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Epithelial CXCR3-B Regulates Chemokines Bioavailability in Normal, but Not in Sjögren's Syndrome, Salivary Glands[1]

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Expression of CXCR3-targeting chemokines have been demonstrated in several diseases, suggesting a critical role for CXCR3 in recruiting activated T cells to sites of immune-mediated inflammation. Sjögren's syndrome (SS) is an autoimmune disease characterized by a mononuclear cell infiltrate of activated T cells around the duct in the salivary gland. Analysis of minor salivary gland biopsy specimens from 20 healthy subjects and 18 patients with primary SS demonstrated that CXCR3, in particular, the B form of this receptor, is constitutively expressed by human salivary gland epithelial cells. Salivary gland epithelial cell cultures demonstrated that CXCR3 participate in removing relevant amount of agonists from the supernatant of exposed cells without mediating calcium flux or chemotaxis while retaining the ability to undergo internalization. Although in normal salivary gland epithelial cells, CXCR3 behaves as a chemokine-scavenging receptor, its role in SS cells is functionally impaired. The impairment of this scavenging function might favor chemotaxis, leading to heightened immigration of CXCR3-positive T lymphocytes. These findings suggest that epithelial CXCR3 may be involved in postsecretion regulation of chemokine bioavailability. They also support a critical role for CXCR3 in the pathogenesis of SS and identify its agonists as potential therapeutic targets. ***The Journal of Immunology,*** **2006, 176: 2581–2589.**

The large family of chemokines encompasses inflammatory chemokines that control the recruitment of effector leukocytes at sites of inflammation, homeostatic chemokines that drive leukocytes under baseline conditions, and dual-function chemokines that participate in immune defense functions and also target noneffector leukocytes, including precursor and resting mature leukocytes. CXCL9, CXCL10, and CXCL11 are dual-function chemokines induced by IFN-γ and produced during Th1-type immune responses that exert their action via an interaction with the CXCR3 (1–3). Although these three ligands are all induced by IFN-γ, they appear to mediate distinct in vivo biological phenomena, probably via a differential activation of CXCR3 (4–7). The expression of CXCR3-targeting chemokines has been demonstrated in several diseases or pathological states, including sarcoidosis, ulcerative colitis, multiple sclerosis, viral infections, allograft rejection, atherosclerosis, delayed-type hypersensitivity skin reactions, chronic skin inflammation, rheumatoid arthritis, diabetes, and glomerulonephritis (8–17). Collectively, these studies suggest a critical role for CXCR3 in the recruitment of activated T cells to sites of immune-mediated inflammation.

Although chemokines were first identified as chemoattractants, their functions extend well beyond their role in leukocyte migration. For instance, IFN-inducible CXC chemokines have been shown to be able to inhibit angiogenesis and endothelial cell chemotaxis; however, they induce migration and proliferation of vascular pericytes. Recent evidence indicates that an alternative splicing variant of the CXCR3 gene, i.e., CXCR3-B, mediates the angiostatic activity of CXCR3-binding chemokines, providing a convincing explanation for the proliferation induced by CXCR3 agonists on endothelial cells that express CXCR3-A and a parallel inhibitory activity on endothelial cells expressing CXCR3-B (18).

Sjögren's syndrome (SS)[3] is an autoimmune disease characterized by mononuclear T cell-rich infiltrate around the ducts in the salivary gland. T cell infiltrates lead to salivary gland destruction and, ultimately, salivary hypofunction. Our preliminary data have shown that CXCR3-positive lymphocytes infiltrate salivary glands in SS, and that, most notably, CXCR3 is also expressed in both SS and normal ductal epithelial cells (19). In this study, we evaluated whether CXCR3 expression by epithelial cells has a role in a physiologic setting and in the pathogenesis of SS. We provide definitive evidence that CXCR3, in particular the B form, is constitutively expressed by salivary gland epithelial cells, where it regulates the bioavailability of chemokines, a function that was found to be impaired in SS.

Materials and Methods

Patients

We studied minor salivary gland (MSG) biopsy specimens from 20 healthy subjects (all female; mean ± SD age, 51.0 ± 20.5 years; range, 20–75) and

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[3] Abbreviations used in this paper: SS, Sjögren's syndrome; MFI, mean log fluorescence intensity; MSG, minor salivary gland.

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18 patients (all female; mean ± SD age, 49.0 ± 15.5 years; range, 29–76) with primary SS fulfilling the new American-European Consensus Group diagnostic criteria for SS (20). MSG biopsy was performed as a routine part of the diagnostic evaluation of SS, and informed consent was obtained from the patients and controls. Control subjects reported sicca symptoms, but were completely negative for all immunologic abnormalities, such as autoantibodies, including anti-SSA, anti-SSB, antinuclear Abs, and rheumatoid factor, and hypergammaglobulinemia. To rule out the possibility of an early, preclinical SS or subclinical infection, we performed oral, ocular, and serologic diagnostic examinations. Test results were normal in all control subjects. With regard to the possibility of a subclinical bacterial or viral infection, periodontitis was excluded in all subjects by a comprehensive oral evaluation. We excluded from the study four subjects in whom CMV genome and mRNA transcript (early and late genes) were detected.

Immunohistochemical analysis

Tissue samples from the labial salivary glands of SS patients were fixed in buffered-formalin, embedded in paraffin, and stained with H&E. For immunostaining, sections were stained using the standard avidin-biotin complex method (Vectastain ABC Kit; Vector Laboratories) as we previously described (12). Briefly, after microwave Ag retrieval procedure, slides were placed in a 2-l glass beaker containing 0.01 M citrate buffer (pH 5.9) and microwaved at full power (800 W for 5 min, three times) before cooling and equilibration in PBS. Anti-CXCR3 mAbs (clone 49801.111 (R&D Systems) or clone 1C6 (BD Biosciences) both used at 1/100) were incubated for 1 h in a humified chamber at 37°C and detected using biotinylated secondary Ab, followed by 30-min incubation with avidin-peroxidase (1/200) and 3,3-diaminobenzidene tetrahydrochloride as chromogen. The intensity of Ab staining was classified in four groups: strong, weak, moderate, and negative. Parallel control slides were prepared either lacking primary Ab or lacking primary and secondary Abs or were stained with normal sera to control for background reactivity. Furthermore, to rule out nonspecific staining, a matching immunohistological assay was performed in which an isotype-negative control was used in place of the antigenically specific primary Ab.

Indirect immunofluorescence analysis

Epithelial cells grown in chamber slides (Nalge; Nunc) were fixed in 2% paraformaldehyde for 10 min and permeabilized in 0.1% Triton X-100 for 4 min. After soaking in PBS containing 1% BSA for 30 min, slides were incubated with the primary Ab at room temperature for 45 min in PBS containing 0.5% BSA. After washing with PBS, slides were stained with FITC-conjugated goat anti-mouse IgG and incubated for 3 min in PBS containing 0.5 μg/ml propidium iodide for nuclear staining before fluorescence microscopy.

Primary cultures of epithelial cells from salivary gland tissues

Primary ductal epithelial cells cultures were established from minor salivary gland biopsies by the explant outgrowth technique (21). Briefly, tissues were minced into fragments of 1 mm^3 and cultured in a mixture (1/3) of DMEM (Invitrogen Life Technologies) and Ham's F-12 (Invitrogen Life Technologies) containing 2.5% FCS, 2 mM L-glutamine (Invitrogen Life Technologies), 0.5 mg/ml insulin (Wako Biochemicals), 0.4 mg/ml hydrocortisone (Sigma-Aldrich), and 10 ng/ml epidermal growth factor (Sigma-Aldrich). When cultures were ~70% confluent, cells were detached using trypsin/EDTA and subcultured in 75-cm^2 tissue culture flask (Nunc). The epithelial cell origin of the primary cultures was confirmed by staining with a cytokeratin-specific Ab (ICN Pharmaceuticals). Experiments were performed after subculture when cells were ~80% confluent. Cells were used from passages 4–6.

Flow cytometric analysis of CXCR3

The expression of CXCR3 on epithelial cells was assessed by flow cytometric analysis using direct immunofluorescence assay. After incubation at 37°C with FITC-conjugated anti-CXCR3 mAbs (R&D Systems) or the matched control mAb, cells were washed and analyzed. For FACS analysis, 3×10^4 cells were scored using a FACSCalibur analyzer (BD Biosciences), and data were processed using CellQuest (BD Biosciences). The threshold of positivity was set at the nonspecific binding observed in the presence of irrelevant control Ab. Mean log fluorescence intensity (MFI) values were obtained by subtracting the MFI of the isotype control sample from the MFI of the positively stained sample. To evaluate whether the differences between the peaks of cells were statistically significant with respect to control, the Kolmogorov-Smirnov test for analysis of histograms was used according to the CellQuest software guide (BD Biosciences), with D/s values >10 considered significant.

Confocal microscopy

Epithelial cells, grown in chamber slides, were incubated with CXCL10 (200 ng/ml) for 0–30 min. Cells were washed three times in HBSS (37°C), fixed with 0.25% glutaraldehyde in PBS for 10 min, blocked with 0.05 M Tris-HCl (pH 7.5) for 20 min, and permeabilized with 0.1% Triton X-100 in PBS for 10 min. The cells were then stained with anti-CXCL10 Abs (clone 49801.111; R&D Systems; 1/150) and Alexa 488 (Sigma-Aldrich)-conjugated goat anti-mouse Ig (1/200) secondary Abs. Samples were analyzed by confocal microscope (2100 Multiphoton; Bio-Rad) with a ×60 objective lens (Nikon), using laser excitation at 488 nm. Images were analyzed using the Adobe Photoshop 7.0 program.

Western blot

Epithelial cells (4×10^5 for each assay) were resuspended and lysed in 62 mM Tris-HCl buffer (pH 6.8) containing 5% glycerol, 0.5% SDS, and 0.5% 2-ME. Samples were then subjected to SDS-PAGE (10% gels), transferred to nitrocellulose membranes, and immunostained with anti-phosphotyrosine (PY-20) mAb (ICN Biotechnology) or anti-human CXCR3 mAb (BD Pharmingen) using an ECL detection system (Amersham Biosciences). The blots were then stripped and reprobed with anti-β-actin mAb (Sigma-Aldrich). Cell extracts from chronic lymphocytic leukemia cells were used as a positive control. Before using these cells in any experiment we evaluated CXCR3 expression by means of flow cytometry, confirming that >95% of leukemic cells were positively stained.

RT-PCR analysis

Total cellular RNA was extracted, using the RNA Mini Kit isolation and purification system (Qiagen), from 3 to 4×10^6 cells. On-column DNase digestion was performed according to the manufacturer's instructions, and RNA was quantified by measuring absorbance at 260 nm. cDNA was synthesized from 1 μg of total RNA at 42°C for 15 min in the presence of AMV reverse transcriptase (2.5 U) using 2.5 mM oligo(dT) primer and reaction conditions described by the manufacturer (Promega).

Real-time quantitative PCR amplification reactions were conducted in an ABI PRISM 7000 sequence detection system (Applied Biosystems) in a 15-μl volume. SYBR Green PCR Master Mix was purchased from Applied Biosystems (P/N 4309155), containing AmpliTaq Gold DNA polymerase and optimized buffer components. A fraction of 5 μM primers and 1.5 μl of cDNA was added to the SYBR Green Master Mix to make a final 15-μl reaction volume. The primers used for CXCR3 and GAPDH amplifications were: *CXCR3-A*: forward, ACC CAg CAg CCA gAg CAC C-3′; reverse, 5′-TCA TAg gAA gAg CTg AAg TTC TCC A-3′; *CXCR3-B*: forward, 5′-TgC CAg gCC TTT ACA CAg C-3′; reverse, 5′-TCg gCg TCA TTT AgC ACT Tg-3′ and 5′-ATg CCA TgA CCA gCT TTC ACT-3′; reverse, 5′-TTA Agg CAg gCC CTC Agg TA-3′; and *GAPDH*: forward, 5′-AAT ggA AAT CCC ATC ACC ATC T-3′; reverse, 5′-CgC CCC ACT TgA TTT Tgg-3′. PCRs were performed under the following conditions: 10-min denaturation at 95°C, followed by 95°C for 15 s and 60°C for 1 s, cycled 50 times. Each quantitation target was amplified in triplicate samples. A no-template control for each Master Mix and three standard curves were generated for GAPDH, CXCR3A, and CXCR3B using cDNA in serial dilutions of 1/1, 1/10, 1/100, and 1/1000. The relative amounts of mRNA were determined by comparison with standard curves. The results of each sample were normalized for GAPDH expression. To distinguish specific amplicons from nonspecific amplifications, a dissociation curve was generated.

Cell cycle analysis

For cell cycle analysis, 1×10^6 epithelial cells were washed twice with PBS, fixed with 70% cold ethanol for 30 min at 4°C, and incubated with a solution containing 50 mg/ml propidium iodide and 1 mg/ml RNase (Sigma-Aldrich). The tubes were placed at 4°C in the dark for 30 min before analysis using a FACSCalibur analyzer (BD Biosciences). For each sample, 10,000 cells were analyzed using CellQuest software (BD Immunocytometry Systems).

Cytosolic calcium measurement

Changes in the intracellular calcium concentration were measured in epithelial cells using the fluorescent indicator fura-2-AM. Briefly, 20×10^6 cells were incubated with a solution containing 2.5 μM fura-2-AM, 20% pluronic acid, and 0.25 mM sulfinpyrazone (to prevent fura-2 release into the medium) at 37°C for 30 min. After the loading procedure, aliquots of the cells (2×10^6) were rapidly washed and resuspended in a magnetically stirred thermostatted cuvette. The incubation medium contained 1 mM $CaCl_2$. Excitation and emission wavelengths were 340 and 500 nm, respectively, the excitation slit width was 5 nm, and the emission slit was 10

nm. Control experiments without sulfinpyrazone gave essentially the same results, except for a slowly increasing baseline due to fura-2 leakage. The chemokines were used at 1 μg/ml; anti-Ig was used at 500 ng/ml. Finally, 1 μM ionomycin was added to obtain the maximal effect.

Chemotaxis of ductal salivary gland epithelial cells

The effects of the CXCR3 ligands, CXCL9–11 and CXCL4, on chemotaxis of epithelial cells were assessed using a 48-well modified Boyden chamber (AC48; NeuroProbe). Different chemotactic stimuli were loaded in the bottom chamber, and cells were added to the top chamber. A polyvinylpyrrolidone-free polycarbonate 8-mm membrane with 3- to 12-μm pores was placed between the chambers. The bottom face of the filter was pretreated with 10 μg/ml fibronectin to maximize the attachment of the transmigrated cells to filters. Fibronectin-treated filters did not induce spontaneous chemotaxis in the absence of chemokines. Briefly, 28-μl aliquots of serum-free HEPES-buffered RPMI 1640 supplemented with 0.1% BSA, with or without ligand (CXCL9–11 and CXCL4; 100, 200, and 500 ng/ml) were dispensed into the bottom wells of the chamber. Fifty-microliter aliquots of epithelial cells (2×10^6 cells/ml) resuspended in HEPES-buffered RPMI 1640 were added to the top wells. Chambers were incubated at 37°C with 5% CO_2 for 2 h. The membrane was then removed, washed with PBS on the upper side, fixed, and stained with DiffQuik (Baxter). Cells were counted microscopically at ×800 magnification in four fields per membrane. All assays were performed in triplicate.

Chemokine assays

Chemokine concentrations in cell-free supernatants harvested from salivary epithelial cells were measured by specific ELISA for CXCL9 (DUOSET DY 392), CXCL10 (DUOSET DY266), and CXCL11 (Quantikine DCX110) purchased from R&D Systems. Detection limits were 60 pg/ml for CXCL9, 30 pg/ml for CXCL10, and 13.9 pg/ml for CXCL11.

Migration of 300-19/hCXCR3 cells toward the supernatants of CXCL10-exposed epithelial cells

The migration of human CXCR3-transfected murine 300-19 B cells (300-19/hCXCR3) was measured in a 48-well modified Boyden chamber (AC48 NeuroProbe). The $CXCR^+$ and $CXCR3^-$ cell lines (300-19 B cells; provided by Dr. B. Moser, Theodor Kocher Institute, University of Bern, Bern, Switzerland) were used as positive and negative controls. Directional migration of 300-19/hCXCR3 was quantified toward 200 ng/ml CXCL10 that had been preincubated with salivary duct epithelial cells. Culture medium and nontreated CXCL10 were used as additional negative and positive controls, respectively. Twenty-eight microliters of supernatant, chemokines, or control medium were added to the bottom chambers, and 50 μl of 5×10^6 cells/ml 300-19/hCXCR3 cells resuspended in RPMI 1640 were added to the top chambers. The chamber was incubated at 37°C in 5% CO_2 for 2 h. The membranes were removed, washed with PBS on the upper side, fixed, and stained with DiffQuik (Dade). Cells were counted in three fields per membrane at ×800 magnification. All assays were performed in triplicate. In blocking experiments, cell suspensions were preincubated before chemotaxis assay for 30 min at 37°C with anti-human CXCR3 mAb at a concentration of 20 μg/ml.

Statistical analysis

Data are expressed as the mean ± SEM, and comparisons between values were made using the ANOVA test. A value of $p < 0.05$ was considered significant.

Results

CXCR3 is expressed by normal salivary gland ductal epithelial cells

To determine whether CXCR3 is expressed in vivo by normal salivary gland epithelial cells, we analyzed MSG biopsy specimens from 20 healthy subjects. The expression of CXCR3 was determined by immunohistochemistry of gland tissues. Histologic examination revealed a moderate expression of CXCR3 on ductal epithelial cells and sometimes on acinar epithelial cells in all subjects (Fig. 1, *A* and *C*). Histological sections from SS patients revealed a similar staining pattern (Fig. 1, *B* and *D*). Additional analyses of MHC class II molecule expression showed that CXCR3 expression did not correlate with the activation state of the epithelial cells. To confirm our findings, we used cultured epithelial cells derived from MSG. Immunocytochemistry clearly demonstrated CXCR3 expression on cultured MSG epithelial cells (Fig. 1, *E* and *F*). To exclude the possibility that detachment of cells induces receptor expression, we also cultured several MSG explants on slide chambers. Epifluorescence microscopy on adherent cells revealed a similar staining pattern (Fig. 1*G*). To exclude the possibility that the addition of FCS to culture medium induces CXCR3 expression, we repeated the analyses before and after 24-h serum starvation. Serum deprivation did not alter CXCR3 expression (data not shown). We then analyzed CXCR3 expression by means of flow cytometry and found that all cells were dimly positive (Fig. 2*A*). To confirm the epithelial origin of cultured cells, an experiment comprised of double staining for CXCR3 and cytokeratins was performed. To confirm our findings, we assessed cultured MSG cells for CXCR3 expression at the mRNA and protein levels using RT-PCR and Western blotting, respectively. Both CXCR3 protein (Fig. 2*B*) and mRNA (Fig. 2*C*) were clearly visible in all cultures. Notably, CXCR3-B, the alternatively spliced variant of CXCR3, was the predominant type of CXCR3 present in the primary cultures of MSG epithelial cells.

CXCR3 expression is not associated with a cell cycle phase

Human microvascular endothelial cells selectively express CXCR3-B, although this expression is limited to the S/G_2-M phase of their cell cycle (22). To test whether CXCR3 on MSG epithelial cells is also associated with certain cell cycle phases, we compared the number of CXCR3-positive cells both from cultures that were far from confluent with a high proliferation rate and from confluent, growth-arrested cultures. However, the proportions of CXCR3-expressing cells were found to be similar, making it unlikely that this chemokine receptor is strictly associated with a certain cell cycle phase. Consistent with these findings, double staining with FITC-conjugated anti-CXCR3 mAbs and propidium iodide analyzed by flow cytometry demonstrated that the presence of CXCR3 was independent of the DNA content.

CXCR3 agonists do not mediate changes in cytosolic Ca^{2+}

Activation of chemokine receptors often leads to elevation of cytosolic Ca^{2+}; for example, in T cells CXCR3 agonists lead to Ca^{2+} mobilization (23). To determine whether epithelial cells express functional CXCR3, we first investigated the ability of CXCL4, -9, -10, and -11 to induce changes in intracellular Ca^{2+}. CXCR3-positive epithelial cells did not respond with calcium flux to any of these four agonists, whereas addition of CXCL12, used as a positive control, resulted in an increase in intracellular calcium (Fig. 3, *A–D*). This is consistent with previous observations that CXCL9, -10, and -11 do not elicit Ca^{2+} mobilization in HMEC-1 CXCR3-B transfectants (18).

CXCR3 ligands activate protein kinases

In T cells, CXCR3 binding activates several protein kinases. The internalization of many GPCRs, including at least two chemokine receptors, CXCR4 and CCR5, is mediated by the phosphorylation of C-terminal residues by the G protein-related kinases (24). We therefore examined the effects of CXCR3 agonists, CXCL10, CXCL9, and CXCL4, on tyrosine phosphorylation in CXCR3-positive epithelial cells. The addition of 200–500 ng/ml CXCL9, CXCL10, or CXCL4 increased intracellular tyrosine phosphorylation, with a peak response at 30 min (Fig. 3, *E* and *C*). Although suggesting that CXCR3 receptors on MSG epithelial cells are able to signal, measurement of the increased tyrosine phosphorylation does not indicate what the functional outcome may be in epithelial cell.

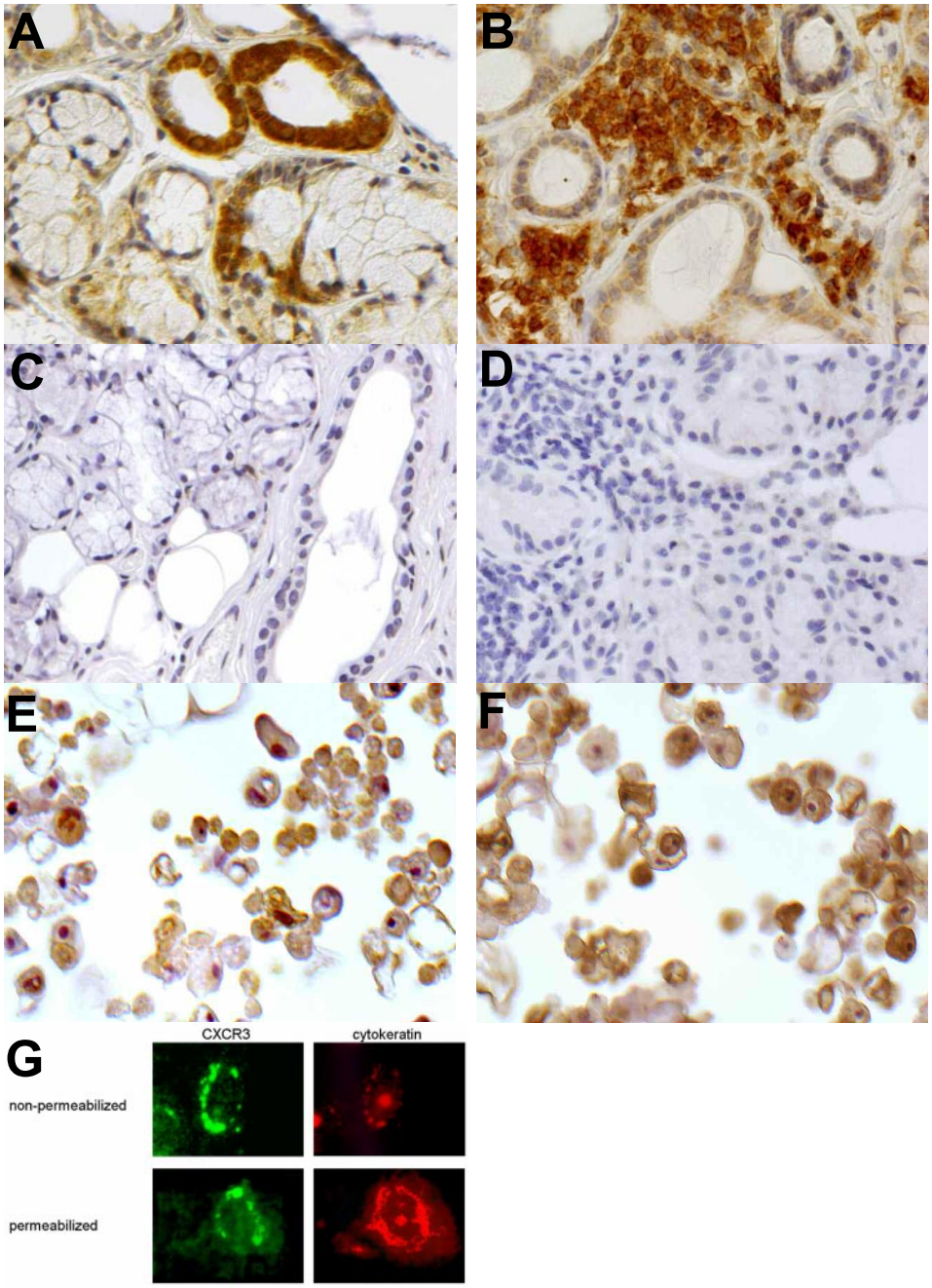

FIGURE 1. CXCR3 is expressed by normal salivary gland epithelial cells. Immunostaining of minor salivary gland sections shows moderate CXCR3 reactivity on ductal and acinar epithelial cells in both healthy subjects (*A*) and SS patients (*B*). *C* and *D*, Matching immunohistological assay in which an isotype-negative control was used in place of the antigenically specific primary Ab. *E*, Immunocytochemistry of primary cultures of MSG ductal epithelial cells reveals strong reactivity with an anti-CXCR3 mAb. *F*, Staining with anti-cytokeratin mAb confirms the epithelial origin of cultured cells. *G*, Epifluorescence microscopy of adherent cells. MSG ductal epithelial cells cultured on a chamber slide were permeabilized (with Triton X-100), or not, and double labeled for CXCR3 (red; Cy3) and cytokeratin (green; FITC). Tissues and cells were stained for CXCR3 with an Ab that recognized both CXCR-A and -B (clone 49801.111; R&D Systems). Original magnifications, ×50 (*A–D*) and ×1000 (*E* and *F*).

Reduced surface expression of CXCR3 upon ligand binding

Upon ligand binding, in most GPCRs the association of phosphorylated receptors with the cytoplasmic adaptor, β-arrestin, ultimately leads to receptor internalization (25). Therefore, we addressed the possibility that CXCL9, -10, and -11 induce CXCR3 internalization in epithelial cells. Results showed that during a 5- to 60-min period of incubation with the agonists, cell surface expression of CXCR3 decreased by 80% (Fig. 3*F*). This indicates that on epithelial cells CXCR3 is connected to an intracellular machinery mediating internalization in a receptor-dependent manner.

CXCR3 agonists do not induce chemotaxis of epithelial cells

To investigate an eventual functional role for the epithelial CXCR3, chemotactic responses were tested. CXCL9–11 and CXCL4 did not induce CXCR3-positive epithelial cell migration (Fig. 3*G*). Taken together, these data show that CXCR3 on epithelial cells is capable of recognizing ligands, but is functionally incapable of activating the two known signaling pathways of chemokine receptors, calcium mobilization and chemotaxis, although they undergo internalization. It was reasoned that CXCR3 on epithelial cells may buffer agonists, behaving as a scavenger receptor.

Scavenging of CXCL10 by salivary gland ductal epithelial cells

We therefore directly assessed the ability of epithelial cells to bind and sequester relevant agonists. The amount of CXCL9, -10, and -11 produced by epithelial cells was first measured. Quiescent cells showed low constitutive levels of CXCL11 (16 pg/ml), whereas CXCL9 and CXCL10 could not be detected in the culture supernatant. Next, exogenous CXCL10 (200 ng/ml) was added to cell cultures, and its disappearance was monitored by ELISA and confocal microscopy. In preliminary experiments supernatants were

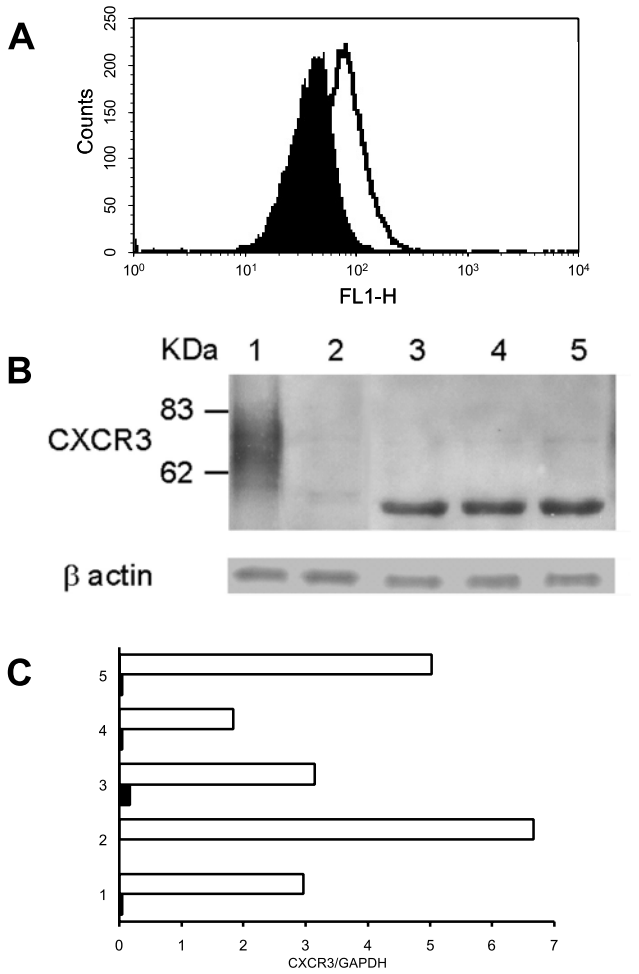

FIGURE 2. Cultured MSG epithelial cells express CXCR3 at the protein and mRNA levels. *A*, Surface expression of CXCR3 on the membrane of MSG ductal epithelial cells as detected by flow cytometric analysis performed with FITC-conjugated anti-CXCR3 mAbs (clone 49801.111; R&D Systems; □). Cells were gated against an isotype control Ab (■). Representative results from 10 independent experiments are shown. Unimodal distribution with a statistically significant shift between the sample data and the subclass control data. To role out false positives, we performed preliminary flow cytometric experiments using both R&D Systems 49801.111 and BD Biosciences 1C6 Abs. Both mAbs showed similar staining patterns even if incubated at 4°C. *B*, Western blotting. Extracts from MSG epithelial cell cultures were run on 10% SDS-PAGE, transferred to nitrocellulose membranes, blotted with the anti-CXCR3 Ab 1C6 (BD Biosciences) developed by chemiluminescence, and detected by autoradiography. The molecular masses of protein standards are indicated on the *left*. The filter was reprobed with anti-β-actin Ab as a loading control. Shown from *left* to *right* are the cell extracts obtained by chronic lymphocytic leukemia cells (250,000 cells; line 1; used as a positive control), normal B cells (250,000 cells; line 2; used as a negative control), and MSG ductal epithelial cells (200,000 cells from three different primary cultures; lines 3–5). *C*, Expression of CXCR3 mRNA in MSG ductal epithelial cell cultures from three healthy controls (nos. 1–3) and two SS patients (nos. 4 and 5). RT-PCR analysis, performed with primers with selective specificity against the two alternative splicing variants (■, CXCR3-A; □, CXCR3-B), shows the predominance of CXCR3-B. The mRNA level was expressed as a ratio to G3PDH mRNA. Results for no RT Control show the absence of genomic DNA contamination in samples.

harvested at different time points (from 0 to 60 min). Because of its very rapid disappearance, it was not possible to obtain accurate clearance curves for CXCL10. However, we observed decreased CXCL10 concentrations to 5% in 5 min (Fig. 4*A*). This high fraction of scavenging (95%) was substantially maintained after 2 h. The anti-CXCR3 mAb 49801.111 was used to assess the contribution of receptor-mediated internalization compared with other scavenging mechanisms. The addition of the blocking Ab inhibited chemokine uptake by 20%. These observations are consistent with the hypothesis that epithelial cells may scavenge CXCR3 agonists and that this function is in part mediated by CXCR3-B. To visualize the uptake of chemokine by MSG epithelial cells, we used immunofluorescence labeling with anti-chemokine Abs. Confocal microscopy demonstrated that most cells internalized the chemokine after 5 min (Fig. 4, *B* and *C*).

CXCL10 scavenging is not due to CXCR3-containing microparticles

Microparticles released from the surface of various cell types contain cell surface protein and cytoplasmic components of the original cell (26). It has been demonstrated that at least the chemokine receptor CCR5 can be released through microparticles (27). To address the possibility that CXCL10 scavenging depends on CXCR3-containing microparticles, we assessed by Western blot analysis the MSG epithelial cell supernatant after centrifugation for 10 min at 500 $\times$ *g*. There was no CXCR3 in the epithelial cell supernatant. This refutes the possibility that CXCL10 scavenge is due to CXCR3-containing microparticles.

Salivary gland ductal epithelial cells inhibit 300-19/hCXCR3 cell migration

In an effort to confirm our results and assess the biological relevance of these findings, we used a chemotaxis assay to quantify directional migration of 300-19 cells transfected with human CXCR3 (300-19/hCXCR3) toward CXCL10. Epithelial cells strikingly down-regulated 300-19/hCXCR3 cell migration. This inhibitory effect was reversed by the addition of anti-CXCR3 mAb 49801.111 (Fig. 4*F*).

SS salivary gland ductal epithelial cells display defective scavenging of CXCL10

Using cultured MSG epithelial cells from SS patients, we investigated whether these cells have the capability of healthy control cells to scavenge CXCL10. In the supernatants from unstimulated SS epithelial cells, low levels of CXCL11 comparable to those observed in controls were detected, whereas CXCR3 protein expression was slightly reduced. In contrast the fraction of scavenging over a period of 2 h was reduced and ranged from 21 to 70% (Fig. 4*A*). Immunofluorescence confocal microscopy confirmed this observation, showing reduced CXCL10 internalization 5 min after loading cultured cells with the chemokine (Fig. 4, *D* and *E*). Moreover, as expected on the basis of these results, chemotaxis assay showed that 300-19/hCXCR3 cells migrate vigorously toward the supernatants of CXCL10 even in the presence of SS epithelial cells (Fig. 4*F*). This indicates that SS ductal epithelial cells have lost their ability to scavenge CXCR3 agonists.

Discussion

Our results have shown that MSG ductal epithelial cells express CXCR3. This receptor is weakly expressed as a cell surface protein by all cells within the homogeneous population of cultured cells. Western blotting experiments confirm CXCR3 expression at the protein level. Notably, the receptor is differently glycosylated in MSG epithelial cells compared with chronic lymphocytic leukemia cells. However, as reported in a recent paper by Ehlert et al. (28),

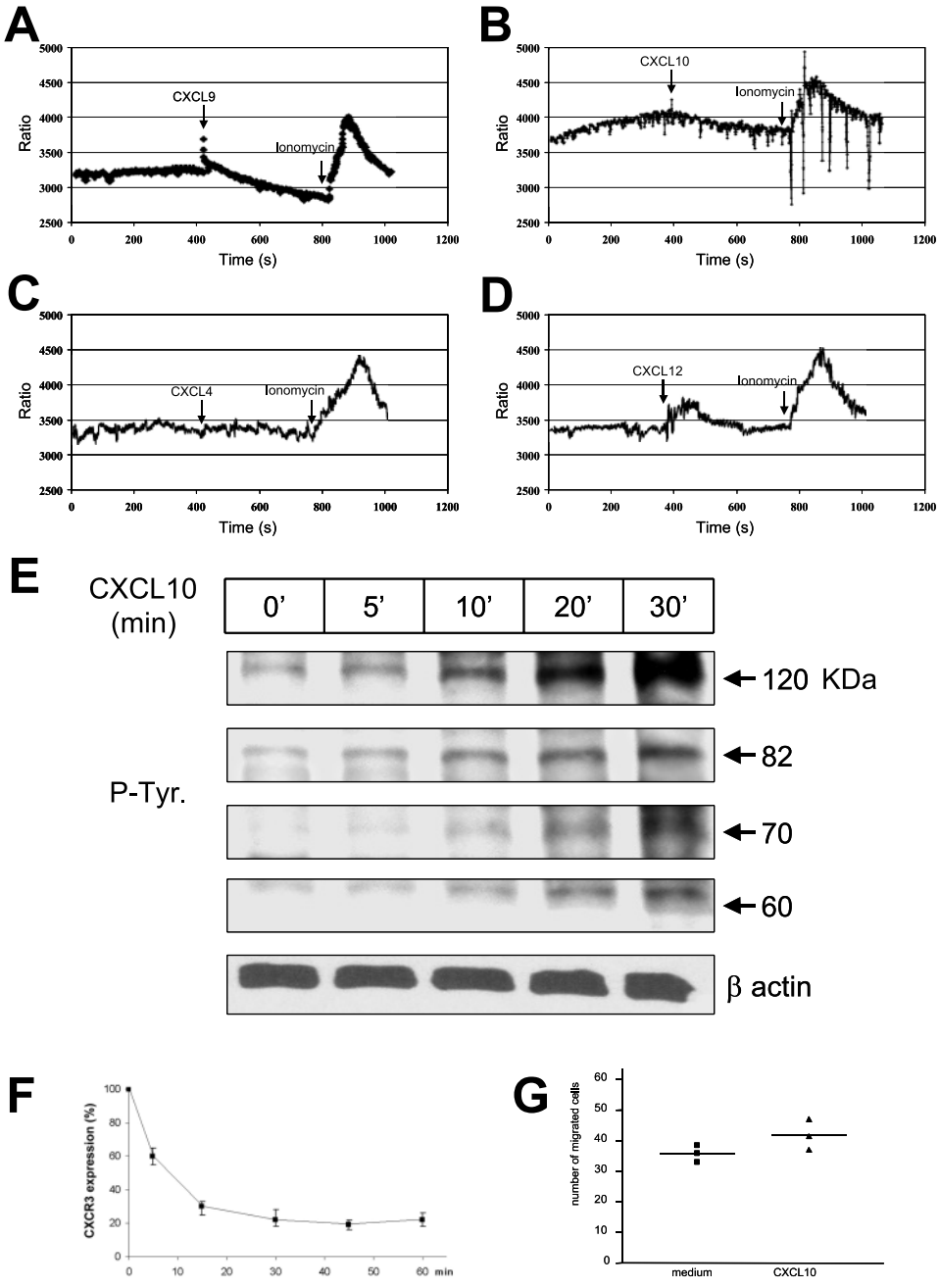

FIGURE 3. CXCL4, CXCL9, and CXCL10 effects on epithelial CXCR3. *A–D*, Calcium flux in MSG epithelial cells in response to CXCL4, CXCL9, CXCL10, and CXCL12. Aliquots of fura-2-AM-loaded cells were stimulated with 1 μg/ml CXCL4, CXCL9, CXCL10, and CXCL12. Results show that CXCL9 (*A*), CXCL10 (*B*), and CXCL4 (*C*) do not induce elevation of intracellular calcium, whereas an increase was observed after CXCL12 addition (*D*). Ionomycin is an ionophore used as a positive control for calcium mobilization. *E*, Analysis of protein tyrosine phosphorylation in MSG ductal epithelial cells. Cells were incubated with 200 ng/ml CXCL10 for the indicated time, then lysed, and the proteins were analyzed by Western blotting and immunostaining with Ab against phosphorylated tyrosine. The inducible bands that develop over time are presented. Time zero represents cells that were not stimulated with CXCL10. The filter was reprobed with anti-β-actin Ab as a loading control. The data are representative of three experiments performed with six different samples. *F*, Time-dependent internalization of CXCR3 on epithelial cells. MSG ductal epithelial cells were incubated with 500 ng/ml CXCL10 for the indicated times. CXCR3 surface expression was expressed as a percentage of baseline expression using the formula: (MFI of tested cells/MFI of untreated cells) $\times$ 100. Data represent the mean $\pm$ SEM of three independent experiments. *G*, MSG ductal epithelial cells from three different primary cultures were assessed for their ability to migrate in response to CXCL10 (200 ng/ml). The dose was chosen because in preliminary experiments it did not greatly affect cell viability. Data points were assessed in triplicate, and each symbol represents the mean of those responses. The bars indicate the mean response from three donors. Statistical evaluation was performed using ANOVA (p = NS).

it is known that CXCR3 can be differentially glycosylated. Concerning the mechanism that allows changing in glycosylation between different cell types, it is likely that malignant transformation may be associated with abnormal glycosylation. Previous studies have demonstrated that human microvascular endothelial cells express CXCR3-B that, if activated by CXCL10, does not mediate calcium flux, but, rather, inhibits endothelial cell migration. This activity is in contrast with the proliferation and chemotaxis induced by CXCL10 on T cells, a response that is mediated by CXCR3-A. Concerning epithelial cells, a CXCR3-A-mediated chemotactic response to CXCL11 has been recently demonstrated in airway epithelial cells (29). Our study extends these observations by demonstrating that MSG ductal epithelial cells constitutively express CXCR3-B. This chemokine receptor is capable of recognizing its ligands, but does not induce calcium mobilization or chemotaxis on epithelial cells, while retaining the ability to undergo postligand internalization.

A number of conditions have been described in which cells expressing chemokine receptor fail to respond to appropriate agonists. There are three chemokine-binding molecules, with homology to chemokine receptors, but defective in signaling function, i.e., D6, DARC, and CCX-CKR, which are classified as silent chemokine receptors (30–32). Despite exhibiting high affinity interactions with chemokines, these receptors are not coupled to the major signaling pathways that are activated by other chemokine receptors upon ligand stimulation and thus do not mediate cell migration. Recently, Fra et al. (33) demonstrated that D6 binds and scavenges most inflammatory chemokine agonists at CCR1 through CXCR5. These data together with the restricted expression of D6 on endothelial cells lining afferent lymphatics suggest that D6 acts as a gatekeeper to prevent excessive transfer of inflammatory chemokine to lymph nodes. Similarly, DARC and CCX-CKR may act as a chemokine buffering and scavenging system, which can internalize their ligands without inducing any detectable signal and target them for degradation (34).

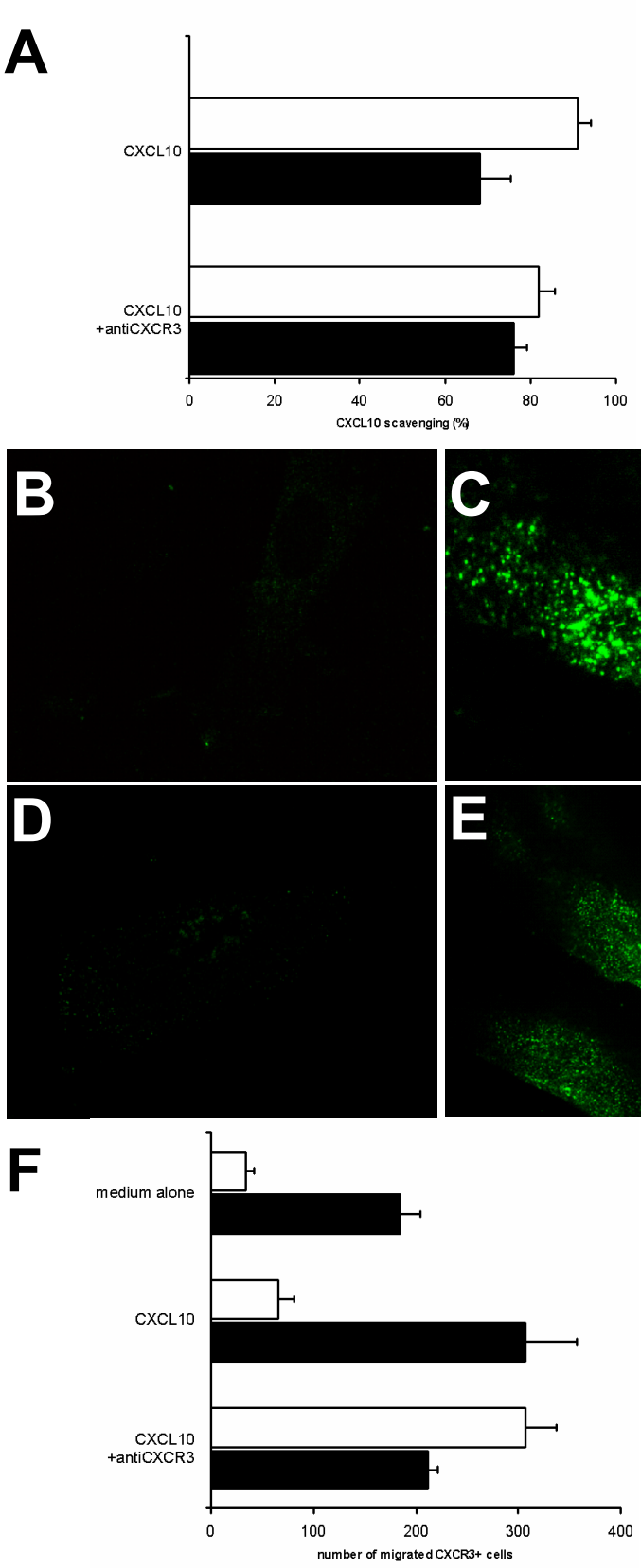

FIGURE 4. Scavenging of CXCL10 and inhibition of 300-19/hCXCR3 cells migration by MSG epithelial cells. *A*, Scavenging of CXCL10 by normal MSG ductal epithelial cells (□) and MSG ductal epithelial cells from SS patients (■). Epithelial cells were either untreated or treated with 10 μg/ml CXCR3-blocking mAbs (clone 49801.111; R&D Systems) 30 min before the addition of 200 ng/ml CXCL10. Scavenging was assessed at 5 min by monitoring the disappearance of CXCL10 by ELISA. Data shown are the mean ± SEM of four separated experiments. *B–E*, Immunofluorescence confocal laser scanning microscopy analysis shows the distribution of CXCL10 in the cytoplasm of normal MSG ductal epithelial cells before (*B*) and 5 min after (*C*) loading of cultured cells with CXCL10 (200 ng/ml). MSG ductal epithelial cells from SS patients (*D* and *E*) display reduced CXCL10 internalization 5 min after loading of cultured cells with the chemokine. The z-plane is a 0.5-μm-thick confocal section, running through the cytoplasm of the cells. *F*, Functional effect on 300-19/human CXCR3 cells chemotaxis of normal MSG ductal epithelial cells (□) and MSG ductal epithelial cells from SS patients (■). The supernatant of MSG ductal epithelial cells, either unexposed or exposed to 200 ng/ml CXCL10 for 5 min, was placed in the lower Boyden chamber. 300-19/hCXCR3 cells were added to the upper chamber and allowed to migrate for 120 min at 37°C. In blocking experiments, epithelial cells were preincubated before the addiction of CXCL10 for 30 min with anti-human CXCR3 mAb (clone 49801.111; R&D Systems) at a concentration of 10 μg/ml. Results are expressed as the number of migrated cells in three fields and are representative of five experiments.

The majority of these decoy receptors are expressed by endothelial cells, where they act as chemokine sink or transporter, thus contributing to maintain the homeostatic levels of chemokines in the surrounding microenvironment. Our data suggest that CXCR3-B expression by epithelium is also involved in the maintenance of tissue homeostasis. Epithelial tissues are continuously exposed to a wide variety of pathogens. The mammalian immune system has developed intricate mechanisms to provide defense at

epithelial surfaces and to control potentially harmful responses to normal symbiotic flora and low virulence pathogens. Epithelial cells are important players in the homeostatic trafficking of lymphocyte subsets through the secretion of tissue-specific chemokines (35). It has been demonstrated that chemokine proteins can be directed to intracellular storage depots. Secretion of these stores enables rapid initiation of chemokine-driven responses without the delay required to initiate transcription (34). Just the opposite, leukocyte recruitment should be tightly regulated by the absorption of chemokines to avoid excessive inflammatory cell infiltration with subsequent damage of the adjacent nondistressed cells.

As the ductal epithelial cells simultaneously express a chemokine receptor and its ligand, we hypothesized that ligand binding may activate an autocrine or juxtacellular loop. Our data demonstrated that CXCR3 on epithelial cells does not mediate cell migration or calcium flux, although it participates in tyrosine phosphorylation. The kinetics of protein tyrosine phosphorylation appear to be slow compared with receptor internalization and CXCL10 scavenging. However, it is known that chemokine receptor internalization and receptor-mediated signaling share only partly common signal transduction steps. In addition, the fate of the receptor after ligand stimulation may affect the length, strength, and type of intracellular signals generated (36). Therefore, the increased intracellular tyrosine phosphorylation, although suggesting that CXCR3 on MSG epithelial cells is able to signal, is not necessarily linked to chemokine trapping and receptor internalization. In this view, because CXCL10, like many others chemokines, binds to cell surface glycosaminoglycans, it is possible that MSG ductal epithelial cells trap CXCL10 on their surface in a receptor-independent manner, thus removing relevant amounts of chemokine. Indeed, it has been recently demonstrated that glycosaminoglycans on CXCR3-expressing cells are not required for ligand binding and signaling, whereas glycosaminoglycans on non-CXCR3-bearing cells may retain and sequestrate CXCL10 close to its site of secretion (37). Finally, because we have shown that the scavenging function is in part reversed by pretreatment of epithelial cells with a CXCR3-blocking Ab (indicating the direct role of epithelial CXCR3 in CXCL10 scavenge), it is also possible that expression of IFN-γ-induced chemokine-scavenging receptors may help the innocent bystander epithelial cells avoid immune attacks by lymphocyte infiltration. The hypothesis that CXCR3-B on epithelial cells acts as a molecular trap for the agonists, down-regulating type 1-dominated inflammatory processes, has been documented in this report by a number of data in both normal subjects and SS patients. We first investigated whether epithelial cells down-regulate the ability of CXCL10 to induce the migration of 300-19 cells transfected with human CXCR3 (300-19/hCXCR3). As expected, normal MSG ductal epithelial cells strikingly down-regulate chemotaxis toward CXCL10. Next, we used cultured MSG ductal epithelial cells from SS patients. The molecular basis for the directed migration of autoreactive T cells leading to epithelitis in SS is presently unknown. It has recently been proposed that IFN-γ stimulates the production of CXCL9, -10, and -11 in the SS ductal epithelium, and that CXCL9, -10, and -11 are involved in the accumulation of T cell infiltrates in the SS salivary glands (38, 39). Using cultured MSG epithelial cells from SS patients we have shown that the scavenging of CXCL10 is reduced, leading to a striking loss of the ability of these cells to inhibit cell migration. Thus, it is possible that the impairment of this scavenging function might favor chemotaxis, leading to an excessive recruitment of CXCR3-positive T lymphocytes. Therefore, we hypothesize that antagonizing this receptor could have great potential to provide novel therapeutic agents for the treatment of SS.

In conclusion, our results show that salivary gland epithelial cells constitutively express CXCR3-B. We also show that this receptor scavenges CXCL10 from the supernatant without mediating calcium flux or chemotaxis. Finally, we demonstrated that in SS, this scavenging function is impaired so that MSG epithelial cells are not able to inhibit chemotaxis.

Disclosures

The authors have no financial conflict of interest.

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