nm-to-280 nm optical density ratios of 1.8–2.0. The RT reaction was performed using Taqman reverse transcription reagents (Applied Biosystems, Foster City, CA). Each 100-μl reaction consisted of 2 μl total RNA, 1× RT buffer, 5.5 mM MgCl2, 2.5 μM random hexamer, 500 μM dNTP, 40 U RNase inhibitor, and 125 U MultiScribe reverse transcriptase. Temperature/time parameters for the RT reaction were 25°C for 10 min followed by 48°C for 30 min. We performed TaqMan PCR using an Applied Biosystems model 7500 real-time PCR system with computer interface. Each 20-μl reaction contained 0.9 μM forward and reverse primers, 0.25 μM probe, and 1× Universal PCR Master Mix (Applied Biosystems), a proprietary preformulated master mix of enzymes, reaction buffer, and dNTPs. Temperature/time parameters for 2-step PCR were as follows: 1) for denaturation, 95°C for 15 s and 2) for annealing/elongation, 60°C for 60 s, 40–45 cycles total. Along with CXCR3-A and CXCR3-B, we measured β-actin expression for normalization purposes using a proprietary FAM-labeled specific probe/primer set (Applied Biosystems). All samples were run in duplicate along with negative RT controls and H2O blanks. To maximize the precision and sensitivity of the PCR data, the threshold was manually centered to the exponential phase of amplification during data analyses. The same setting was used for all experiments.

The following primers and probes were developed. For the two human CXCR3 splice variants, CXCR3-A and -B: CXCR3-A (accession no. NM_001504.1): FAM probe is 5′-CATGTTGTCGAGTGGTGCACCACC-3′, forward primer is 5′-CCCAGGCACGCA- GAGCACCC-3′, and reverse primer is 5′-TCATTAGGAGAAGTGGTTCCT-CCA-3′; for CXCR3-B (accession no. AF469635): FAM probe is 5′-CCGTTCCGCCTCCATACGG-3′, forward primer is 5′-TGCCAGGCTTTACACGGC-3′, and reverse primer is 5′-TGGGCTATTTGACCTTG-3′.

The efficiency of the primer pairs was assessed, and the absolute amount of CXCR3-A and -B cDNA in the PCR phase of the reaction was determined by constructing standard curves relating PCR crossing points to known starting concentrations of CXCR3-A and -B cDNA (Fig. 1, insets). These standard curves were developed using plasmids encoding CXCR3-A or CXCR3-B cDNA sequences, respectively. pCMV containing CXCR3-A (i.e., pCMV-CXCR3-A) was purchased from American Type Culture Collection and cloned. pTarget containing CXCR3-B cDNA (i.e., pTarget-CXCR3-B) was obtained from Dr. Paola Romagnani (Univ. Florence, Florence, Italy) as a kind gift, cloned, and sequenced. The CXCR3-A and -B primer sets

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displayed similar amplification efficiencies and selective specificities when tested on pCMV-CXCR3-A and pTarget-CXCR3-B. Therefore, CXCR3-A and -B were expressed as picograms cDNA per 100 ng total RNA.

**Cell proliferation protocol.** NHBEC were plated at 25,000 cells in 1 ml BEGM on 12-well plates. After 24 h, cells were washed and transferred to a “depleted” medium (DBEGM), lacking the EGF and BPE supplements to induce growth arrest (24). After an additional 48 h, cells were then treated with fresh DBEGM containing 0, 1, 10, 100, or 1,000 ng/ml I-TAC in PBS plus 0.1% BSA for 48 h. The vehicle concentration was identical for all treated wells. As a positive control to stimulate cell growth, some wells were treated with BEGM (i.e., complete medium containing both EGF and BPE). In each experiment, at least three wells were used for each condition.

**Cell proliferation assay.** Cells were harvested at 0 or 48 h after I-TAC treatment. Cells were washed with PBS and then lysed by freezing at −70°C for at least 30 min. Cell number was determined from measurement of DNA concentration (CyQuant assay, Molecular Probes, Eugene, OR) in a fluorimeter (Victor 2, model 1420, Perkin-Elmer, Downs Grove, IL) at 555 nm wavelength. The CyQuant assay was scaled up from 200 µl to 1 ml reaction volume to allow use with a 12-well plate cell culture. Standard curves were generated for each cell lot to relate DNA fluorescence to cell number.

**Thymidine incorporation.** Twenty-four hours before harvest, I-TAC-stimulated NHBEC were pulsed with [3H]thymidine (2 µCi/well). At harvest, cells were washed with PBS and then collected by scraping and repipetting through a 27-gauge needle in 250 µl 0.5% SDS. Each well was then washed with an equal volume of 0.1% SDS, which was combined with the original lysate in a polyethylene tube. Chilled 20% TCA (500 µl) was then added to each tube, and the tubes were kept on ice for 30 min. The lysates were then transferred by vacuum filtration to a manifold fitted with GFC filter disks (model M-48R Cell Harvester, Brandel, Gaithersburg, MD). The tubes were rinsed with 2× 500 µl 5% TCA, and the filters were washed with 4× 5 ml 5% TCA followed by 10 ml of chilled methanol. The filters were then removed from the manifold, air-dried in the hood, and liquid scintillation counted with 5 ml of scintillation cocktail.

**Flow cytometry.** Cells at 80–100% confluence were washed in PBS and then harvested with Versene (Sigma-Aldrich) (15–20 min at 37°C). To label CXCR3 expressed on the cell surface, dissociated cells were centrifuged and resuspended in PBS plus 0.5% BSA, pH 7.4 (FACS buffer), and Fc III/II receptors were blocked with anti-human CD16/CD32 (0.5 µg/ml) (BD Biosciences, San Diego, CA). The samples were then labeled for 30 min at 4°C with a fluorochrome-conjugated antibody against human CXCR3 (FITC-conjugated mouse IgG, 100 001.111) (15 µg/ml) (R&D Systems, Minneapolis, MN). In some experiments, a mouse anti-human CXCR3-B IgG1 (5 µg/ml) (a kind gift of Dr. P. Romagnani) was also used in conjunction with a Cy2-conjugated donkey, anti-mouse secondary antibody (Jackson ImmunoResearch, West Grove, PA). In all cases, naive mouse IgG1 MAb was used as matching isotype controls in place of the primary antibodies.

To label CXCR3 intracellularly as well as on the surface, cells were permeabilized before addition of the anti-CXCR3 antibody. Cells were fixed and permeabilized in a proprietary mixture of saponin and 4% paraformaldehyde (Cytofix/Cytoperm buffer, BD Biosciences) for 30 min at 4°C. The cells were then washed in 10× dilute Cytofix/Cytoperm buffer, followed by FACS buffer (BD Biosciences) (15). To relate surface expression of CXCR3 to phases of the cell cycle, CXCR3-labeled cell samples were washed in FACS buffer and then fixed and permeabilized as described above. Ten minutes before the FACS assay, cell samples were treated with 7-AAD (50 µg/ml; 15 µl/10⁶ cells) to label DNA nucleotides for cell cycle analysis. Fluorescence readings were acquired using a FACS Calibur flow cytometer (Becton-Dickenson, San Jose, CA). Quantitation of cell numbers in G₁ and S + G₂/M phases was performed using ModFit LT software (Verity House, Topsham, ME) (19). In some experiments, Summit software (Dako Cytomation, Fort Collins, CO) was used and yielded identical results.

**Immunofluorescence microscopy.** Immunocytochemistry for CXCR3 was performed on NHBEC (passage 2) and 16-HBE cells seeded onto chamber slides (Lab-tek, Naple/Nunc, Naperville, IL). The latter were serum-starved for 24 h before study. Surface expression of CXCR3 was determined in nonpermeabilized cells fixed in 4% paraformaldehyde. A mouse anti-CXCR3, clone 49801.111 (R&D Systems) (15 µg/ml) was used in conjunction with either Cy2- or Cy3-conjugated donkey, anti-mouse secondary antibodies (Jackson ImmunoResearch). To characterize intracellular and surface CXCR3 expression, cells were fixed in 4% paraformaldehyde and then permeabilized with 0.1% Triton X-100 in PBS and blocked with 10% donkey serum in PBS, before addition of antibody. To further examine the correlation between CXCR3 surface expression and cells in mitosis, 16-HBE cells were double labeled with anti-CXCR3 and antibodies for two markers of mitosis, either cyclin B₁, which is temporally confined to the G₂/M phase (27, 34), or tubulin, the main structural component of the mitotic spindle. Phcoerythrin-conjugated mouse anti-human cyclin B₁ (20 µl/× 10⁵ cells) (BD Biosciences) was used to identify the G₂/M phase of the cell cycle. A FITC-conjugated mouse anti-human α-tubulin (7.5 µg/ml) (Sigma-Aldrich) was employed to label mitotic spindles. For each marker, cells were first surface labeled with anti-CXCR3 and then permeabilized as described above, before addition of either anti-α-tubulin or anti-cyclin B₁.

To check for nonspecific staining, matching isotype controls were used in place of the primary antibodies, in all experiments. Nuclei were stained with 4',6'-diamidino-2-phenylindole dilactate or with propidium iodide. An FITC-conjugated, mouse IgG1 anti-cytokeratin (20 µl/× 10⁵ cells) (clone J1B3; Beckman-Coulter, Brea, CA) was also used to validate the epithelial identity of successive cell lots. A fluorescence microscope (Eclipse E800; Nikon, Tokyo, Japan) with digital video interface (DEI-750 CE Digital Output; Optronics, Goleta, CA) was used to image cells. Cells were also visualized by laser scanning confocal microscopy (Fluoview microscope system; Olympus America, Melville, NY). All images were processed using Adobe Photoshop CS (Adobe Systems, San Jose, CA).

**Assay for P38, ERK1/2, and JNK phosphorylation in cultured NHBEC.** NHBEC were cultured as described above in 35-mm dishes to 90% confluence and then transferred to basal growth medium (BEBM) for 24 h. Cells were then stimulated with 100 ng/ml I-TAC for 0, 1, 5, and 10 min and 1, 8, 18, and 24 h. At the specified time points, cells were washed twice with ice-cold PBS and lysed in 1% SDS, 6 mM Na₂VO₄, 6 mM NaF, 2 mM PMSF, leupeptin (4 µg/ml), aprotinin (4 µg/ml), and pepstatin (4 µg/ml), by scraping and repipetting through a 27-gauge needle. Protein concentrations were determined using the DC protein assay kit (Bio-Rad, Hercules, CA), and samples were stored at −80°C until immunoblotting (see below).

**Western blotting.** NHBEC lysates (100 µg) were electrophoresed by SDS-PAGE on a 10% acrylamide gel and immunoblotted onto nitrocellulose membranes, as previously described (5). The nitrocellulose membranes were washed, blocked with 5% nonfat milk in 1× TBS and 0.1% Tween 20 for 1 h while it was shaken at 25°C, and then incubated overnight with rabbit polyclonal antibodies for phospho-p38,-ERK1/2, and -JNK MAPK proteins (1:1,000 dilution), as directed by the manufacturer (Cell Signaling Technology, Beverly, MA). Membranes were washed and then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG and visualized by chemiluminescence (ECL, Amersham, Piscataway, NJ) on X-ray film. The blots were subsequently stripped and reacted with rabbit polyclonal antibodies for total p38, ERK1, and JNK MAPK proteins (1:1,000 dilution) (Cell Signaling Technology) and visualized as described above.

**Statistical analysis.** Group data are expressed as means ± SE. Statistical significance of differences in group mean data was assessed using one-way and rank-order ANOVA, X², and Student's t-tests, with a P value <0.05 considered statistically significant.
RESULTS

CXCR3 mRNA expression assessed by real-time RT-PCR. Expression of the CXCR3 mRNA splice variants, CXCR3-A and -B, was quantitated by real-time RT-PCR. NHBEC and 16-HBE cells expressed both CXCR3-A and -B (Fig. 1) \( n = 3 \) in agreement with our previous findings (18). However, expression of CXCR3-B mRNA considerably exceeded (i.e., 6 to 39-fold) that of CXCR3-A in both NHBEC and 16-HBE cells (Table 1).

Mean cDNA concentrations for CXCR3-A and -B variants in NHBEC and 16-HBE cells along with variant ratios for each cell type are shown in Table 1.

CXCR3 expression assessed by flow cytometry. CXCR3 expression on the cell surface was measured in nonpermeabilized cells. On the other hand, the total CXCR3 receptor pool, that is, those receptors located intracellularly as well as those on the surface, was measured under permeabilizing conditions. CXCR3 was expressed on the cell surface in 37 ± 11% of NHBEC and 29 ± 5% of 16-HBE cells \( n = 4 \) or 5. In contrast, under permeabilizing conditions, most NHBEC and 16-HBE cells (80 ± 15% and 91 ± 5%, respectively) stained positively for CXCR3 \( n = 4 \) or 5. Representative histograms are shown in Fig. 2.

Similar results were obtained with the anti-CXCR3-B antibody. A subset of NHBEC (23 ± 5%) and 16-HBE (22 ± 5%) cells showed CXCR3-B surface expression, whereas a majority of NHBEC (72 ± 10%) and 16-HBE (76 ± 16%) cells were positive for CXCR3-B under permeabilizing conditions \( n = 3 \). These results suggest that CXCR3 and the CXCR3-B variant are present in virtually all epithelial cells but are expressed on the cell surface in only a subset.

To determine cell cycle dependence of CXCR3 surface expression, we used a DNA stain, 7-AAD, to characterize the cell cycle. 7-AAD staining revealed a heterogeneous cell population whose DNA content was compatible with the G0/G1, S, and G2/M phases of the cell cycle in 16-HBE (Fig. 3A) and NHBEC (Fig. 3B). Of interest, the cells staining positively for CXCR3 on their surface were almost exclusively in the late S + G2/M phases of the cell cycle. In contrast, cells that did not stain for CXCR3 on the surface were largely in G0/G1, with lesser numbers in the S phase. Group mean data \( n = 8 \) – 10 are shown in Fig. 3C. Significantly more CXCR3-positive cells were in S + G2/M than in G1 for both 16-HBE and NHBEC \( P < 0.001 \) by \( \chi^2 \). In contrast, most of the CXCR3-negative cells were in G1 \( P < 0.001 \) by \( \chi^2 \).

CXCR3 expression assessed by immunocytochemistry. To confirm that CXCR3 expression on the cell surface was related

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<th>16-HBE</th>
<th>NHBEC</th>
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<tr>
<td>Crossing Point, cycle no.</td>
<td></td>
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<tr>
<td>CXCR3-B</td>
<td>27.6±0.1</td>
<td>28.6±0.1</td>
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<tr>
<td>cDNA, pg/100 ng RNA</td>
<td>6.4±0.2</td>
<td>3.9±0.3</td>
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<tr>
<td>CXCR3-A</td>
<td>31.5±0.6</td>
<td>35.4±0.6</td>
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<tr>
<td>cDNA, pg/100 ng RNA</td>
<td>1.0±0.3</td>
<td>0.1±0.0</td>
</tr>
<tr>
<td>cDNA Ratio (CXCR3-B/CXCR3-A)</td>
<td>6.4</td>
<td>39</td>
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Values are means ± SE. NHBEC, normal human bronchial endothelial cells.

Table 1. Mean cDNA concentrations for CXCR3-A and -B variants in NHBEC and 16-HBE cells along with variant ratios for each cell type.
to the cell cycle, cells were examined by immunofluorescence microscopy. As shown in Fig. 4, only a subset of 16-HBE (Fig. 4A) and NHBEC (Fig. 4B) expressed CXCR3 on the cell surface (n = 3 for each). The cells that showed the most intense staining for CXCR3 demonstrated “mitotic figures” (i.e., condensed chromatin), indicative of mitosis. This was confirmed by confocal microscopy (n = 2) (Fig. 4C). In contrast, CXCR3 immunoreactivity was visible in all permeabilized cells (Fig. 5) (n = 4–6).

Cyclin B1 immunoreactivity was most pronounced in 16-HBE cells showing mitotic figures and was colocalized around the condensed chromatin (n = 3). These cells also exhibited the most intense CXCR3 surface staining (Fig. 6A). Similarly, α-tubulin-labeled 16-HBE cells, which displayed prominent mitotic spindles, also displayed the most intense CXCR3 surface staining (Fig. 6B; n = 4).

CXCR3-stimulated cell proliferation and thymidine incorporation. Cell proliferation and thymidine incorporation assays were performed to determine the influence of CXCR3 activation on cell growth. In NHBEC, I-TAC/CXCL11 dose dependently stimulated cell proliferation over 48 h (P = 0.014 by ANOVA, n = 5) (Fig. 7). Proliferative responses to I-TAC were biphasic, with a maximum at a concentration of 100 ng/ml (125 ± 8 SE% of control, i.e., cells maintained in EGF and BPE-depleted medium alone) (P < 0.05 for comparison with control). In comparison, cells stimulated for 48 h with
EGF and BPF demonstrated cell numbers that were $133 \pm 12$ SE% of control ($P < 0.05$ for comparison with control) (Fig. 7).

I-TAC also dose dependently increased $[^{3}H]$thymidine incorporation ($P < 0.001$ by rank-order ANOVA, $n = 5$) (Fig. 8). Like proliferation, thymidine incorporation responses to I-TAC were biphasic, with a maximum at 100 ng/ml ($148 \pm 15$ SE% of control) ($P < 0.05$).

CXCR3-stimulated activation of the MAPK pathway. To examine the potential signaling pathways underlying CXCR3-induced cell proliferation, we assessed the effect of I-TAC on phosphorylation of the MAPKs, ERK1/2, p38, and JNK. I-TAC (100 ng/ml) stimulated phosphorylation of ERK1/2 (Fig. 9A) and p38 (Fig. 9B) in NHBEC ($n = 3$). The peak of ERK phosphorylation occurred at 5 min and declined thereafter. In contrast, a peak of p38 phosphorylation was detected at 5 min but was biphasic and occurred again at 18–24 h. JNK phosphorylation was unchanged in response to I-TAC (data not shown).

DISCUSSION

The chemokine receptor, CXCR3, originally described in activated T lymphocytes (21, 22), is expressed by a variety of structural cells (e.g., airway epithelial cells, endothelial cells, renal tubular epithelial cells, keratinocytes, mesangial, trophoblastic, and microglial cells) (4, 6, 17–19, 25, 30, 32). In structural cells, CXCR3 induces a number of cell type-specific
responses important for angiogenesis/angiostasis, tissue repair, and remodeling (3, 9, 28–30). Differing cellular responses to activation of CXCR3 appear to be explained by expression of at least two receptor splice variants, i.e., CXCR3-A and -B (19). CXCR3-A codes for a protein of 368 amino acids. The more recently described CXCR3-B is formed by alternative splicing within the single intron and codes for a larger protein of 416 amino acids. CXCR3-B differs from CXCR3-A in the first 52 amino acids of its longer NH2-terminal extracellular domain (19). It has been suggested that these two proteins signal through different pathways. CXCR3-A couples to G\textsubscript{i} and increases intracellular calcium, whereas CXCR3-B couples to G\textsubscript{s} and activates adenylyl cyclase (19).

We have recently demonstrated that human airway epithelial cells constitutively express CXCR3 in vivo and in vitro, which when activated by I-TAC, induces robust airway epithelial cell chemotaxis (18). CXCR3-induced chemotactic responses are mediated by both MAPK and phosphatidylinositol 3-kinase signaling pathways (33a).

Our previous study demonstrated mRNA for both CXCR3 splice variants (i.e., CXCR3-A and -B) by qualitative RT-PCR.

Fig. 5. Immunofluorescence microscopy of permeabilized airway epithelial cells labeled for CXCR3 (green, Cy2). Field 1, 16-HBE cells; field 3, NHBEC. Negative controls are shown in fields 2 and 4. Cell nuclei are stained with PI. Note that, unlike cells labeled for permeabilization (Fig. 4), nearly all cells show CXCR3 expression. Magnification = ×400.

Fig. 6. Immunofluorescence microscopy of cyclin B\textsubscript{1}, α-tubulin, and CXCR3 surface expression in airway epithelial cells. A: fluorescence microscopy showing cyclin B\textsubscript{1} (orange, PE = phycoerythrin) (fields 1 and 2) and surface expression of CXCR3 (green, Cy2) (fields 4 and 5) in 16-HBE cells. Cells were stained for CXCR3 before permeabilization and labeling for cyclin B\textsubscript{1} (see METHODS). Isotype controls are shown for cyclin B\textsubscript{1} (field 3) and CXCR3 (field 6). Cell nuclei are visualized with DAPI (blue). Note that CXCR3 expression is most intense on cells staining strongly for cyclin B\textsubscript{1}. Magnification = ×600. B: fluorescence microscopy showing α-tubulin (green, FITC) (fields 1 and 2) and surface expression of CXCR3 (orange, Cy3) (fields 4 and 5) in 16-HBE cells. Cells were stained for CXCR3 before permeabilization and labeling for α-tubulin (see METHODS). Isotype controls are shown for α-tubulin (field 3) and CXCR3 (field 6). Note that CXCR3 expression is most intense in cells staining strongly for α-tubulin and showing mitotic spindle formation. Magnification = ×600.
In the present study, the relative abundance of CXCR3-A and -B transcripts was assessed by real time RT-PCR and compared with a standard curve developed with plasma constructs of CXCR3-A and -B in the PCR reaction. The results of the present study confirm that both NHBEC and the 16-HBE cell line express the A and B splice variants of the CXCR3 chemokine receptor. However, both airway epithelial cell types primarily express CXCR3-B mRNA, with considerably less CXCR3-A mRNA.

These results obtained in human airway epithelial cells differ from those obtained in human T cells, renal mesangial cells, and microvascular endothelial cells. For example, T cells cultured in phytohemagglutinin and IL-2 to produce an activated Th1 phenotype demonstrate a high level of CXCR3-A expression with relatively little CXCR3-B expression (19). In contrast, microvascular endothelial cells express only CXCR3-B and no CXCR3-A, whereas renal mesangial cells express only CXCR3-A and no CXCR3-B (19). The results of the present study indicate, therefore, that airway epithelial cells have a unique pattern of CXCR3 subtype expression.

A third CXCR3 splice variant, termed CXCR3-alt, has been described recently by Ehlert and colleagues (11). This splice variant codes for a truncated receptor of 260 amino acids. Rather surprisingly, given the lack of several transmembrane domains, CXCR3-alt is functionally active and mediates chemotaxis. The CXCR3-alt receptor appears to be coexpressed with the classic CXCR3-A receptor, albeit at a very low level (5% of CXCR3-A mRNA) (11). The present study did not test for the presence of this splice variant, since CXCR3-A was expressed at low abundance in airway epithelial cells.

In the present study, flow cytometry indicated that virtually all NHBEC and 16-HBE cells expressed CXCR3 when permeabilized. However, only a subset of epithelial cells (20–40%) expressed CXCR3 on the cell surface. These results suggested that CXCR3 is largely contained in a cytoplasmic pool and is present on the surface in only a minority of cells. Similar findings have been obtained for CXCR1 and CXCR2 in human mast cells and T lymphocytes (20). Experiments using an antibody selective for CXCR3-B indicated that at least some of the CXCR3 expressed on the surface and within the cytoplasmic pool is the B variant. No antibody selective for CXCR3-A is presently available.

The possibility that CXCR3 was expressed at the cell surface during a portion of the cell cycle, as is the case with human endothelial cells (29), was confirmed using two immunocytochemical approaches. First, cells were costained for cyclin B1, which is selectively expressed during the G2/M phase of the cell cycle (27, 34).
Second, cells were costained for α-tubulin to detect mitotic spindle formation. These approaches demonstrated that cells expressing cyclin B1 and manifesting mitotic spindles, also preferentially expressed CXCR3 on the cell surface.

In the present study, stimulation of NHBEC for 48 h with I-TAC, the most potent of the CXCR3 ligands (8, 14), induced DNA synthesis, as reflected in thymidine incorporation and cell proliferation. Of interest, the response to I-TAC was nearly as great as the combined effects of EGF and BPE, two powerful mitogenic factors, used as a positive control (12, 36). To our knowledge, the present study is the first showing that CXCR3 activation increases proliferation in a structural cell in the human respiratory tract.

The proliferative response of NHBEC to CXCR3 stimulation by I-TAC resembles the mitogenic responses of T cells and renal mesangial cells to IP-10 (19, 35). In contrast, the response of NHBEC differs from that of human endothelial cells, which undergo apoptosis in response to IP-10 (19, 29). The apoptotic response of endothelial cells to CXCR3 ligands is believed to be due to selective activation of CXCR3-B, the only CXCR3 variant expressed by these cells (19). Although NHBE express both CXCR3 variants, our findings of a proliferative rather than an apoptotic response to I-TAC may be explained by marked differences in affinity of I-TAC for the two receptor variants. I-TAC has an ~100-fold greater affinity for CXCR3-A than for -B receptor, with approximate IC50 values of 0.4 and 32 nM, respectively (19). Accordingly, I-TAC in the concentration range of 1–100 ng/ml would be expected to induce a largely CXCR3-A-mediated response. On the other hand, an I-TAC concentration of 1,000 ng/ml, the highest used in our experiments, might be sufficient to induce a CXCR3-B-mediated response and hence explain the biphasic shape of the mitogenic and DNA synthesis responses observed in this study.

I-TAC stimulation induced activation of both the ERK1/2 and p38 arms of the MAPK pathway. Phosphorylation of p38 demonstrated two peaks, an early peak at 5 min and a later peak at 18–24 h. Activation of ERK and/or p38 may contribute to the proliferative response of airway epithelial cells to I-TAC. Others have shown that activation of ERK in response to CXCR3 activation mediates the mitogenic response to IP-10 in renal mesangial cells (6).

Airway epithelial cells not only express CXCR3 but also its ligands, the interferon-γ-inducible CXC chemokines, IP-10, MIG, and I-TAC (31, 33). Moreover, production of these chemokines by human airway epithelial cells is markedly increased by the proinflammatory cytokines IFN-γ, TNF-α, and IL-1β, which are elevated in patients with chronic obstructive pulmonary disease (1, 10, 33). The results of the present study indicate that human airway epithelial cells express both CXCR3-A and -B splice variants, thus having interesting functional implications. First, the results suggest the possibility of autocrine or paracrine effects of these chemokines on epithelial cell growth. Second, they suggest that epithelial responses to the CXCR3 ligands may depend on ligand concentration, which in turn likely depends on the inflammatory milieu in the airway. For example, under normal conditions in which the airway is not inflamed and CXCR3 ligand concentration is relatively “low,” the predominant effect on the epithelium may be stimulation of cell proliferation. On the other hand, when the airway is inflamed and ligand concentrations are “high,” the predominant effect may be inhibition of epithelial cell proliferation leading to airway mucosal denudation and damage. Further studies examining the functional response of airway epithelial cells to selective activation of the two CXCR3 subtypes are needed to test these possibilities.

GRANTS
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REFERENCES