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CXC Chemokine Receptor 4 Expression and Function in Human Astrogliaoma Cells

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Chemokines constitute a superfamily of proteins that function as chemoattractants and activators of leukocytes. Astrocytes, the major glial cell type in the CNS, are a source of chemokines within the diseased brain. Specifically, we have shown that primary human astrocytes and human astrogliaoma cell lines produce the CXC chemokines IFN-γ-inducible protein-10 and IL-8 and the CC chemokines monocyte chemoattractant protein-1 and RANTES in response to stimuli such as TNF-α, IL-1β, and IFN-γ. In this study, we investigated chemokine receptor expression and function on human astrogliaoma cells. Enhancement of CXC chemokine receptor 4 (CXCR4) mRNA expression was observed upon treatment with the cytokines TNF-α and IL-1β. The peak of CXCR4 expression in response to TNF-α and IL-1β was 8 and 4 h, respectively. CXCR4 protein expression was also enhanced upon treatment with TNF-α and IL-1β (2- to 3-fold). To study the functional relevance of CXCR4 expression, stable astrogliaoma transfectants expressing high levels of CXCR4 were generated. Stimulation of cells with the ligand for CXCR4, stromal cell-derived factor-1α (SDF-1α), resulted in an elevation in intracellular Ca2+ concentration and activation of the mitogen-activated protein kinase cascade, specifically, extracellular signal-regulated kinase 2 (ERK2) mitogen-activated protein kinase. Of most interest, SDF-1α treatment induced expression of the chemokines monocyte chemoattractant protein-1, IL-8, and IFN-γ-inducible protein-10. SDF-1α-induced chemokine expression was abrogated upon inclusion of U0126, a pharmacological inhibitor of ERK1/2, indicating that the ERK signaling cascade is involved in this response. Collectively, these data suggest that CXCR4-mediated signaling pathways in astrogliaoma cells may be another mechanism for these cells to express chemokines involved in angiogenesis and inflammation. The Journal of Immunology, 2001, 166: 2695–2704.
pathogenesis of neurologic diseases such as multiple sclerosis and HIV-1-associated dementia (for review, see Refs. 10–12), as well as in animal models of CNS disease, including experimental allergic encephalomyelitis, mechanical injury/trauma, ischemia, virus-induced demyelination, and SIV-induced encephalitis (13–16). Astrocytes, the major glial cell type in the CNS, have been shown to be a predominant source of chemokine production within the diseased brain (17–19). A growing literature also indicates that astrocytes are activated in vitro to produce chemokines such as RANTES, IL-8, IP-10, MCP-1, MIP-1α, and MIP-1β (20–23). Thus, astrocytes serve as an important source of chemokines within the CNS.

CXC chemokines bind to seven-transmembrane domain receptors (CXCR1 to CXCR5) that are coupled to heterotrimeric G proteins (for reviews, see Refs. 3 and 24). Signaling by CXC chemokines leads to an increase in intracellular calcium, tyrosine phosphorylation, and activation of mitogen-activated protein kinases (MAPKs) (25–28). Little is known about the role of CXC chemokines in the CNS, although several CXC chemokine receptors are expressed in the brain; among these is CXCR4 (29–33). CXCR4 is involved in the directional migration of immune cells in response to its exclusive ligand SDF-1. Furthermore, CXCR4 functions as a coreceptor with CD4 in the binding and fusion of HIV-1, specifically interacting with the viral protein gp120 (34–36). CXCR4 is expressed in the CNS by neurons, astrocytes, endothelial cells, and microglia (27, 30, 32, 37–42). CXCR4 is overexpressed in astrogloma tumors, and SDF-1 and CXCR4 expression is colocalized when both are expressed (43–45). Furthermore, SDF-1 and CXCR4 expression increases with increasing tumor grade (45). Mice with a targeted mutation in CXCR4 or SDF-1 have a defect in migration of cerebellar granule cells, indicating that CXCR4 signaling can mediate migration of neuronal progenitors (46, 47). SDF-1 binding to CXCR4 leads to a number of functional effects on cells of the CNS, including calcium mobilization and microglia migration (37–41).

In this study, we have examined the expression of CXCR4 by human astrogliaoma cells in response to a variety of stimuli, including LPS, TNF-α, IL-1β, IFN-γ, IL-6, IL-4, and IL-10. Our results demonstrate that TNF-α or IL-1β treatment of astrogliaoma cells induces CXCR4 expression at both the mRNA and protein level, leading to increased responsiveness to SDF-1α. To assess the functional importance of CXCR4 expression, astrogliaoma transfectants were generated that expressed high levels of CXCR4. Stimulation of these cells with SDF-1α led to intracellular Ca2+ concentration ([Ca2+]i) mobilization and selective activation of the extracellular signal-regulated kinase 2 (ERK2) MAPK pathway. Importantly, SDF-1α treatment also resulted in the induction of MCP-1, IL-8, and IP-10 chemokine gene expression in CXCR4 stable transfectants. CXCR4-mediated signaling in astrogliaoma cells provides another pathway for these cells to express chemokines involved in angiogenesis and inflammation.

### Materials and Methods

#### Cells

CRT-J cells, a subclone of the CRT-MG human astrogliaoma cell line, were maintained in RPMI media with 2 mM l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated FBS (HI-FBS), as described previously (48). U87-MG human astrogliaoma cells were maintained in HAM’s/F-12 DMEM medium with 2 mM l-glutamine, 100 U/ml g/ml streptomycin, and 10% heat-inactivated FBS (HI-FBS), as described previously (48). U87-MG human astrogliaoma cells were maintained in HAM’s/F-12 DMEM medium with 2 mM l-glutamine, 100 U/ml

#### FIGURE 1. TNF-α and IL-1β induction of CXCR4 mRNA expression by human astrogliaoma cells. A, CRT-J cells were incubated with medium alone (lane 1), LPS (1 μg/ml; lane 2), TNF-α (10 ng/ml; lane 3), IL-1β (4 ng/ml; lane 4), IFN-γ (200 U/ml; lane 5), H-IL-6 (10 ng/ml; lane 6), IL-4 (10 ng/ml; lane 7), or IL-10 (10 ng/ml; lane 8) for 10 h, then RNA was isolated and analyzed for CXCR4 mRNA expression by RPA. B, Values for CXCR4 mRNA were normalized to GAPDH mRNA levels for each experimental condition. Quantification of the experiment shown in A is depicted. Constitutive expression of CXCR4 mRNA was set at 1, and cytokine treatments were compared with control levels to arrive at the fold induction value. Representative of three experiments. C, CRT-J cells were incubated with medium alone (lane 1) or with TNF-α (10 ng/ml; lanes 2–7) for various time periods (1–24 h). RNA was analyzed by RPA for CXCR4 and GAPDH mRNA expression. D, Quantification of the experiment shown in C is depicted. Constitutive expression of CXCR4 mRNA was set at 1, and TNF-α treatment was compared with control levels to arrive at the fold induction value. Representative of three experiments.
penicillin, 100 μg/ml streptomycin, and 10% HI-FBS, as previously described (23). For passage, monolayers were rinsed with PBS, and then dislodged by trypsinization (0.25% trypsin, 0.02% EDTA).

**Reagents**

Human recombinant TNF-α and IL-1β were purchased from Genzyme (Cambridge, MA), and human recombinant IL-4, IL-10, and SDF-1α were purchased from R&D Systems (Minneapolis, MN). Hybrid-IL-6 (H-IL-6) was the generous gift of Dr. S. Rose-John (University of Mainz, Mainz, Germany). Human rIFN-γ was the generous gift of Biogen (Cambridge, MA). LPS was from Sigma (St. Louis, MO). P.c.fusin plasmid containing human CXCR4 cDNA was generously provided by the National Institutes of Health AIDS Research and Reference Reagent Program (Rockville, MD) (49). Mouse anti-human CXCR4 mAb and anti-human SDF-1α-neutralizing Ab were purchased from R&D Systems. Mouse IgG2a and goat anti-mouse IgG2a-FITC were purchased from Southern Biotechnology Associates (Birmingham, AL). Rabbit anti-ERK2, anti-p38 MAPK, and anti-c-Jun N-terminal kinase (JNK)/stress-activated protein kinase Ab were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The MAP/ERK kinase 1 (MEK1) inhibitor U0126 was from Promega (Madison, WI). Pertussis toxin (PTX) and cholera toxin (CTX) were purchased from Sigma. Myelin basic protein (MBP) and GST-c-jun for the in vitro kinase assays were from Sigma and Stratagene (La Jolla, CA), respectively. HRP-conjugated secondary anti-rabbit IgG Ab and ECL kits were purchased from Amersham-Pharmacia Biotech (Little Chalfont, U.K.).

**FIGURE 2.** TNF-α and IL-1β induction of CXCR4 protein expression. CRT-J cells and U87-MG cells were incubated with medium alone, TNF-α (10 ng/ml), IL-1β (4 ng/ml), or both cytokines for 72 h. The cells were then trypsinized, and CXCR4 protein expression was assessed by FACS analysis. A representative experiment of three experiments is shown.

**FIGURE 3.** Measurement of [Ca²⁺], in cultured CRT-J astrogliaoma cells in response to SDF-1α. CRT-J cells were incubated without or with TNF-α (10 ng/ml) for 72 h. The cells were trypsinized and loaded with 2 μM fura 2-AM for 60 min at 37°C, and then SDF-1α (50 ng/ml) was added to the cell suspension (the time of addition is indicated by arrows). [Ca²⁺], measurements were determined as described in Materials and Methods. A representative experiment of three is shown.

**RNA isolation, riboprobes, and RNase protection assay (RPA)**

Total cellular RNA was isolated from cell monolayers that were incubated for various time periods with the different cytokines/chemokines. Briefly, cells were lysed directly in the culture dish. RNA was extracted with guanidinium isothiocyanate and phenol, and precipitated with ethanol, as previously described (23).

Linearized human chemokine/chemokine receptor multiprobe sets (hCK-5, catalog 45035P and hCR-6, catalog 45376P; PharMingen, San Diego, CA) were in vitro transcribed with T7 RNA polymerase, resulting in 10 antisense RNA probes. The probes generated from the kits are as follows: for the hCK5 set, lymphotactin, RANTES, IP-10, MIP-1β, MIP-1α, MCP-1, IL-8, I-309, L32, and GAPDH. For the hCR6 set, CXCR1, CXCR2, CXCR3, CXCR4, BLR-1, BLR-2, V28, L32, and GAPDH.

RPA was conducted with a RPA kit according to the manufacturer’s instructions (PharMingen), as previously described (23). Briefly, 20 μg of total cellular RNA was hybridized with hCK5 riboprobes (3.1 × 10⁵ cpm) or hCR6 riboprobes (2.1 × 10⁵ cpm) and treated with RNase A/T1 (1/200 dilution in 200 μl of RNase digestion buffer) at 30°C for 1 h, then RNA was precipitated and analyzed by 5% denaturing (8 M urea) PAGE. The gels were exposed to x-ray film, and quantification of the protected RNA fragments was performed by scanning with the PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Values for each chemokine/chemokine receptor mRNA were normalized to GAPDH mRNA levels for each experimental condition.

**FIGURE 4.** Stable expression of CXCR4 in CRT-J and U87-MG astrogliaoma cells. CXCR4 cDNA was subcloned into the pcDNA3 expression vector and was transfected into CRT-J or U87-MG cells by either electroporation or lipofectamine. G418-containing medium was used for selection of transfected cells. CRT-J13 and U87-T37 stable transfectants were chosen based on CXCR4 expression, as determined by flow cytometry. Profile 1 in A and B shows the isotype control, and profile 2 in A and B shows positively stained cells with anti-CXCR4 Ab. A representative experiment of five experiments is shown.
Analysis of CXCR4 receptor expression by immunofluorescence flow cytometry

Human CRT-J and U87-MG astroglia cells (2 × 10^6/well) were plated in six-well (35 mm²) plates (Costar, Cambridge, MA) and grown to ~80% confluency. The cells were incubated with medium alone, TNF-α, IL-1β, or a combination of both cytokines for 72 h. Cells were trypsinized, suspended in PBS containing 1% BSA and 0.05% azide, blocked with normal rabbit serum (1 μg/ml) for 15 min at room temperature, and stained with mouse anti-human CXCR4 (1/10 dilution) mAb for 1 h on ice, washed twice, and then were stained with FITC-conjugated goat anti-mouse Ab (1/50 dilution). After washing three times, cells were fixed in 1% paraformaldehyde and analyzed on the FACSstar (Becton Dickinson, Mountain View, CA) for CXCR4 expression. Negative controls were incubated with an isotype-matched (mouse IgG2a) control mAb. Ten thousand cells were analyzed for each sample.

Stable transfection and FACS analysis

Human CXCR4 cDNA was subcloned into the BamHI sites of the pcDNA3 expression vector. CRT-J and U87-MG (1 × 10^5 cells) were transfected by either lipofectamine or electroporation with pcDNA3 plasmid containing CXCR4 cDNA and the geneticin (G418)-resistant marker (20 μg). The CXCR4 transfectants were grown in medium containing 0.5 mg/ml G418 (Life Technologies, Rockville, MD). Mock cDNA, which has only the pcDNA3 plasmid, was used as a negative control. Cell surface expression of CXCR4 on the transfectants was confirmed by FACS analysis, as described above. The CRT-J13 and U87-T37 transfectants showed the best response to SDF-1α, as assessed by an increase in [Ca^{2+}], (data not shown), and were selected from more than 60 clones grown through the limiting dilution method for further analysis.

Measurements of [Ca^{2+}]_i

Parental U87-MG astroglia cells and U87-T37 stable transfectants (1 × 10^6 cells/ml) were resuspended in HBSS supplemented with 1% FBS and 1.25 mM CaCl_2, and then incubated with 2 μM fura 2-AM (Molecular Probes, Eugene, OR) for 30 min at 37°C. Subsequently, excess fura 2-AM was removed by washing the cells twice, and the cells were resuspended in 2 ml of HBSS containing 1% FBS and 1.25 mM CaCl_2 to a final concentration of 1 × 10^6 cells/ml. [Ca^{2+}]_i was measured using a spectrofluorometer (PTI System QM-1) with alternating excitation at 340 and 380 nm, and fluorescence emission at 510 nm. After a baseline level of [Ca^{2+}]_i was established, the cells were treated with various concentrations of SDF-1α or bradykinin (BK; 2 μg/ml) as a positive control. When a stable peak of [Ca^{2+}]_i in response to SDF-1α was achieved, the cells were permeabilized with 0.1% digitonin. EGTA was then added to a final concentration of 5 mM. The digitonin and EGTA measurements were used to calibrate [Ca^{2+}]_i vs fura 2-AM fluorescence in each sample using a calibration equation originally described by Grynkiewicz et al. (50).

Measurement of chemokine production

CRT-J13 and U87-T37 cells (3 × 10^5/well) were incubated with medium alone or various concentrations of SDF-1α for 24 h, then supernatants were collected, centrifugated, and stored at ~70°C until use. MCP-1 and IL-8 in culture supernatants were quantitated using a dual-Ab solid-phase ELISA (Biosource International, Camarillo, CA), as previously described (23). The minimal detection limit for the MCP-1 ELISA is 20 pg/ml, and for IL-8 is 10 pg/ml.

In vitro kinase assay

Soluble lysates (100–300 μg) were used to phosphorylate MBP or GST c-jun, as previously described (51). Lysates were incubated with 1 μg of anti-p38, anti-ERK2, or anti-JNK/stress-activated protein kinase MAPK Ab overnight at 4°C, followed by an additional 1-h incubation with protein A/G gel beads (Pierce, Rockford, IL). The immunocomplexes were washed, then incubated for 20 min at 30°C in 20 μl of kinase buffer containing 20 μM ATP, 5 μCi [γ-32P]ATP, and 10 μg MBP as the substrate for ERK and p38, and 2 μg of GST c-jun for JNK. Substrate phosphorylation was stopped by boiling in sample buffer, followed by 12% SDS-PAGE and autoradiography. To assess for changes in the activation of each protein kinase, levels of phosphorylated proteins were normalized relative to the total MAPKs detected in each sample. The density of the bands was quantified by a UMAXScan desktop scanner (Fremont, CA) using National Institutes of Health Image software.

Statistical analysis

Levels of significance for comparisons between samples were determined using Student’s t test distribution.

Results

CXCR4 expression is enhanced by the cytokines TNF-α and IL-1β

We were interested in determining whether CXCR4 receptor expression on astroglia cells could be modulated by cytokines known to be present in the diseased CNS. To initiate this study,
FIGURE 6. SDF-1α activates ERK MAPK, but not p38 or JNK MAPK. A, CRT-J13 cells (2 × 10⁶) were stimulated with SDF-1α (50 ng/ml) for various time periods (0–60 min), lysed, and immunoprecipitated with anti-human ERK2, anti-human JNK Abs, and then used for in vitro kinase assay using MBP as the substrate for ERK and p38, or GST c-jun for JNK, as described in Materials and Methods. Representative of two experiments. B, CRT-J13 cells (2 × 10⁶) were pretreated without or with PTX (0.1 μg/ml) or CTX (0.1 μg/ml) for 16 h at 37°C, then stimulated with SDF-1α (50 ng/ml) for 20 min, lysed, immunoprecipitated with anti-human ERK2 Abs, and then used for the in vitro kinase assay using MBP as the substrate for ERK. Representative of two experiments.

two human astroglioma cell lines, CRT-J and U87-MG, were treated with the proinflammatory cytokines TNF-α, IL-1β, and IFN-γ; the antiinflammatory cytokines IL-4 and IL-10; the hybrid molecule of the soluble IL-6R and IL-6 called H-IL-6 (52); or LPS for 10 h. The cells were harvested and CXCR4 mRNA expression was analyzed by multiprobe RPA. As shown in Fig. 1A, low constitutive CXCR4 mRNA expression was detected (lane 1), and expression increased ~2-fold upon TNF-α or IL-1β treatment (lanes 3 and 4). However, CXCR4 mRNA expression was not influenced by LPS or the other cytokines (IFN-γ, H-IL-6, IL-4, and IL-10) (lanes 2 and 5–8). The kinetics of TNF-α-enhanced CXCR4 mRNA expression were next examined. As shown in Fig. 1, C and D, CXCR4 mRNA expression is enhanced at 2 h, peaks 8 h after TNF-α treatment (~5-fold), and remains elevated 12–24 h after exposure to TNF-α. Comparable results were seen in U87-MG cells (data not shown). Astroglioma cells did not constitutively express mRNA for any of the other CXC receptors (CXCR1, CXCR2, CXCR3), nor was expression induced upon cytokine or LPS treatment (data not shown).

CXCR4 protein expression upon TNF-α or IL-1β treatment on astroglial cells was examined by flow cytometry (Fig. 2). CRT-J and U87-MG cells were treated with TNF-α, IL-1β, or a combination of both cytokines for 72 h, then CXCR4 surface expression was analyzed. In unstimulated CRT-J and U87-MG cells, 9.6% and 23.8%, respectively, were CXCR4 positive. Upon TNF-α treatment, 41.2% of CRT-J and 60.6% of U87-MG cells became CXCR4 positive. IL-1β stimulation also enhanced CXCR4 expression (28.8% for CRT-J and 41.6% for U87-MG cells). The combined treatment of CRT-J cells with TNF-α plus IL-1β for 72 h had an additive effect on CXCR4 induction (61.9% positive for CXCR4). In contrast, in the U87-MG cells, TNF-α and IL-1β did not exhibit an additive effect. CXCR4 protein expression was also detected by immunofluorescence analysis (data not shown).

To determine whether CXCR4 expressed on astroglial cells is functional, we tested the modification of [Ca²⁺]i concentration in U87-T37 cells, whereas no increase was detected in U87-MG parental cells or U87-mock-transfected cells. CRT-J13 cells (2 × 10⁶), were chosen based on high CXCR4 expression, as determined by flow cytometry (see Fig. 4). Ca²⁺ mobilization following ligand stimulation is a characteristic of most chemokine receptors. The effect of SDF-1α on intracellular Ca²⁺ modification was determined in U87-T37 and CRT-J13 cells. SDF-1α increased the [Ca²⁺]i concentration in U87-T37 cells, whereas no increase was detected in U87-MG parental cells or U87-mock-transfected cells (Fig. 5A). CRT-J13 cells also showed a similar pattern as that of U87-T37 cells (data not shown). As a positive control for Ca²⁺
influx, cells were treated with BK. The effect of SDF-1α was dose dependent, with a clear increase in [Ca$^{2+}$], observed at a SDF-1α concentration of 5 ng/ml, and optimal effects noted at 50 ng/ml (Fig. 5B). To establish the specificity of this effect, neutralizing Ab to SDF-1α was used, which abolished the SDF-1α-mediated Ca$^{2+}$ response (Fig. 5C). Anti-human TGF-β1 Ab had no effect in this system (Fig. 5C), indicating that the increase in [Ca$^{2+}$], was induced by SDF-1α through CXCR4.

The CXCR4 receptor couples to Gαi proteins, and SDF-1-induced responses can be inhibited by PTX (27, 28, 37, 53). Thus, we tested whether PTX treatment would inhibit SDF-1α-induced Ca$^{2+}$ mobilization in U87-T37 cells. As shown in Fig. 5D, PTX pretreatment blocked SDF-1α-induced Ca$^{2+}$ mobilization, while CTX had no effect. These results indicate that Gαi proteins couple to the CXCR4 receptor in astroglioma cells to control intracellular Ca$^{2+}$ homeostasis.

**SDF-1α-mediated activation of the MAPK signaling cascade**

We next determined whether SDF-1α stimulation could induce the activation of ERK1/2, p38, and/or JNK MAPK in the CXCR4 stable transfectants. CRT-J13 or U87-T37 cells were stimulated with SDF-1α for various periods of time (0–60 min), cells were lysed, and subjected to immunoprecipitation with anti-ERK2, anti-p38, or anti-JNK Ab, and then in vitro kinase assays were performed, as previously described (51). MBP was used as the substrate for ERK2 and p38, and GST c-jun as the substrate for JNK. Fig. 6A illustrates that phosphorylation of MBP by ERK2 kinase was seen after 1 min of stimulation with SDF-1α (lane 2), reached a maximum level after 30 min (lane 5), and was sustained for 60 min (lane 7). SDF-1α did not activate p38 or JNK MAPK. Pretreatment of cells with PTX, but not CTX, inhibited SDF-1α-induced ERK2 activation (Fig. 6B, lanes 3 and 4), indicating that this signaling was mediated by Gαi proteins.

**SDF-1α induction of chemokine expression in astroglialoma cells**

Recent studies have suggested that MAPKs play an important role in the signal cascades that lead to the induction of various inflammatory molecules, including chemokines (54). As we demonstrated that SDF-1α stimulation of astroglialoma cells led to the activation of ERK2 MAPK, we wished to further determine whether SDF-1α stimulation could induce chemokine expression in these cells. CRT-J13 cells were stimulated with SDF-1α for various time periods (0–12 h), then chemokine mRNA expression was assessed using multiprobe RPA. As shown in Fig. 7A, mRNA induction of IP-10 and IL-8, two CXC chemokines, was detected as early as 1 and 2 h (lane 2 for IL-8, and lane 3 for IP-10); reached a maximum at 4 h (lane 4 for IL-8) and 8 h (lane 5 for IP-10); and was sustained until 12 h (lane 6). mRNA encoding MCP-1, a CC chemokine, was constitutively expressed (lane 1), and upon stimulation with SDF-1α, MCP-1 mRNA expression was enhanced, reaching maximum levels at 8 h (lane 5). Quantification of the data is shown in Fig. 7, B–D. Similar results were observed in U87-T37 cells (data not shown). The astroglialoma cells did not constitutively express mRNA for the other chemokines that can be detected by this multiprobe RPA kit (lymphotactin, RANTES, MCP-1, and IP-10).

**FIGURE 7.** SDF-1α induction of chemokine expression. A, CRT-J13 cells were incubated with medium alone (lane 1) or with SDF-1α (50 ng/ml; lanes 2–6) for various time periods (1–12 h). RNA was analyzed by RPA for IP-10, MCP-1, IL-8, and GAPDH mRNA expression. B, C, and D, Values for each chemokine mRNA were normalized to GAPDH mRNA levels for each experimental condition. Quantification of the experiment shown in A is depicted. Constitutive expression of chemokine mRNA was set at 1, and SDF-1α treatment was compared with control levels to arrive at the fold induction value. Representative of three experiments.
MIP-1α, MIP-1β), and SDF-1α treatment did not induce expression of these chemokines (data not shown).

The ERK1/2 inhibitor U0126 abrogates SDF-1α induction of chemokine expression

To test the involvement of MAPK activation in SDF-1α-induced chemokine expression, we examined the effect of U0126, an inhibitor of MEK1/2, a MAPK that phosphorylates ERK1/2 (55). The cells were pretreated for 20 min with the indicated amounts of U0126 and stimulated with SDF-1α for an additional 4 h, and chemokine mRNA expression was analyzed using multiprobe RPA. SDF-1α-induced IP-10, MCP-1, and IL-8 mRNA expression was inhibited by U0126 in a dose-dependent manner, with inhibition observed using 0.005–5 μM of U0126 (Fig. 8, lanes 4–8). Optimal inhibition was detected using 5 μM of U0126 (lane 8). The diluent DMSO had a negligible effect on chemokine expression (lane 3).

To test whether the results at the mRNA level were reflected at the protein level, cells were stimulated with SDF-1α for 24 h, then supernatants were analyzed for IL-8 and MCP-1 protein expression by ELISA. The chemokines were induced at the protein level, and pretreatment of the cells with U0126 inhibited IL-8 and MCP-1 protein secretion (Fig. 9). Similar results were obtained for IP-10 expression (data not shown). These results collectively indicate that the ERK signaling pathway regulates SDF-1α-induced IP-10, IL-8, and MCP-1 gene expression.

Discussion

In this study, we investigated CXCR4 regulation by cytokines known to be present in the diseased CNS, the functional properties of CXCR4 on astroglioma cells, and the signal transduction pathways activated upon SDF-1 engagement of CXCR4. In the human astrogloma cell lines CRT-J and U87-MG, TNF-α and IL-1β were the only cytokines capable of up-regulating CXCR4 expression, as shown by RPA and flow cytometry. It has also been reported that IL-1β can up-regulate CXCR4 expression on primary human fetal astrocytes (12, 56), indicating that this finding is relevant for both primary astrocyte cultures as well as astrogloma cell lines. Cytokine regulation of CXCR4 expression appears to be cell-type specific; TNF-α also up-regulates CXCR4 expression on PBMCs and eosinophils, while for endothelial cells, TNF-α inhibits its expression in a time-dependent manner (53, 57–59). The cytokine IL-4 strongly up-regulates CXCR4 expression on CD4+ T cells, specifically Th2 cells (57, 60), and inhibits expression on eosinophils (53). Our results indicate that IL-4 has no effect on CXCR4 expression on astrogloma cells, although these cells express functional IL-4R (61). Expression of IL-1 and TNF-α is elevated in astrogloma tumors (for review, see Ref. 62). The ability of TNF-α and IL-1β to up-regulate CXCR4 expression on astrogloma cells (this study) may be important for the growth of these cells because Sehgal et al. (43) have shown that SDF-1 induces proliferation of astrogloma cells.

Engagement of CXCR4 by its ligand SDF-1 leads to a diverse array of biological effects, including chemotaxis, adhesion molecule expression, apoptosis, activation of ion channels, and angiogenesis (37, 45, 63–66). We wished to discern the functional consequences of CXCR4 expression on astroglial cells; thus, stable CXCR4 astrogloma transfectants were generated. The function of CXCR4 was first confirmed by SDF-1 stimulation of [Ca2+]i elevation. Indeed, [Ca2+]i levels were elevated in response to SDF-1α, and abrogated by the inclusion of anti-SDF-1α Ab, demonstrating the specificity of the response. As mentioned previously, a wide array of intracellular signaling events occurs upon SDF-1/CXCR4 interaction. These include tyrosine phosphorylation of FAK, Pyk2, and paxillin (26); activation of phosphatidylinositol 3-kinase (26, 38); activation of MAPKs (26, 27, 63); NF-κB activation (26); and activation of the JAK/STAT signaling pathway (28). Regarding MAPK, we observed selective activation of ERK2 MAPK by SDF-1α in the astroglial cells, with no effects on p38 or JNK MAPK. Activation of MAPKs by SDF-1α also occurs in a cell-type-specific manner; in rat cerebrocortical cultures, SDF-1α activates the p38 MAPK pathway (63).

Given that MAPK activation contributes to the induction of genes involved in inflammation and angiogenesis, including chemokines, we next examined whether SDF-1 stimulation led to the induction of chemokine gene expression in astroglial cells. Indeed, our results demonstrated that SDF-1α enhanced expression of three chemokines, IP-10, IL-8, and MCP-1, in the CXCR4 stable transfectants. SDF-1α-mediated expression of IP-10, IL-8, and MCP-1 was inhibited by U0126, a very potent and highly selective MEK1/2 inhibitor, indicating an involvement of the ERK MAPK pathway in this response. It appears that the signaling pathways ultimately leading to chemokine expression vary depending on the stimulus used. In this regard, we have recently demonstrated that HIV-1 Tat is a potent inducer of MCP-1, IL-8, and IP-10 expression in astrocytes (67). U0126 completely blocked IL-8 induction by Tat, and partially abrogated MCP-1 expression; however, no inhibitory effect was observed on IP-10 expression. In contrast, the p38 MAPK-specific inhibitor SB202190 suppressed IP-10 induction by Tat (67). Thus, numerous signaling pathways can be activated, ultimately leading to chemokine expression.

In this study, we have identified SDF-1 as an important inducer of chemokine expression in astroglioma cells. Regarding potential CNS sources of SDF-1, astrocytes and monocyte-derived macrophages (MDM) constitutively express SDF-1 mRNA transcripts, with higher levels of expression in astrocytes (38). Interestingly, regulation of SDF-1 expression differs in these two cell types; in
MDM, SDF-1 expression was inhibited after HIV-1 infection or LPS treatment of the cells, while in astrocytes, SDF-1 expression was enhanced upon LPS treatment or exposure of cells to conditioned media from HIV-1-infected MDM (38, 39). We have also observed enhanced SDF-1 expression in human astroglia cells upon LPS treatment (data not shown). SDF-1 is detected at low levels in brains from normal controls, is overexpressed in astroglia tumor tissue, and increases with increasing tumor grade (45). SDF-1 was localized to astroglia cells, neurons, and phagocytic cells around vessels in the tumors (45). These findings indicate that astroglia cells can serve as an autocrine source of SDF-1, and other CNS cells (neurons, microglia) also contribute to SDF-1 expression within the brain.

SDF-1/CXCR4 engagement on astroglia cells leads to diverse biological effects, ranging from cell proliferation, chemokine induction, and angiogenesis. There are several possibilities for the participation of SDF-1/CXCR4 in the angiogenic process. SDF-1 stimulation of human endothelial cells induces the production of the angiogenic factor vascular endothelial growth factor (68), while in this study, we have shown that SDF-1 induces IL-8 production by astroglia cells. Both of these angiogenic factors are increased in brain tumors (69, 70). At present, the contribution of IL-8 to CNS angiogenesis is unclear. IL-8 stimulates angiogenesis in the rat and rabbit cornea, suggesting the possibility that IL-8 may also participate in glioma neovascularization (71). Interestingly, the ERL + CXC chemokine IP-10 has been reported to have angiostatic effects to angiogenic factors such as IL-8 on endothelial cells (72). IP-10 is also induced upon SDF-1α stimulation of astroglia cells; however, little is known about the functional relevance of IP-10 in astroglia tumors. It will be interesting to determine whether in fact IL-8 and IP-10 have opposing effects on the angiogenic process.

The other chemokine induced upon SDF-1α stimulation of astroglia cells is MCP-1. MCP-1 is a CC chemokine that induces the migration of monocytes, memory T cells, and NK cells. MCP-1 is expressed by human astroglia in vivo and in vitro (73, 74). Infiltrating macrophages are a common feature of human gliomas, and the extent of macrophage infiltration correlates with the level of MCP-1 expression (74). The functional significance of infiltrating macrophages is not known; however, given the ability of macrophages to produce a wide array of tumoricidal mediators, this may be a mechanism to provoke an immune attack on gliomas.

The importance of CXCR4 in glioma proliferation is controversial. One group has shown that blockade of CXCR4 (by CXCR4 Abs or antisense CXCR4) inhibited the proliferation of human glioblastoma tumors (43, 44), suggesting an important role for CXCR4 in cell transformation. However, Rempel et al. (45) determined by immunohistochemical analysis that SDF-1/CXCR4 expression, and that of the proliferation marker MIB-1, were mutually exclusive. We are currently conducting studies to assess the ability of SDF-1α to induce proliferation of the CXCR4 astroglia transfectants, which should be informative with respect to the role of CXCR4 in astroglia proliferation.

Our results highlight another functional role for SDF-1/CXCR4, that leading to chemokine expression. In this regard, two recent studies have documented the ability of SDF-1 to activate chemokine expression: SDF-1 up-regulated production of the CXC chemokines IL-8 and GRO-α by human colon epithelial cells (75) and MCP-1, IL-8, and GRO-α by human retinal pigment epithelial cells (76). These findings suggest that SDF-1 activation of chemokine expression may occur in a cell-type-specific manner. It is clear, then, that chemokines have broader functional properties than initially anticipated, and with respect to the CNS, are important for both inflammatory and angiogenic events within this organ.

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References


