Interleukin-6 and cAMP Induce Stromal Cell-derived Factor-1 Chemotaxis in Astroglia by Up-regulating CXCR4 Cell Surface Expression

IMPLICATIONS FOR BRAIN INFLAMMATION*

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The chemokine stromal cell-derived factor-1 (SDF-1) and its receptor CXCR4 control the migration of neurons and microglial cells in the central nervous system. Although functional CXCR4 is also expressed by astroglia, recent studies have failed to observe a chemotactic response of these cells to SDF-1. Here, we demonstrate that SDF-1-dependent chemotaxis can be induced by treating cultured cortical astroglia with either dibutyryl cyclic AMP (dbcAMP; 10^{-4} M) or interleukin-6 (IL-6; 10 ng/ml). Flow cytometric analysis revealed that both the dbcAMP- and IL-6-induced onset of SDF-1-dependent chemotaxis of astroglia are due to the increased cell surface expression of CXCR4. In addition, dbcAMP and IL-6 also increased CXCR4 transcript levels, further suggesting that both treatments primarily affect CXCR4 surface expression in astroglia by stimulation of gene expression. Moreover, unlike the case with IL-6 and dbcAMP, which allowed for an optimal chemotactic response to SDF-1 only after 48 h, a similar chemotactic response, associated with an increase in CXCR4 cell surface expression, already occurred after 24 h when astroglial cultures were maintained with medium conditioned by IL-6- or dbcAMP-pretreated astrocytes, indicating that the stimulatory effects of IL-6 and cAMP on CXCR4 cell surface expression involve a secondary mechanism. The findings that elevated extracellular levels of IL-6 or factors positively coupled to cAMP result in increased CXCR4 cell surface expression levels and subsequent SDF-1-dependent chemotaxis in central nervous system astrocytes point to a crucial role of this chemokine during reactive gliosis and human immunodeficiency virus-mediated dementia.

Chemokines are a family of structurally related low molecular mass cytokines crucial to the development of lymphoid tissue and the migration of leukocytes (1). Four subfamilies, designated C, CC, CXC, and CX3C chemokines are distinguished based on the relative position of conserved cysteine residues (2, 3). The CXC chemokine stromal cell-derived factor-1 (SDF-1), which exists in three splice variants, SDF-1α, SDF-1β, and SDF-1γ (4–6), was originally isolated as a pre-B cell stimulatory factor (7). Subsequent studies revealed that SDF-1 plays a critical role in the regulation of trafficking, transendothelial migration, proliferation, and differentiation of hematopoietic cells (3, 8). SDF-1 selectively activates CXCR4, a chemokine receptor initially identified in leukocytes and known to serve as a co-receptor for the entry of T-cell-tropic and dual-tropic HIV into CD4^+ lymphocytes (9, 10). CXCR4 transcripts are abundantly present in hematopoietic cells and have been detected also in non-hematopoietic organs such as lung, heart, and brain (11, 12). In the brain, CXCR4 is constitutively expressed by various neural cell types, including neurons, microglia, and astrocytes (13–15). Recently, great attention was attracted by the capability of CXCR4 to mediate neuronal cell death upon binding of the HIV envelope protein, gp120, a process believed to underlie HIV dementia (16, 17). The understanding of the physiological functions of CXCR4/SDF-1 in the central nervous system (CNS) is just beginning to emerge (18). One crucial role seems to consist in the control of neuronal cell migration. During brain development, CXCR4 is highly expressed in migrating neuroepithelial cells (19). Moreover, inactivation of the genes encoding either CXCR4 or SDF-1 perturbs the migration of granule cells within the developing cerebellum from the outer external to the inner granule layer (20–22). In addition, SDF-1 acts as a potent chemottractant for microglial cells (14), further pointing to a modulatory role of this chemokine during neuroinflammation. Inflammation of the brain leads to reactive gliosis, a process characterized by proliferation and migration of astroglia and the subsequent formation of a glial scar (23). The participation of chemokines in these reactive processes has recently been suggested (18). However, the role of SDF-1 remains elusive. Whereas SDF-1 activates distinct signaling pathways in astroglia (14, 24, 25) and promotes astroglial proliferation (26), a previous study failed to demonstrate a chemotactic response of astrocytes toward SDF-1 (14). In lymphocytes, SDF-1-dependent chemotaxis is modulated via CXCR4 cell surface expression levels (27, 28). Intriguingly, in these cells, CXCR4 cell surface expression level and SDF-1-dependent chemotaxis are enhanced with interleukin-6 (IL-6) and factors positively coupled to cAMP, signals.
previously also found to increase at the site of reactive gliosis (23).

In the present study, we have assessed the putative role of SDF-1/CXCR4 in reactive gliosis by determining how IL-6 and the cell membrane-permeable cAMP analog, dibutylryl cAMP (dbcAMP) affect CXCR4 cell surface expression and SDF-1-dependent chemotaxis in cultured cortical astroglia.

EXPERIMENTAL PROCEDURES

Glial Cultures—Astroglial cultures were initiated from the cerebral hemispheres of postnatal (P1) mouse pups obtained by either mating fos-lacZ transgenic mice (generously provided by Dr. J. Morgan, St. Jude Children’s Research Hospital, Memphis, TN) or fos-lacZ transgenic animals with BALB/c mice (Charles River, Sulzfeld, Germany). A detailed protocol of the cultivation procedure has been recently published elsewhere (29). These cultures predominantly consist of astrocytes (90–95%) as well as small populations of oligodendrocytes and glial progenitor cells and are virtually free of neurons and microglial cells. Briefly, dissected tissue pieces were incubated for 20 min in Ca2+ and Mg2+-free Dulbecco’s phosphate-buffered saline (Invitrogen) containing 0.1% trypsin and 0.02% EDTA. Trypsin action was terminated by transferring tissue pieces to Hank’s balanced salt solution (Invitrogen) supplemented with 10% fetal calf serum (Invitrogen). The tissue was gently dissociated by trituration through a plastic pipette. Cells were plated into 100-mm culture dishes (Costar, Cambridge, MA) coated with poly-L-ornithine (0.1 mg/ml; molecular mass 30–70 kDa; Sigma). Upon reaching confluence, cultured cells were trypsinized and replated. After the second passage, cells obtained from the offspring of c-Fos-lacZ/Fos-lacZ crosses were seeded into 48-well cluster plates (Costar) for subsequent c-Fos assay. Cells obtained from the offspring of c-Fos-lacZ/BALB/c mice were seeded into 100-mm culture dishes; these cells were subsequently used for RT-PCR analysis and migration assay. In both cases, cultures were further maintained with serum-free N2 medium additionally supplemented with the indicated concentration of IL-6 (Prope Tech, Rocky Hill, NJ), dbcAMP (Sigma), or conditioned medium (CM).

c-Fos Assay—Functional SDF-1 signal transduction coupling was assessed by analyzing SDF-1-included c-Fos-lacZ expression as described previously (30). Glial cultures were stimulated for 3 h with SDF-1α (100 ng/ml; PropeTech) and fixed with 2% paraformaldehyde for 30 min. β-Galactosidase activity was visualized by incubating cultures for 24 h at 37 °C with X-Gal (1 mg/ml; Bachem, Heidelberg, Germany).

Chemotaxis—The chemotactic response of cortical glia to chemokines was evaluated using a modified 48-well Boyden chamber (Neuro Probe, Cabin John, MD), as previously described (31). For cell harvest, astrocytes (90–95%) were collected using the PeqGold isolation kit (Peqlab, Schwalbach, Germany) according to the manufacturer’s instructions.

Total RNA isolation and RT-PCR analysis—Total RNA was isolated from cortical glial cultures using the PeqGold isolation kit (Peqlab, Schwalbach, Germany) according to the manufacturer’s instructions.

Total RNA concentration was measured by spectrophotometric absorbance at 260 nm. A total of 5 μg of RNA was reverse transcribed using 200 units/ml Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) and 2 μg of random hexamer primers (Thermo Hybaid, Ulm, Germany). Obtained templates were amplified in a final volume of 50 μl. For initial detection of CXCR4 expression in astroglia by conventional RT-PCR analysis, the following primers were designed: sense, 5’-GTA CAA TGA CCT CCT CTT TGT C-3’; and antisense, 5’-CAA CAG CAG CAG GGC-3’. The expected size of the reaction product is 458 bp. Amplification was carried out with 30 PCR cycles of 58 °C for 1 min in the presence of 15 pmol of each primer. Reaction products were separated in an agarose gel and visualized with ethidium bromide. RNA levels were quantified by real time PCR using the following primers: CXCR4, sense (5’-ACG GCC TGA CCT CCT CTT TGT C-3’) and antisense (5’-GCC CAC ATA GAC TGC CTT TTC-3’); CCR5, sense (5’-AGA ATG AGA AGA GGC ACA-3’) and antisense (5’-AGA TGA CAG GGT TTA GGC ACG-3’); GAPDH, sense (5’-CCG TTG TCC TAC CCC CAA TG-3’) and antisense (5’-GTC CAC CAC CCT GGT GCT GTA-3’) (all from Thermo Hybaid, Ulm, Germany). Thermocycling for each reaction was done in a final volume of 10 μl containing 1 μl of cDNA sample (or standard), 1 μl of LightCycler-DNA Master SYBR-Green I (Roche Diagnostics), MgCl2 (2.5 mM), and sense and antisense primers (10 pmol each). The thermal cycling conditions were 95 °C for 30 s followed by 45 cycles of 58 °C for 5 s and 72 °C for 10 s. Data were collected using the LightCycler instrument (Roche Diagnostics). To confirm the amplified products, melting curves were performed at the end of the amplification by cooling samples at 20 °C/s to 65 °C for 15 s and then increasing the temperature to 95 °C at 0.1 °C/s with continuous fluorescence measurement. Each sample was tested in duplicate. For generation of standard curves, PCR products (CXCR4, CCR5, GAPDH) were purified using QIAquick (Qiagen) spin columns (Qiagen). One-fifth of the amplified products was deproteinized with 0.1% sodium azide and subsequently incubated for 20 min at 4 °C with either goat anti-rat CXCR4 antibodies (1:200; Santa Cruz Biotechnol- ogy, Inc., Santa Cruz, CA) or anti-god antibodies (1:200; R&D Systems, Minneapolis, MN), as an isotype control, and for another 20 min with fluorescein isothiocyanate-labeled secondary antibodies (1:500; Jackson Laboratories, West Grove, PA). Flow cytometric analysis was performed with a FACScan (Becton Dickinson). The forward angle light scatter was used to exclude dead and aggregated cells.

Preparation of Conditioned Medium from IL-6- and dbcAMP-treated Astrocytes—Astroglial cultures were maintained for 48 h with N2 medium in the presence or absence of IL-6 (10 ng/ml) or dbcAMP (10−4 M). After extensively rinsing three times with minimal essential medium/F-12 medium (1:1; v/v), cultures were subsequently incubated with N2 medium (200 μl/well) in the absence of IL-6 or dbcAMP. After 24 h, the CM was collected and centrifuged at 3000 × g to remove cells and membrane fragments. The CM was aliquoted and stored at −70 °C.

RESULTS

Cultured Cortical Astrocytes Express Functional CXCR4—RT-PCR of RNA isolated from second passage rat cortical glia with primers specific for CXCR4 allowed the amplification of a single product with the expected molecular size of 458 bp (Fig. 1A). Subsequent restriction analysis of the amplified product resulted in fragments of the predicted size (data not shown). This confirms that CNS glia express CXCR4 (14, 24, 32). To further determine whether glia express CXCR4 at their cell surface, cultured glial cells were stained with anti-CXCR4 an-tiserum and subjected to flow cytometric analysis (Fig. 1B). In three independent experiments, an average of 27 ± 6% (mean ± S.D.) of the cultured cells showed CXCR4 surface expression.

In the next step, we set out to determine whether CXCR4 expressed by cultured glia are functionally active. To this end, we exploited the fact that activation of many growth factor and
cytokine receptors leads to the rapid and transient expression of immediate early genes, most prominent among them, c-fos (33). SDF-1-induced c-fos expression was monitored in second passage cortical glia initiated from the offsprings of fos-LacZ × fos-lacZ crosses. This transgenic approach allows the selective and sensitive detection of c-fos expression (33, 34) and further favors the rapid phenotypic characterization of c-fos-positive and thus CXCR4-expressing cells by double labeling with antibodies against cell type-specific markers (30). Transgene expression was assessed after exposing cortical glia for 3 h to SDF-1 (100 ng/ml). We have previously demonstrated that this stimulation protocol leads to maximal transgene expression (30). Subsequent staining of the stimulated cultures for β-galactosidase histochemistry revealed that SDF-1 induced transgene expression in 33.6% of the cultured cells (β-galactosidase-positive cells, 15,350 ± 4050 cells/cm², n = 18 wells pooled from three independent experiments; total cell number, 45,730 ± 8150 cells/cm²) (Fig. 2). In untreated controls, 4450 ± 950 β-galactosidase-positive cells were present. Fibroblast growth factor-2 (25 ng/ml), which served as a positive control (30), induced β-galactosidase activity in 29,150 ± 7250 cells/cm². To further confirm that the SDF-1-responsive/CXCR4-expressing cells are astrocytes, the predominant cell type in our glial cultures (29), cells were double-labeled with antibodies against the astrocytic marker, GFAP. The majority (>95%) of the β-galactosidase-expressing cells were immunoreactive for GFAP (Fig. 2). The relative number of astrocytes responding to SDF-1 with c-fos expression remained unaffected when astroglial cultures were initiated from the offsprings of fos-LacZ × BALB/c crosses (data not shown), cultures used for all following experiments. Together these findings reveal that cultured cortical astrocytes show functional signal transduction coupling for SDF-1.

Exposure of Cortical Glia to either IL-6 or dbcAMP Is Necessary to Induce Chemotaxis toward SDF-1—The chemotactic response of astrocytes to SDF-1 was assessed in a migration assay using a modified Boyden chamber. Exposure of second passage cortical glia to SDF-1 at 100 ng/ml completely failed to induce cell migration (Fig. 3A). A migratory response was, however, detectable with glia previously maintained with either IL-6 or the cell membrane-permeable cAMP analog, dbcAMP (Fig. 3A). Specific cell migration toward SDF-1, as indicated by a migration index of >1, first occurred after a 24-h pretreatment with either IL-6 or dbcAMP, although this increase in the migration index was statistically not significant. With both substances, the migration index peaked after 48 h and declined up to 72 h. The combined pretreatment of cultured cortical glia with IL-6 and dbcAMP for up to 72 h did not further increase the number of migrating cells beyond that seen with the single factors. Maintaining cortical glia for 48 h with serial dilutions of IL-6 or dbcAMP further demonstrated that the inductive effects on SDF-1-dependent cell migration were concentration-dependent and required IL-6 and dbcAMP at concentrations of >1 ng/ml and >10⁻⁵ M, respectively (Fig. 3, B and C). A similar concentration dependence applied for SDF-1-induced chemotactic response (Fig. 3D). In glial cultures maintained for 48 h with dbcAMP (10⁻⁴ M), SDF-1 at concentrations up to 50 ng/ml failed to evoke glial cell migration, whereas a maximal migratory response was induced by SDF at 100–500 ng/ml. Similar SDF-1 concentrations have been previously reported to induce maximal chemotactic responses in a variety of other cell types, including neurons, microglial cells, late streak glaustrae, lymphocytes, and monocytes (14, 16, 19, 27, 35). Increasing SDF-1 concentrations to ≥750 ng/ml resulted in a decline in the number of migrating glial cells. Since our glial cultures also contained small numbers of oligodendrocytes and glial precursors in addition to the large portion of astrocytes (≥90%) (29), polycarbonate filters were fixed after incubation and stained with antibodies against GFAP. The vast majority of the migrated cells (99 ± 2%; n = 18 wells pooled from two independent experiments) expressed immuno-
reactivity for GFAP, thus confirming that SDF-1 preferentially induced the migration of astrocytes. Together, these findings demonstrate that IL-6 or the activation of cAMP-dependent signaling pathways is required for the onset of a migratory response of astroglia to SDF-1.

MIP-1α is a chemokine recently shown to act as a chemoattractant for astrocytes (14). In apparent contrast to SDF-1, cultured cortical glia specifically migrated toward MIP-1α at 10 ng/ml, a concentration previously reported to induce maximal chemotactic response in glial cells (14), without prior exposure to IL-6 or dbcAMP (Fig. 4). Treatment of cortical glial cultures with either IL-6 or dbcAMP alone or a combination of both substances for 6–48 h did not further modulate the MIP-1α-indcated migratory response of cortical astroglia. Taken together, these findings reveal that IL-6 and dbcAMP specifically modulate the chemotactic response of cortical astroglia to SDF-1 but not to MIP-1α.

The Inductive Effects of IL-6 and dbcAMP on SDF-1-dependent Chemotaxis Correlate with an Increase in CXCR4 Cell Surface Expression Levels—Since both IL-6 and dbcAMP modulate CXCR4 cell surface expression levels in lymphocytes (27, 28), we determined whether similar regulatory influences apply to SDF-1-induced Chemotaxis of Astroglia

FIG. 3. A, time course of the inductive effects of IL-6 and dbcAMP on SDF-1-dependent chemotaxis in cortical glia. Cortical glia were maintained for the indicated time in N2 medium supplemented either with IL-6 (10 ng/ml) or dbcAMP (10−4 M) alone or a combination of both factors and were subsequently tested for SDF-1 (100 ng/ml)-dependent chemotaxis in a modified Boyden chamber assay. Data were normalized by calculating the migration index, which was defined as the ratio of the number of cells migrating in the presence and absence of the chemokine. Data represent the mean ± S.D. from 24 wells pooled from three independent experiments. SDF-1 failed to elicit a migratory response in untreated controls. The migration index significantly increased in cultures maintained for 48 h (p < 0.001; Student’s unpaired t test) or 72 h (p < 0.001) with either IL-6 or dbcAMP. The effects of IL-6 and dbcAMP on the migratory response of glia toward SDF-1 were not additive. Dose-dependence of the inductive effects of IL-6 (B) and dbcAMP (C) on SDF-1-dependent chemotaxis in cortical glia is shown. Cultured cortical glia were treated for 48 h with serial dilutions of either IL-6 or dbcAMP and assayed for their migratory response toward SDF-1 (100 ng/ml). Each bar represents the mean ± S.D. from 12 wells pooled from two independent experiments. Glial cell migration was induced with IL-6 at 10 ng/ml (p < 0.001; Student’s unpaired t test) and dbcAMP at 10−4 M (p < 0.001). D, chemotaxis of dbcAMP-treated astroglia in response to different SDF-1 concentrations. Cultured cortical glia were exposed for 48 h to dbcAMP (10−4 M) and assayed for cell migration in the presence of the indicated concentrations of SDF-1. Data represent the mean ± S.D. from 12 wells pooled from two independent experiments. The number of migrating glial cells significantly increased with SDF-1 at concentrations of ≥100–500 ng/ml (degrees of freedom = 30, F-value = 10.09, p < 0.0001; analysis of variance) and declined with SDF-1 at ≥750 ng/ml.

FIG. 4. Analysis of MIP-1α-induced chemotaxis in cortical glia. In contrast to SDF-1, untreated cortical glia showed a significant migratory response to MIP-1α (10 ng/ml) as indicated by a migration index of >1 (p < 0.001; Student’s unpaired t test). Maintaining glial cultures for 6–48 h in N2 medium supplemented with dbcAMP (10−4 M), IL-6 (10 ng/ml), or a combination of both factors did not lead to detectable changes of the migration index. Data are the mean ± S.D. from 24 cultures pooled from three independent experiments.
astrocytes. Flow cytometry demonstrated that treatment of cortical glia with either dbcAMP or IL-6 for 48 h up-regulated CXCR4 cell surface expression (Table I and Fig. 5). CXCR4 cell surface expression levels did not further increase in cultures co-treated with IL-6 and dbcAMP. These findings suggest that IL-6 and dbcAMP induce SDF-1-dependent chemotaxis by up-regulating CXCR4 cell surface expression in glia.

Increases in cell surface expression levels of chemokine receptors are either due to inhibited receptor internalization, increased translocation of receptors from intracellular pools to the cell surface, or enhanced receptor synthesis (27, 28, 36, 37). To distinguish whether IL-6 and dbcAMP affect CXCR4 synthesis or receptor compartmentalization, both substances were tested for their effects on CXCR4 mRNA expression in cortical glial by real time RT-PCR. Treatment of cortical astroglial cultures with either IL-6 or dbcAMP for 6 h did not affect CXCR4 mRNA expression (Fig. 6). However, both substances increased CXCR4 transcript levels after 24 and 48 h. In dbcAMP-treated cultures, CXCR4 mRNA levels were still elevated after 72 h, whereas CXCR4 transcripts had returned to control levels in cultures maintained for 72 h with IL-6. Control experiments further demonstrated that, in contrast to CXCR4, IL-6 and dbcAMP did not interfere with the expression of CCR5 (data not shown). Moreover, none of the treatments affected GAPDH expression used as an internal standard (Fig. 6B). Together, these findings indicate that both IL-6 and dbcAMP primarily increase CXCR4 cell surface expression levels by stimulating RNA synthesis and further suggest that prolonged IL-6 exposure additionally regulates CXCR4 cell surface expression by affecting receptor compartmentalization and/or RNA stability.

The Stimulatory Effects of IL-6 and dbcAMP on CXCR4 Cell Surface Expression Involve a Secondary Mechanism—The observation that a 48-h treatment with either IL-6 or dbcAMP was necessary to up-regulate CXCR4 cell surface expression in cultured astrocytes prompted us to investigate whether these inductive effects involve a direct or indirect mechanism. To address this issue, astrocytes were treated for 48 h with IL-6 (10 ng/ml) or dbcAMP (10⁻⁴ M), rinsed, and subsequently used for the preparation of CM. In contrast to the direct treatment with IL-6 or dbcAMP, exposure of astroglial cultures to CM derived from these IL-6- or dbcAMP-pretreated astrocytes for 24 h was sufficient to induce SDF-1-dependent chemotaxis.

### Table I

| Treatment | No. of gated cells % |
|-----------|---------------------|
| None      | 27 ± 5              |
| dbcAMP (10⁻⁴ M) | 54 ± 9*            |
| IL-6 (10 ng/ml) | 48 ± 8*            |
| dbcAMP (10⁻⁴ M) + IL-6 (10 ng/ml) | 48 ± 7* |

Fig. 5. Representative fluorescence-activated cell sorting analysis of IL-6- and dbcAMP-treated astrocytes for CXCR4 cell surface expression. Cortical glial cultures were maintained for 48 h with dbcAMP (10⁻⁴ M), IL-6 (10 ng/ml), or a combination of both factors and analyzed for CXCR4 cell surface expression by flow cytometry as described in the legend to Fig. 1. Both IL-6 and dbcAMP up-regulated CXCR4 cell surface expression. Data are summarized in Table I. FL1-Height, fluorescence intensity.

Fig. 6. A, effects of dbcAMP and IL-6 on glial CXCR4 mRNA expression. Total RNA was isolated from cortical glia maintained for the indicated time with either dbcAMP (10⁻⁴ M) or IL-6 (10 ng/ml). CXCR4 mRNA levels were quantified by real time PCR as described under “Experimental Procedures.” Standard curves were produced by determining the crossing point of each standard and plotting them against the logarithmic value of concentration (CXCR4 standard curve: slope, \(y = -3.2943 \pm 22.747; R² = 0.9987\); GAPDH standard curve: slope, \(y = -3.3857 \pm 27.029; R² = 0.9953\)). CXCR4 levels of samples were determined by setting their crossing points to the standard curve and were normalized to GAPDH. Data are the mean ± S.D. from three to four independent experiments. Dibutyryl cAMP significantly increased CXCR4 mRNA levels between 24 and 72 h (\(p < 0.01\)). In IL-6-treated cultures, significant increases in CXCR4 transcript levels occurred between 24 and 48 h (\(p < 0.02\)). B, IL-6 and dbcAMP do not affect the expression of GAPDH used as an internal standard. GAPDH mRNA levels in IL-6 (10 ng/ml) and dbcAMP (10⁻⁴ M) treated cultures were determined by real time PCR as described under “Experimental Procedures.” Data represent the mean ± S.D. from three independent experiments.
ent experiments.

Data represent mean ± S.D. from 24 wells pooled from three independent experiments. Both dbcAMP- and IL-6-CM, but not control-CM, significantly increased CXCR4 cell surface expression levels. *, p < 0.01; treatment versus control; Student’s unpaired t test.

FIG. 7. IL-6 and dbcAMP induce CXCR4-dependent chemotaxis of astroglia by a secondary mechanism. CM was prepared from IL-6- and dbcAMP-pretreated cultures as described under “Experimental Procedures” and added to astroglial cultures. After 24 h, cells were analyzed for SDF-1 (100 ng/ml)-dependent migration. Chemotactic response was detectable in cultures exposed to CM obtained from IL-6-pretreated (IL-6-CM; p < 0.05; Student’s unpaired t test) or dbcAMP-pretreated (dbcAMP-CM; p < 0.002) astrocytes but was absent in cultures exposed to CM from untreated control astrocytes (control-CM). Data represent mean ± S.D. from 24 wells pooled from three independent experiments.

A similar chemotactic response was undetectable in cultures maintained for 24 h with medium conditioned by untreated controls. To further assess whether the induction of SDF-1-dependent chemotaxis by CM correlates with an increase in CXCR4 cell surface expression, astroglial cells were maintained for 24 h with CM and subjected to flow cytometric analysis. CXCR4 cell surface expression levels increased ∼2-fold upon treatment with CM obtained from dbcAMP-pretreated astrocytes and 1.4-fold upon treatment with CM derived from IL-6-pretreated astroglia (Fig. 8, Table II). Together these findings suggest that both IL-6 and dbcAMP induce CXCR4 cell surface expression in astroglia and the subsequent onset of SDF-1-dependent chemotaxis by a secondary mechanism.

DISCUSSION

Only recently, the chemokine SDF-1 and its receptor CXCR4 have been recognized to control the migration of CNS neurons and microglial cells (14, 21, 22). In contrast, similar regulatory influences have not yet been observed for the migration of CNS astrocytes (14), although these cells are known to express CXCR4 (14, 15, 24–26, 32). We now demonstrate that SDF-1-dependent migration occurs upon exposure of CNS astroglia to IL-6 or upon activation of cAMP-dependent signaling pathways, treatments that increase the cell surface expression of CXCR4. We suggest that SDF-1 is crucially involved in reactive gliosis and might control the building of the glial scar and the restoration of the blood-brain barrier at the site of injury.

In accordance with previous studies (14, 15, 24–26, 32), CXCR4 transcripts and protein were detectable in highly enriched astroglial cultures of the rat cortex. Exposure of these cultures to SDF-1 induced the expression of the immediate early gene, c-fos, in a subpopulation of the cells, confirming that these receptors are functionally active (14, 24–26). Double staining of c-fos-positive, and thus CXCR4-expressing, cells for GFAP further allowed us to demonstrate that in these cultures CXCR4 is almost exclusively (>95%) expressed by astroglia. Despite all signs for functional signal transduction machinery, cortical astroglia did not chemotactically respond to SDF-1 unless cells were treated with IL-6 or dbcAMP, conditions leading to an increase in CXCR4 cell surface expression levels. Further evidence for a direct correlation between up-regulation of CXCR4 cell surface expression and the onset of SDF-1-de-

pendent chemotaxis comes from recent studies with fibroblast growth factor-2. Fibroblast growth factor-2 activates several signaling molecules/pathways, including cAMP-dependent signaling (58). Unlike dbcAMP, fibroblast growth factor-2 fails to affect CXCR4 expression and does not allow for SDF-1-dependent chemotaxis, thus excluding the possibility that cAMP signaling directly interferes with the mechanism of cell migration. 2 Along the same line, we observed that dbcAMP and IL-6 failed to affect MIP-1α-dependent chemotaxis of astroglia (see below). Moreover, similar to our findings with astroglia, up-regulation of CXCR4 cell surface expression is required for the induction of SDF-1-dependent chemotaxis in several other cell types such as endothelial and dendritic cells (38–40). Taken together, these observations strongly favor the assumption that, with the exception of differentiating B cells (41), SDF-1-dependent chemotaxis of most cell types is tightly controlled by CXCR4 expression levels. An important future issue will be to identify the molecular mechanism(s) underlying these regulatory influences. The previous observation that the absence of SDF-1-dependent chemotaxis in unstimulated (control) astroglia is not due to a failure of SDF-1 to mobilize intracellular free Ca2+ (14, 24) currently implies that these regulatory in-

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fluences occur downstream of Ca\(^{2+}\). Under normal conditions, the regulation of SDF-1-dependent chemotaxis by CXCR4 cell surface expression levels might be essential to prevent aberrant cell migration in response to SDF-1, a chemokine constitutively expressed in all organs yet examined, including the brain (5, 15, 22, 42–44). In support of this notion, we observed that cultured cortical astroglia chemotactically responded to MIP-1α without prior manipulations of cell surface receptor expression levels. Unlike SDF-1, MIP-1α is a chemokine not constitutively expressed in the brain (45). It is of note that, in contrast to MIP-1α, distinctly higher concentrations of SDF-1 were necessary to induce a chemotactic response in cultured astrocytes. This is in good agreement with the observations that CXCR4 expressed by neural cells exhibit a \(K_d\) value of \(54 \times 10^{-9}\) M (46), whereas MIP-1α binds to neural as well as to nonneural cells with a \(K_d\) of 1.4–0.3 \(\times 10^{-9}\) M (47, 48).

Cell surface expression of chemokine receptors is either regulated by gene expression or by the recirculation of the receptor between the cell surface and internal compartments. Previous studies with lymphocytes revealed that the different modes of CXCR4 regulation are governed by distinct extracellular signals. As with most G protein-coupled receptors, CXCR4 cell surface expression decreases upon binding of the ligand and the subsequent internalization of the ligand-receptor complex (36, 37). In addition, CXCR4 receptor internalization in lymphocytes is induced by mitogens via activation of protein kinase C-dependent signaling pathways (36, 37). Recent work by Orsini et al. (49) provided evidence that the SDF-1-mediated internalization uses both an arrestin- and dynamin-dependent pathway, whereas the protein kinase C-induced internalization process only depends on dynamin. An up-regulation of lymphocyte CXCR4 cell surface expression occurs with IL-6 or factors signaling through cAMP/protein kinase A (27, 28). We observed that IL-6 and activation of cAMP protein kinase A-dependent signaling pathways also increased CXCR4 surface expression in CNS astrocytes. However, unlike with lymphocytes, these stimulatory effects seem to depend on a secondary mechanism, as suggested by the observation that a 48-h treatment with either IL-6 or dbcAMP was required to induce maximal SDF-1-dependent chemotaxis of cultured cortical astroglia, whereas an increase in CXCR4 cell surface expression and a subsequent optimal chemotactic response to SDF-1 already occurred after 24 h when astroglia were maintained with CM obtained from IL-6- or dbcAMP-pretreated astrocytes. Since highly purified astroglial cultures were used as an assay system, we would suggest that IL-6 and dbcAMP lead to the enhanced synthesis of a releasable factor(s) in a subpopulation of astroglia, which in turn promotes astroglial CXCR4 cell surface expression in an autocrine and/or paracrine manner. The distinct temporal differences in the onset of SDF-1-dependent chemotaxis in astroglia directly treated with IL-6 and dbcAMP (48 h) or exposed to CM obtained from IL-6- or dbcAMP-pretreated astrocytes (24 h) further indicate that the CM effects are not due to residual IL-6 or dbcAMP. The additional observations that IL-6 and dbcAMP interfere with CXCR4 cell surface expression by slightly different mechanisms currently favor the assumption that the stimulatory effects of IL-6 and dbcAMP on CXCR4 cell surface expression are mediated by different factors. In cultures exposed to dbcAMP, CXCR4 transcript levels were elevated for up to 72 h, implying that the cAMP-induced secondary processes primarily increase CXCR4 mRNA expression levels via stimulation of gene expression. In contrast, IL-6 only resulted in a transient increase in CXCR4 mRNA levels, lasting up to 48 h. This indicates that the IL-6-induced secondary processes initially increase CXCR4 surface levels by stimulating its synthesis and subsequently affect CXCR4 surface expression levels by inhibiting receptor internalization and/or by altering the balance of CXCR4 expressed in intracellular versus extracellular compartments (27, 36).

Attempts to further characterize the subcellular distribution of CXCR4 under the various conditions by the use of cell fractions failed so far, since it turned out that in our hands none of the CXCR4 antibodies currently available are suitable for immunoblot analysis. An important future issue will be to identify the factors mediating the effects of IL-6 and cAMP on glial CXCR4 expression. Potential candidates are IL-1β and tumor necrosis factor α, proinflammatory cytokines previously found to stimulate CXCR4 expression in astrogliaoma cells (57).

A pathomechanism eventually leading to an IL-6- and/or cAMP-induced onset of SDF-1-dependent chemotaxis in CNS astroglia represents reactive gliosis. Reactive gliosis occurs as a consequence of injury or inflammation of the nervous system and is characterized by astrocytic hypertrophy, the increased expression of the intermediate filament protein, GFAP, as well as by astrocytic proliferation and migration (23). A major objective of reactive gliosis is to prevent CNS tissue from secondary damage by building an astrocytic barrier around the site of injury. In addition, reactive gliosis seems to initiate the restoration of the lesioned site by inducing microvessel regeneration. In this respect, it must be pointed out that in the brain, microvessels are typically contacted by astrocytic endfeet, representing an integral part of the blood-brain barrier. One major regulator of these reactive processes is IL-6, which increases at the site of gliosis (45). In addition, a similar increase occurs for yet unknown extracellular signals positively coupled to cAMP (50). Hence, we suggest that upon up-regulation of CXCR4 in reactive astrocytes, SDF-1 will control the formation of the glial scar and the restoration of the blood-brain barrier in the injured brain. At present, no data are available on whether local SDF-1 gradients form after brain injury, which would direct cell migration. The recent observations that SDF-1 expression locally increases in brain material of patients with glioblastoma multiforme tumors as well as after peripheral nerve lesion (6, 51), however, strongly suggest this possibility.

CXCR4 plays a critical role in HIV infection and can mediate CD4-dependent and -independent HIV entry via binding of the HIV-1 envelope protein, gp120 (9, 10). In addition, gp120/CXCR4 induces neuronal cell death (16, 52), a process thought to contribute to HIV-associated dementia. Interestingly, recent in vitro studies revealed that neurotoxicity occurs upon binding of gp120 to various cell types, including macrophages/microglia, neurons, and astroglial cells (16, 17, 53–55). The exact cellular and molecular mechanism(s) underlying these neurotoxic effects are at present not completely understood. Initial findings suggest that the neuronal cell death induced by activation of neuronal as well as glial CXCR4 results from the increased release of glutamate and the subsequent overstimulation of neuronal glutamate receptors of the N-methyl-D-aspartate type (54–56). Since HIV infection is regularly associated with inflammatory processes of the CNS, the cytokine- and/or cAMP-induced increase in CXCR4 cell surface expression levels on reactive astroglia, as proposed by our findings, could represent a major predisposition for the onset and/or acceleration of neuronal cell death associated with HIV dementia.

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