TNF-α mediates SDF-1α–induced NF-κB activation and cytotoxic effects in primary astrocytes

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Received for publication February 27, 2001, and accepted in revised form June 4, 2001.

Stromal-derived cell factor-1α (SDF-1α; CXCL12) and its receptor, CXCR4, are constitutively expressed on neuroepithelial cells and are believed to be involved in both development and pathological processes, such as AIDS-associated neurologic disorders. Here, we demonstrate that SDF-1α activates NF-κB, stimulates production of chemokines and cytokines, and induces cell death in primary astrocytes, effects that depend on ongoing secretion of TNF-α. SDF-1α upregulated TNF-α mRNA and protein secretion, as well as TNF receptor 2 expression. TNF-α treatment mimicked SDF-1α induction of NF-κB, IL-1α/β, and RANTES, as well as cell death; neutralizing antibodies against TNF-α opposed these responses. We also found that SDF-1α activated Erk1 and Erk2 (Erk1/2) MAPK in a biphasic fashion. Early Erk1/2 activation was stimulated directly by SDF-1α and late activation was mediated by TNF-α. PD98059 suppression of early Erk1/2 activation correlated with reduction of SDF-1α–induced TNF-α expression. Late Erk1/2 activation was involved in TNF-α–stimulated NF-κB activation and cytokine induction. SDF-1α was induced in reactive CXCR4-positive astrocytes near axotomized spinal cord motor neurons, consistent with autocrine SDF-1/CXCR4 signaling in these cells. We propose that these novel effects of SDF-1α are relevant to the pathogenic and developmental roles of SDF-1α in the CNS.

J. Clin. Invest. **108**:425–435 (2001). DOI:10.1172/JCI200112629.

Introduction

Stromal-derived cell factor-1 (SDF-1) was first described as a factor produced by bone marrow stromal cells that induces proliferation of B cell progenitors and regulates B cell maturation (1, 2). Two isoforms (SDF-1α and SDF-1β) are encoded by a single gene and arise by alternative splicing (2). SDF-1 is the unique biological ligand for CXCR4, a high-affinity seven-transmembrane G protein–coupled receptor (3–5). Acting on CXCR4, SDF-1 exerts chemoattractant effects on target cells and mediates critical retentive functions towards pre-B cells, maintaining their presence in an appropriate microenvironment for development (6). Among chemokine/chemokine-receptor pairs, SDF-1 and CXCR4 exhibit atypical expression patterns: SDF-1 is constitutively produced in many organs and CXCR4 is found widely in both hematopoietic and other tissues (3–5, 7), indicating functions beyond inflammation and hematopoietic development. During organogenesis, SDF-1 and CXCR4 have dynamic and complementary expression patterns in neural, vascular, hematopoietic, and craniofacial systems (7). These expression patterns are consistent with organ-specific roles in development, as revealed by studies of gene-targeted mice (6, 8–11). CXCR4 is a coreceptor for T-tropic HIV, and SDF-1α inhibits CXCR4-mediated HIV-1 infection in vitro (3–5). Signaling molecules associated with the response to SDF-1α in hematopoietic cells have recently been clarified; these include Erk1/2 mitogen-activated protein kinase (Erk1/2 MAPK), Pyk-2, paxillin, Crk, protein kinase B, phosphatidylinositol 3-kinase, and NF-κB (12–17). The Janus kinase (JAK) and signal transducer and activator of transcription (STAT) system has also been implicated in this pathway (18).

In the CNS, SDF-1α has been detected predominantly in astrocytes. CXCR4 is expressed on both neurons and glial cells (19–24). Beyond their roles in cerebellar development, SDF-1α and CXCR4 appear to be involved in diverse pathological processes, including AIDS-associated neurologic disorders and proliferation of glioblastoma tumor cells (25). HIV-associated dementia (HAD) is a common complication of the late stages of viral infection characterized pathologically by reactive astrogliosis, macrophage activation and accumulation, neuron loss, and defective blood-brain barrier (25–27). Increased astrocyte cell death in the CNS of HAD patients was recently shown to be associated with severe neurologic impairment (28). It is believed that neuronal cell death is caused indirectly by soluble factors secreted by HIV-infected or HIV-activated macrophages and glial cells. Most attention

has focused on HIV-1 proteins (HIV gp120 and Tat) and proinflammatory cytokines (TNF-α and IL-1). However, SDF-1α expression is upregulated in the brains of patients dying with HAD, and binding of SDF-1α to CXCR4 elicits neuronal apoptosis in vitro (15, 29), implicating pathological roles of SDF-1α in development of HAD.

TNF-α is expressed during immune-mediated CNS inflammation and AIDS dementia (25). TNF-α expression is regulated mainly at the level of transcription, and the diverse biological functions of TNF-α are mediated through complex intracellular signaling pathways (30). In general, TNF-α activates two signaling pathways, one leading to the activation of gene transcription and the other leading to cell death. Cytotoxic effects of TNF-α involve receptor-dependent apoptosis mediated through the action of death-domain-containing proteins. TNF-α–induced gene transcription events are primarily dependent on the activation and translocation of NF-κB. In some experiments, activation of NF-κB can mediate antiapoptotic actions of TNF-α (31, 32).

The transcription factor NF-κB plays a critical role in immune and inflammatory responses. NF-κB, a prototypic heterodimer of p50 and p65 (Rel-A), is sequestered in the cytoplasm through association with cellular inhibitors, members of the IκB family of proteins (IκBs). Upon exposure to a wide variety of agents, most notably TNF-α and IL-1, IκBs are phosphorylated by IκB kinases and then undergo ubiquitination and degradation in proteosomes, a rapid process independent of protein synthesis that allows the subsequent nuclear translocation of NF-κB (33, 34). Once in the nucleus, NF-κB binds a cognate *cis*-acting regulatory element (κB site) and activates gene expression through cooperative interactions with other factors.

Erk1 and Erk2 (Erk1/2) MAPK mediates signals for many growth factors and G protein–coupled receptors. Activation of Erk1/2 MAPK by G protein–coupled receptors occurs via the Raf-MEK1/2-Erk1/2 cascade; the process can be blocked by a specific chemical inhibitor, PD98059 (35, 36).

Given the diverse physiopathological roles of SDF-1α and CXCR4 in the CNS, ligand-induced and receptor-associated intracellular signaling and gene products in neuroepithelial cells merit characterization. Here, we analyzed SDF-1α–induced signaling in primary astrocytes. Our results revealed novel effects of SDF-1α and demonstrated an essential role of TNF-α in SDF-1α–elicited biological functions in primary astrocytes.

Methods

Reagents and treatments. Recombinant murine SDF-1α and purified polyclonal anti-murine TNF-α antibodies were obtained from PeproTech Inc. (Rocky Hill, New Jersey, USA); recombinant human TNF-α from Becton Dickinson Labware (Bedford, Massachusetts, USA); and genistein from Life Technologies Inc. (Grand Island, New York, USA). PD98059 was obtained from Calbiochem-Novabiochem Corp. (La Jolla, California, USA). Poly-D-lysine (PDL), pertussis toxin (PTX), cycloheximide (CHX), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were obtained from Sigma Chemical Co. (St. Louis, Missouri, USA). Rabbit anti–Erk2 MAPK, anti–NF-κB (p50, p52, p65, c-Rel, and Rel-B), anti–IκB-α, anti–IκB-β, anti-STAT3, anti–SDF-1α, and anti-CXCR4 were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, California, USA). Anti–phospho-Erk1/2 monoclonal antibody was obtained from New England Biolabs Inc. (Beverly, Massachusetts, USA), and monoclonal anti-murine IL-1α and IL-1β antibodies were from BD PharMingen (San Diego, California, USA). Cells were treated with 100 ng/ml of SDF-1α or 5 ng/ml of TNF-α in the presence or absence of PTX (100 ng/ml) or PD98059 (50 μM), respectively, unless stated otherwise.

Astrocyte isolation and culture. Astrocytes were purified from P0-P3 mouse cerebrum by differential adhesion, as described previously (37). Nonadherent oligodendrocyte and microglial cells were removed by intensive shaking and washing. The purity of the astrocyte population was >95%, as determined by indirect immunofluorescence assay with anti-glial fibrillary acidic protein (GFAP) antibodies. Astrocytes were cultured for a total of 16–22 days.

Immunohistochemistry. Ventral root avulsion was performed as previously described (38). Mice were sacrificed by intracardiac perfusion with 4% paraformaldehyde under deep anesthesia. Colocalization of GFAP and SDF-1α or CXCR4 immunoreactivity was performed by dual-label immunofluorescence histochemistry and confocal microscopy, as described (39).

RNA isolation and RNase protection assay. Total cellular RNA, isolated using TRIzol reagent (Life Technologies Inc.), was analyzed by RNase protection assay (RPA) with Multi-probe Template Sets and the In Vitro Transcription Kit (BD PharMingen) according to the manufacturer's protocol. Each analysis was repeated more than three times using RNA from independent astrocyte cultures.

SDS-PAGE and Western blotting. Cell extract preparation and Western blotting were performed as described previously (40). After anti–phospho-Erk1/2, anti–IκB-α, or anti–IκB-β immunoblotting, membranes were stripped and neutralized, and then incubated with rabbit anti-Erk2 or anti-STAT3 antibodies for loading control, respectively.

Electrophoresis mobility shift assays. Electrophoresis mobility shift experiments were performed with whole-cell extracts as described previously (40). For antibody blocking experiments, astrocytes were treated with SDF-1α for 2 hours in the presence or absence of 5 μg/ml of anti-murine TNF-α or 2 μg/ml of anti-murine IL-1α and IL-1β before cell extracts were prepared. Electrophoresis mobility shift assay (EMSA) probe sequence derived from the κB2 elements of the IP-10 gene, 5′-GAG CAG AGG GAA ATT CCG TAA CTT-3′ (41), or the FcγRI gene, 5′-

GTA TTT CCC AGA GGG GGA AC-3′ (40), respectively, were end-labeled with [γ-^{32}P]ATP, with aliquots equivalent to ~25,000 cpm used per reaction. Complexes were separated on 5% nondenaturing acrylamide gels in 0.5× TBE and detected by autoradiography. For competition experiments, unlabeled oligonucleotides were added in molar excess and incubated at room temperature for 15 minutes before the addition of radiolabeled probe. For supershift experiments, 0.1 μg of anti–NF-κB antibodies or normal control serum were incubated with the cell extract for 30 minutes on ice before the addition of radiolabeled probe to the reaction mixture.

Transient transfection and luciferase activity assay. The luciferase-reporter plasmid, p6κB-luc, containing six copies of κB2 elements of the IP-10 gene, was a gift from George R. Stark (Cleveland Clinic Foundation). Murine TNF-α promoter constructs [pGL3-mTNF (–1260)] were provided by Dmitry V. Kuprash and Sergei A. Nedospasov (42). Astrocytes at about 80% confluence in 12-well plates were cotransfected with 0.25–0.5 μg of reporter plasmid DNA (expressing *firefly* luciferase) and 0.05 μg of pRL-TK plasmid DNA (expressing *renilla* luciferase) for 3 hours at 37°C, using FuGENE 6 reagents according to the manufacturer's protocols (Roche Molecular Biochemicals, Indianapolis, Indiana, USA). Cells were washed with PBS and treated with the indicated stimuli in 1 ml of DMEM at 37°C for 6 hours. Luciferase activity was analyzed using the Dual-Luciferase assay kit, according to the manufacturer's protocol (Promega Corp., Madison, Wisconsin, USA), in a luminometer (Dynatech Laboratories, Chantilly, Virginia, USA). *Firefly* luciferase activity was normalized to *renilla* luciferase activity. More than 60% of transfected astrocytes expressed reporter gene proteins, as shown by cotransfection with β-gal expression vectors in preliminary experiments. Results presented in this article were obtained from 4–5 experiments with separate astrocyte cultures.

ELISA. Murine TNF-α was quantitated by Cytokine Direct Murine TNF-α ELISA kit (Intergen Co., Purchase, New York, USA) according to the manufacturer's protocol.

Cell death assay. Confluent astrocytes were incubated with SDF-1α (100 ng/ml) or TNF-α (5 ng/ml) for 3 days, or exposed to H_2O_2 (100 μM) containing DMEM for 30 minutes and then washed and incubated with normal DMEM for 3 days. Fresh SDF-1α and TNF-α were added to cells again 1 day before the MTT activity assay. For antibody blocking experiments, 2 μg of anti–TNF-α antibodies were added to cells simultaneously with SDF-1α. Under these conditions, no cell proliferation occurred (43). MTT activity was measured using a colorimetric assay: after treatment, cells were washed twice with PBS and incubated with 200 μl of 2 mg/ml MTT in DMEM at 37°C for 2 hours. MTT-containing medium was removed, and cells were gently washed and extracted with 200 μl of a 1:1 mixture of DMSO and ethanol, and the absorbance was measured on a plate reader, using a test wavelength of 595 nm and a reference wavelength of 655 nm. MTT reduction activity was expressed as a percentage of control, with untreated cells set as 100%.

Results

SDF-1α activates NF-κB in stimulus-specific fashion in specific cell types. SDF-1α activates NF-κB and STAT proteins in human T cells and murine pro-B cells (12, 18). Astrocytes express SDF-1α and functional CXCR4 in vitro (14, 22) and in vivo (19–21, 23, 24). Signaling via CXCR4 demonstrates selective effects in astrocytes as compared with other cell types, including neurons (44). To address the possibility that autocrine effects mediated by SDF-1α/CXCR4 signaling might occur in vivo, we analyzed SDF-1α expression in the mouse spinal cord after ventral root avulsion, which produces neuronal necrosis and glial reaction without breaching the blood-brain barrier (38). Using dual-label immunofluorescence histochemistry and confocal microscopy, we found that the great majority of cells expressing SDF-1α and CXCR4 in response to ventral root avulsion were reactive astrocytes (Figure 1, a–f). The pattern of immunoreactivity for SDF-1α closely paralleled the pattern of reactive astrocytosis in areas of motor neuron injury. Taken together with the established expression of CXCR4 by astrocytes, this result indicated that all the required components of autocrine SDF-1α/CXCR4 signaling in astrocytes were present in vivo. To determine whether NF-κB and STAT proteins are involved in intracellular signaling in primary astrocytes, EMSAs were performed with κB and IFN-γ–activated site (GAS) elements (40, 41, 45). As shown in Figure 1g, a DNA-protein complex was detected in astrocytes treated with SDF-1α for 2 hours using IP-10 κB2 probe (lane 5). SDF-1α–stimulated κB2 binding complex migrated identically to the complex induced by TNF-α (Figure 1g, compare lane 5 with lane 6), and was efficiently competed with by unlabeled κB2 wild-type oligonucleotide, but not by the mutated oligonucleotide (Figure 1h). Supershift analysis with Rel-family antibodies showed that the complex was completely shifted with anti-p50 (Figure 1i, lane 3) and anti-p65 (Figure 1i, lane 5); antibodies to other Rel-family proteins and preimmune serum (PS) were without effect (Figure 1i, lanes 4 and 6–8). Further analysis in the ventral root avulsion model indicated astrocyte expression of NF-κB p65 in vivo (data not shown). To assess the functional relevance of this NF-κB activation, astrocytes were transiently transfected with κB-reporter. SDF-1α treatment resulted in about 15-fold induction of reporter activity (Figure 1j). Primary astrocytes also express functional CCR1 (37), a receptor for CCL5/RANTES. Treatment of astrocytes with RANTES did not stimulate NF-κB activation (data not shown). These data indicated that SDF-1α selectively activated NF-κB in primary astrocytes. We then determined whether SDF-1α activation of NF-κB was mediated by Gαi-dependent signaling. PTX did not

block either SDF-1α–induced NF-κB activation (Figure 1k) or SDF-1α–induced κB-reporter activity (Figure 1j), indicating that NF-κB activation by SDF-1α was independent of Gαi-mediated signaling.

EMSA with an FcγR I-GAS probe (which prefers binding STAT1 and STAT3) did not detect specific STAT protein DNA-binding activity in SDF-1α–treated astrocytes (Figure 1g, lane 2). In contrast, the same probe detected a STAT1 homodimer DNA complex in cells treated with IFN-γ (Figure 1g, lane 3 and data not shown). STAT protein activation by SDF-1α in primary astrocytes was also explored with EMSA using high-affinity SIE elements (hSIE, which efficiently bind STAT3), β-casein–GAS (has high affinity to STAT5) and anti-STAT protein immunoprecipitation, and Western blot analyses. None of these assays detected activated STAT proteins (data not shown). Consistent with these observations, JAK tyrosine phosphorylation was not observed with immunoprecipitation and Western blot analysis in astrocytes treated with SDF-1α (data not shown). Given that JAK and STAT protein activation by SDF-1α is readily demonstrated in lymphocytes, our observations indicate that SDF-1α activation of the JAK-STAT pathway is cell-type specific.

SDF-1α induction of NF-κB activity is indirect but correlates with degradation of IκB-α and IκB-β. Kinetic analysis showed that SDF-1α activation of NF-κB was delayed (Figure 2a, top panel) in comparison to that by other NF-κB activators such as TNF-α (Figure 2a, bottom panel), IL-1, and LPS (data not shown). Activation of NF-κB was observed after SDF-1α treatment for 1 hour, and reached maximum by 4 hours, remaining unchanged for at least 8 hours in the continued presence of SDF-1α (Figure 2a, top panel). In contrast, activation of NF-κB was detected after TNF-α treatment for 15 minutes (Figure 2a, bottom panel). This slow, sustained activation of NF-κB by SDF-1α suggested an indirect process, requiring de novo or ongoing protein synthesis. To address this possibility, NF-κB activation was tested for sensitivity to protein-synthesis inhibition by CHX. Pretreatment of primary astrocytes with CHX almost completely

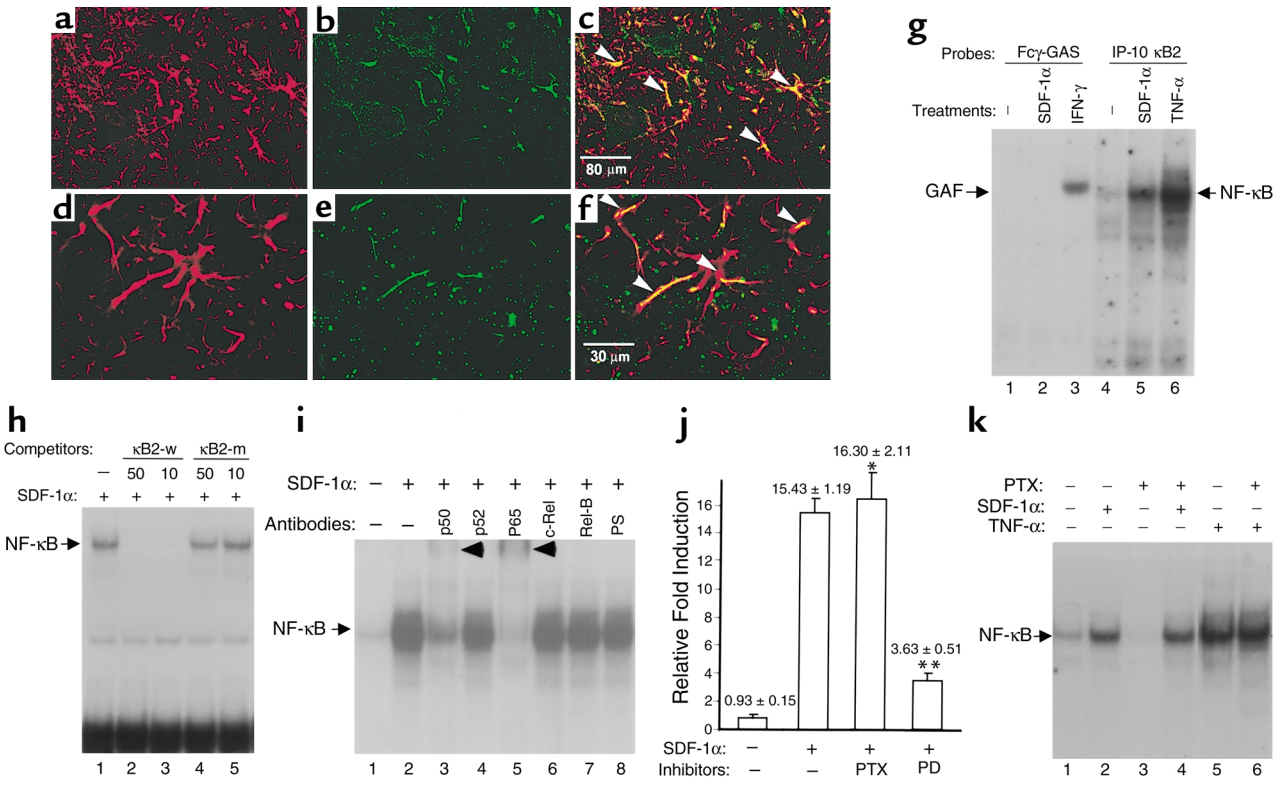

Figure 1

SDF-1α activates NF-κB in primary astrocytes. (**a**–**f**) Immunofluorescence staining of ventral lumbar spinal cord section: anti-GFAP (**a** and **d**, red), anti–SDF-1α (**b**, green), and anti-CXCR4 (**e**, green). Colocalization of GFAP and SDF-1α (**c**) or CXCR4 (**f**) within astrocytes (arrowheads). (**g**–**i**) SDF-1α induces NF-κB DNA binding activity. Primary astrocytes were incubated with medium alone or SDF-1α (2 hours) or IFN-γ (250 U/ml; 15 minutes) or TNF-α (30 minutes). Shown are results of EMSA with Fcγ-GAS (**g**) and IP-10 κB2 probes (**g**–**h**). SDF-1α selectively induces NF-κB DNA binding complex (g) competed by wild-type but not mutant κB elements (**h**) or supershifted by anti-p50 and anti-p65 antibodies (**i**). (**j**) SDF-1α stimulates NF-κB reporter in luciferase activity assay. Primary astrocytes were cotransfected with p6κB-luc and pRL-TK constructs and treated with SDF-1α for 6 hours in the presence or absence of pertussis toxin (PTX) or PD98059 (PD). Shown are mean ± SD (Student's *t* test: *PTX + SDF-1α vs. SDF-1α, $P = 0.5689$; **PD + SDF-1α vs. SDF-1α , $P < 0.001$). (**k**) PTX does not suppress SDF-1α- or TNF-α–stimulated activation. Primary astrocytes were treated with SDF-1α for 2 hours or TNF-α for 30 minutes in the presence or absence of PTX. Shown are results of EMSA with IP-10 κB2 probes. Data represent three or more than three experiments (**g**–**k**).

abolished SDF-1α–stimulated but not TNF-α–stimulated NF-κB DNA binding activity (Figure 2b), suggesting that SDF-1α activation of NF-κB was indirect.

Activation of NF-κB requires sequential phosphorylation, ubiquitination, and degradation of IκBs (33, 34). We compared NF-κB activation and degradation of IκB-α and IκB-β in extracts from SDF-1α–treated cells. IκB-α was reduced after 1 hour of SDF-1 stimulation, with maximal decay between 1 and 2 hours (Figure 2a). Reaccumulation of IκB-α was observed at 4 hours (Figure 2a, middle panel). In contrast, degradation of IκB-β occurred in 1 hour, with maximal degradation in 2 hours and no re-expression at 8 hours (Figure 2a, middle panel). PTX did not block degradation of IκB-α or IκB-β (data not shown). These results demonstrated that SDF-1α activates NF-κB by inducing IκB degradation.

SDF-1α induced expression of TNF-α, IL-1, RANTES, and TNF receptor 2 in primary astrocytes. To identify factors that could mediate SDF-1α–induced NF-κB activation, RNase protection assays (RPAs) were performed with a probe set comprising TNF-α, TNF-β, IL-1α, IL-1β, IL-2, IFN-γ, and RANTES. A time course from 30 minutes to 8 hours was selected for treatment, because maximal NF-κB activation occurred 4–8 hours after SDF-1α stimulation (Figure 2a). SDF-1α selectively stimulated expression of TNF-α, IL-1α, IL-1β, and RANTES, but not TNF-β, IL-2, or IFN-γ (Figure 3a). Induction of TNF-α was detected within 30 minutes, and reached maximum by 4 hours after starting incubation with SDF-1α (Figure 3a). Induction of IL-1α and IL-1β was detected after SDF-1α treatment for 1 hour, and reached maximum by 4 hours (Figure 3a). Induction of RANTES by SDF-1α was detected by 2 hours and reached maximum by 8 hours (Figure 3a). PTX pretreatment did not affect SDF-1α–induced expression of TNF-α, IL-1α, IL-1β, or RANTES mRNA, demonstrating Gαi independence. Because TNF-α induction occurred earliest and could plausibly account for IL-1 and RANTES expression by astrocytes, we characterized further TNF-α induction by SDF-1α. We first examined TNF-α protein in astrocytes and culture medium by Western blot (data not shown) and ELISA to determine whether increased mRNA expression was associated with protein expression and secretion. TNF-α was detected in culture medium after SDF-1α treatment for 30 minutes (data not shown), reached maximum by 8 hours, and remained unchanged for at least 16 hours in the continued presence of SDF-1α (Figure 3b). To determine whether SDF-1α upregulated TNF-α by activation of its promoter, primary astrocytes were transiently transfected with a murine TNF-α promoter–luciferase construct, and reporter activity was monitored after SDF-1α treatment. SDF-1α stimulated reporter activity by about 20-fold, an effect that was insensitive to PTX pretreatment (see Figure 7b), indicating Gαi-independent transcriptional regulation.

We also addressed whether SDF-1α modulated expression of receptors for TNF-α and IL-1 in primary astrocytes. Primary astrocytes expressed IL-1 receptor 1 (IL-1R1) and TNF-α receptors 1 and 2 (TNFR1/2), with TNFR1 being more abundant (Figure 3c). SDF-1α treatment selectively increased TNFR2 expression (Figure 3c and data not shown). These observations demonstrated SDF-1α–induced components required for autocrine response to TNF-α, including TNF-α protein and enhanced TNFR2 mRNA.

TNF-α mediates SDF-1α–induced IL-1 and RANTES expression and NF-κB activation in primary astrocytes. These observations prompted us to determine whether SDF-1α–mediated cytokine/chemokine gene induction was mediated by TNF-α. For initial experiments, we treated astrocytes with CHX before SDF-1α stimulation and RPA analysis. CHX treatment almost completely abolished expression of IL-1α, IL-1β, and RANTES mRNA, but only slightly affected TNF-α mRNA expression, indicating that induction of IL-1α, IL-1β,

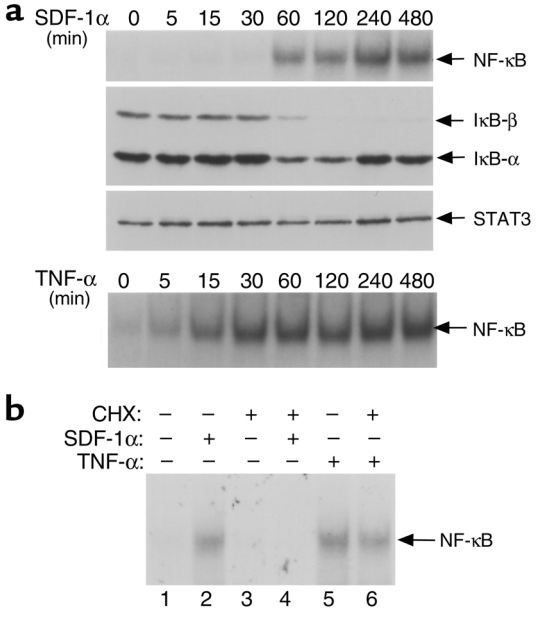

Figure 2
SDF-1α induction of NF-κB activity is indirect but correlates with degradation of IκB-α and IκB-β. (**a**) SDF-1α induction of NF-κB activity is slow but correlates with degradation of IκB-α and IκB-β. Murine primary astrocytes were incubated with medium alone or with SDF-1α or TNF-α for the indicated periods of time. Whole-cell extracts were then prepared and subjected to NF-κB DNA binding activity assays by EMSA with IP-10 κB2 probe (top panel: SDF-1α treatment; bottom panel: TNF-α treatment). Additional aliquots of the same whole-cell extracts were subjected to anti–IκB-α and anti–IκB-β Western blot (middle panel). After stripping, the same membrane was blotted with anti-STAT3 (middle panel) to monitor protein loading for each sample. Data represent three experiments. (**b**) CHX suppresses SDF-1α–induced but not TNF-α–induced NF-κB activation. Murine primary astrocytes were treated with SDF-1α (lane 2) for 2 hours, TNF-α (lane 5) for 30 minutes, CHX (50 μg/ml, lane 3) for 1 hour, or CHX for 1 hour followed by either SDF-1α (lane 4) for 2 hours or TNF-α (lane 6) for 30 minutes. Whole-cell extracts were then prepared and analyzed by EMSA with the IP-10 κB2 probe. Data represent three experiments.

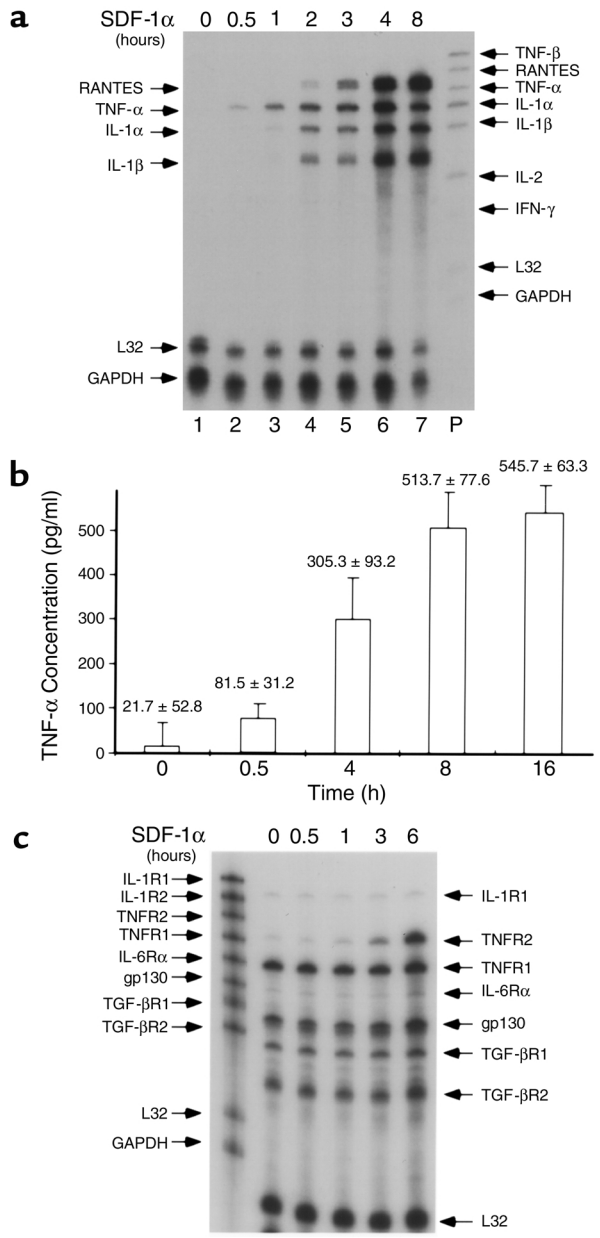

Figure 3
SDF-1α induces expression of TNF-α, IL-1, RANTES, and TNFR2 in primary astrocytes. (**a**) SDF-1α induces TNF-α, IL-1, and RANTES mRNA expression in primary astrocytes. Astrocytes were incubated with or without SDF-1α for the indicated periods of time. RNA was prepared and analyzed by RPA using cytokine and chemokine probe sets. Unprotected probe bands are indicated with arrows on the right and protected bands are shown with arrows on the left. Data represent four experiments. (**b**) SDF-1α stimulates TNF-α protein expression in primary astrocytes. Astrocytes in 12-well plates were incubated with or without SDF-1α for the indicated periods of time. TNF-α concentration in the culture medium was quantitated using the Mu TNF-α Cytokine Direct ELISA Kit. TNF-α concentration obtained from triplicate wells is shown as the mean ± SD. Data represent three independent experiments. (**c**) SDF-1α induces TNFR2 expression in primary astrocytes. Astrocytes were incubated with or without SDF-1α for the indicated periods of time. RNA was prepared and analyzed by RPA. Unprotected probe bands are indicated with arrows on the left and protected bands are shown with arrows on the right. Data represent three experiments.

and RANTES, but not TNF-α, depends on ongoing protein synthesis (Figure 4a).

To determine whether TNF-α could also mediate NF-κB activation by SDF-1α, astrocytes were treated with SDF-1α for 2 hours in the presence or absence of anti-murine TNF-α or IL-1 antibodies, and NF-κB activation was monitored by EMSA. As shown in Figure 4b, anti–TNF-α (lane 2) but not anti–IL-1α or anti–IL-1β antibodies (lane 3) blocked SDF-1α–induced NF-κB DNA binding activity, by 55% (Figure 4b, compare lane 2 to lane 4). Because of the difficulty of abrogating autocrine response with antibody, we further analyzed NF-κB activation using conditioned medium (CM) prepared from SDF-1α–treated astrocytes. This CM contained an activity that strongly induced NF-κB. This activity was almost completely removed by preincubation of CM with anti-murine TNF-α but not anti–IL-1 antibodies (data not shown). These data demonstrated that SDF-1α–induced TNF-α mediates NF-κB activation in primary astrocytes.

Taken together, these results suggested that exposure to SDF-α directly induced TNF-α expression in primary astrocytes; it was proposed that the subsequent response to TNF-α accounted for upregulation of IL-1, RANTES, and (by autoregulatory mechanisms) further TNF-α expression. In support of this hypothesis, SDF-1α–induced expression of IL-1α, IL-1β, and RANTES mRNA could be markedly suppressed by anti-TNF antibodies (data not shown).

TNF-α mediates SDF-1α–induced cell death in astrocytes. SDF-1α stimulated neuronal apoptosis in vitro, indicating a direct cytotoxic effect on neural cells (19, 29). Primary astrocytes expressed TNFR1/2 and increased TNFR2 expression after treatment with SDF-1α (Figure 3). MTT assays were used to evaluate cytotoxic effects of SDF-1α and TNF-α on astrocytes. H_2O_2-induced astrocyte death was used as a positive control. For these experiments, confluent astrocyte cultures were incubated with 100 ng/ml of SDF-1α, 5 ng/ml of TNF-α, or 100 μM of H_2O_2 for 30 minutes, then washed and incubated in complete medium. Figure 5 shows that TNF-α, H_2O_2, and SDF-1α treatment each caused significant cell death. Anti–TNF-α antibodies significantly reduced SDF-1α–induced cell death (Figure 5). These data indicate that SDF-1α exerts cytotoxic effects on primary astrocytes, and that these effects are mediated at least in part by SDF-1α–induced TNF-α.

Biphasic Erk1/2 MAPK activation in response to SDF-1α stimulation. Previously, we reported that SDF-1α rapidly and transiently stimulates phosphorylation of Erk1/2 MAPK in primary astrocytes (14). Additional detailed kinetic analysis of Erk1/2 activation revealed that SDF-1α had a biphasic effect on Erk1/2 phosphorylation (Figure 6a). Phosphorylation of Erk1/2 was increased after treatment with SDF-1α for 5 minutes and reached maximum by 15 minutes. It then gradually declined to the basal level of phosphorylation by the 1 hour point (early activation), followed by

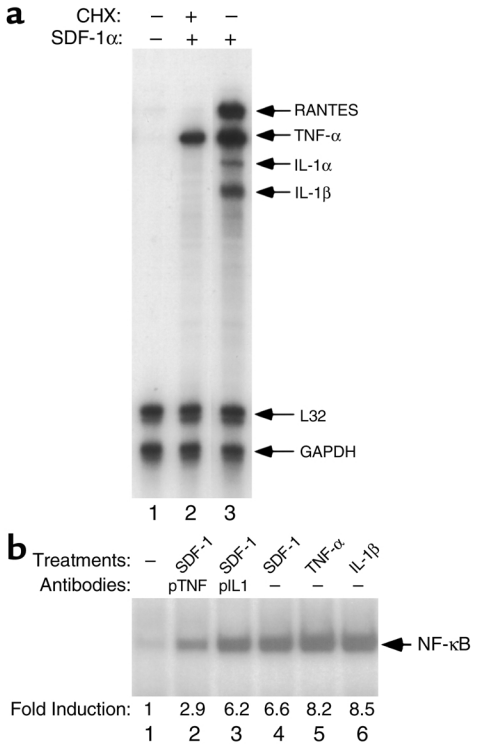

Figure 4
TNF-α mediates SDF-1α–induced IL-1 and RANTES expression and NF-κB activation in primary astrocytes. (**a**) CHX suppresses SDF-1α–induced gene expression in astrocytes. Astrocytes were incubated with or without CHX (50 μg) for 1 hour followed by SDF-1α stimulation for 2 hours. RNA was then prepared and analyzed with RPA. Protected bands are shown with arrows on the right. Data represent four experiments. (**b**) TNF-α mediates SDF-1α–induced NF-κB activation. Astrocytes were incubated with either SDF-1α (lanes 2–4) for 2 hours in the presence or absence of 5 μg/ml of anti-murine TNF-α (pTNF, lane 2), 2 μg/ml of anti-murine IL-1α and IL-1β (pIL1, lane 3), or 2 μg/ml TNF-α (lane 5), or 2.5 ng/ml IL-1β (lane 6). Whole-cell extracts were then prepared and analyzed by EMSA with the IP-10 κB2 probe. Data represent three experiments.

a subsequent rephosphorylation (late activation) in the continued presence of SDF-1α (Figure 6a, lanes 7 and 8). Rephosphorylation of Erk1/2 lasted for at least 2 hours. Early activation of Erk1/2 by SDF-1α was specifically inhibited by the Erk1/2 MAPK pathway inhibitor PD98059, but not by PTX or CHX (Figure 6b and data not shown), indicating a Gαi-independent, MEK1/2-mediated activation. However, late phosphorylation of Erk1/2 was blocked by both PD98059 and CHX (data not shown), and by anti–TNF-α antibodies (Figure 6c). These results suggest that early Erk1/2 activation is stimulated directly by SDF-1α, and late phosphorylation is mediated indirectly by SDF-1α–induced TNF-α.

SDF-1α–induced Erk1/2 activation is involved in TNF-α induction and NF-κB activation. The TNF-α promoter contains MAPK-dependent and -independent regulatory elements that are used in a cell-type-specific fashion (46). We asked whether Erk1/2 MAPK was involved in SDF-1α–stimulated TNF-α induction and NF-κB activation in primary astrocytes. PD98059 pretreatment of astrocytes inhibited TNF-α mRNA expression by 40–60% (with experimental variation) (Figure 7a); PD98059 also inhibited SDF-1α–induced TNF-α promoter reporter activity (Figure 7b) and TNF-α accumulation in culture medium (data not shown). In addition, PD98059 treatment reduced SDF-1α–induced NF-κB DNA binding activity (Figure 7c) and κB reporter activity (Figure 1j), and almost completely abolished induction of IL-1α, IL-1β, and RANTES (Figure 7a). PD98059 treatment also suppressed TNF-α–induced NF-κB DNA binding activity and expression of IL-1α, IL-1β, and RANTES (data not shown). These data demonstrate that Erk1/2 activity is required for TNF-α induction and NF-κB activation in response to SDF-1α.

Discussion

CXCR4 and SDF-1α play critical roles in cerebellar development and some pathological processes of the CNS (8, 9, 11, 25). The current studies demonstrate that SDF-1α activates NF-κB, stimulates chemokine and cytokine production, and induces cell death in primary astrocyte culture. These effects are mediated primarily by newly synthesized and secreted TNF-α. Biphasic Erk1/2 MAPK activation by SDF-1α provided evidence that Erk1/2 MAPK is important for SDF-1α–stimulated TNF-α induction and TNF-α–induced NF-κB activation. These cascades are summarized in Figure 8. These data documented both cell-type-specific and conserved mechanisms of response to SDF-1α. A spinal root avulsion model showed that CXCR4, SDF-1α, and NF-κB are expressed in vivo by reactive astrocytes (Figure 1, a–f, and data not shown), suggesting autocrine

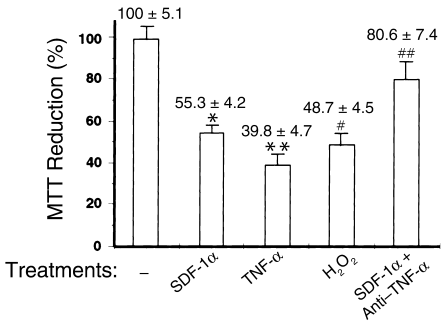

Figure 5
TNF-α mediates SDF-1α–induced astrocyte cell death. Confluent astrocytes in 12-well plates were incubated with SDF-1α, TNF-α, or SDF-1α plus anti–TNF-α antibodies (2 μg/ml) for 3 days; or exposed to H_2O_2 (100 μM) for 30 minutes followed by incubation with normal medium for 3 days; and then an MTT activity assay was performed. Mean ± SD of MTT activity obtained from triplicate treatments is shown (Student's *t* test: *SDF-1α treatment vs. untreated control, $P < 0.001$; **H_2O_2 treatment vs. untreated control, $P < 0.001$; #TNF-α treatment vs. untreated control, $P < 0.001$; ##treatment with anti–TNF-α antibody + SDF-1α vs. SDF-1α alone, $P = 0.0058$). Untreated control was plotted as 100%. Data represent three experiments.

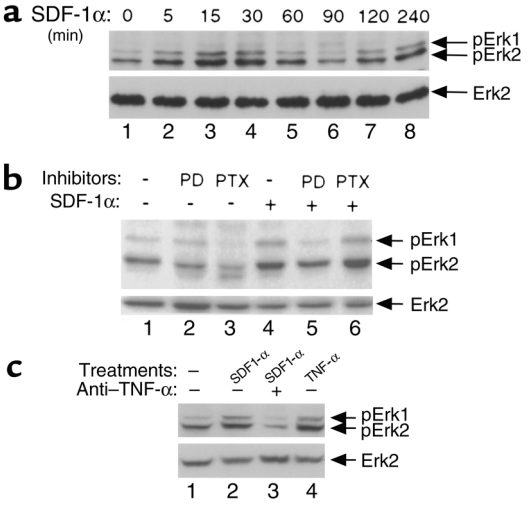

Figure 6
SDF-1α activates Erk1/2 MAPK. (**a**) Kinetic analysis of SDF-1α–induced Erk1/2 MAPK phosphorylation. Whole-cell extracts were prepared after SDF-1α treatment of primary astrocytes for the indicated periods of time. Erk1/2 MAPK phosphorylation was then analyzed by anti–phospho-Erk1/2 Western blot (top panel). After stripping, the same membrane was blotted with anti-Erk2 antibodies (bottom panel). Data represent three experiments. (**b**) PD98059, but not PTX, suppresses SDF-1α–stimulated Erk1/2 MAPK phosphorylation. Murine primary astrocytes were first incubated with PD98059 for 1 hour or with PTX for 20 hours, and then stimulated with SDF-1α for 15 minutes. Whole-cell extracts were then prepared and subjected to anti–phospho-Erk1/2 Western blotting (top panel). After stripping, the same membrane was blotted with anti-Erk2 antibodies (bottom panel). Data represent three experiments. (**c**) TNF-α mediates SDF-1α–induced late Erk1/2 activation. Murine primary astrocytes were incubated with SDF-1α in the presence (lane 3) or absence (lane 2) of anti–TNF-α (5 μg/ml) for 2 hours or treated with TNF-α alone (lane 4) for 30 minutes. Whole-cell extracts were then prepared and subjected to anti–phospho-Erk1/2 Western blotting (top panel). After stripping, the same membrane was blotted with anti-Erk2 antibodies (bottom panel). Data represent three experiments.

signaling. Importantly, both SDF-1α and CXCR4 are also expressed in the brains of patients dying with HAD, in which increased astrocyte cell death was recently shown to be associated with severe neurologic impairment (28). Our data suggest that SDF-1α may exert a pathogenic role in HAD by several mechanisms: induction of TNF-α (which could transactivate HIV gene expression); cytotoxicity towards astrocytes and neurons (15, 19, 29); and generation of inflammatory cytokines and chemokines.

Selective NF-κB activation by SDF-1α. In primary astrocyte cultures, SDF-1α induced NF-κB DNA binding activity and activated κB reporter activity (Figures1b–1e). Although primary astrocytes also express functional CCR1 (37), treatment of astrocytes with RANTES did not stimulate NF-κB activation (data not shown), indicating a selective response to SDF-1α. Pretreatment of cells with PTX did not block SDF-1α–induced IκB degradation (data not shown), NF-κB DNA binding activity (Figure 1k), or κB reporter activity (Figure 1j), indicating Gαi-independent activation of NF-κB.

The JAK-STAT pathway mediates responses to many cytokines and growth factors (47). SDF-1α binding to CXCR4 was shown to cause activation of JAK2/3 and STAT1/2/3/5b in T cell lines (18). We did not observe JAK or STAT activation using either EMSA or immunoprecipitation–Western blot analyses (Figure 1g and data not shown). Astrocytes from STAT1 knockout and wild-type mice showed comparable responses to SDF-1α stimulation, including Erk1/2 and NF-κB activation as well as

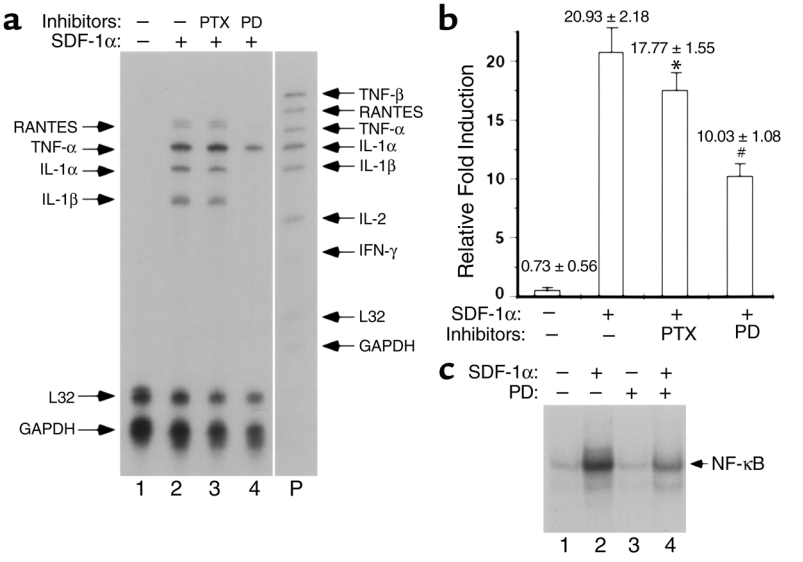

Figure 7
SDF-1α–induced Erk1/2 activation is involved in TNF-α induction and NF-κB activation. (**a**) PD98059, but not PTX, suppresses SDF-1α–induced cytokine and chemokine mRNA expression. Murine primary astrocytes were incubated with medium alone, with PTX for 20 hours, or with PD98059 for 1 hour. Incubation was followed by SDF-1α stimulation for 2 hours. RNA was then prepared and analyzed by RPA. Unprotected probe bands are indicated with arrows on the right, and protected bands are shown with arrows on the left. Data represent four experiments. (**b**) PD98059, but not PTX, suppresses SDF-1α–induced TNF-α promoter reporter activity. Primary astrocytes in the presence or absence of PTX or PD98059 were transiently cotransfected with pGL3-mTNF (–1260) (0.5 μg) and pRL-TK (0.05 μg) constructs followed by SDF-1α stimulation for 6 hours. Cells were then lysed and subjected to luciferase activity assay. After normalization, mean ± SD relative luciferase activity was obtained from triplicate independent transfection (Student's *t* test: *treatment with PTX + SDF-1α vs. SDF-1α alone, $P = 0.1097$; #treatment with PD + SDF-1α vs. SDF-1α alone, $P = 0.0015$). Data represent three independent experiments. (**c**) PD98059 suppresses SDF-1α–induced NF-κB activation. Murine primary astrocytes were treated with SDF-1α (lane 2) for 2 hours, with PD98059 for 3 hours (lane 3), or with PD98059 for 1 hour followed by SDF-1α for 2 hours (lane 4). Shown are results of EMSA with the IP-10 κB2 probes. Data represent three experiments.

cytokine induction (data not shown), suggesting that JAK-STAT activation by SDF-1α is specific to cell type and is dispensable for biological functions of SDF-1α in primary astrocytes.

SDF-1α induction of TNF-α, IL-1, RANTES, and TNFR2. The induction of expression of TNF-α, IL-1α, IL-1β, RANTES (Figure 3a), and several other chemokines (data not shown), but not TNF-β, IL-2, or IFN-γ, by SDF-1α (Figure 3a) highlights a regulatory role of SDF-1α/CXCR4 in cytokine and chemokine expression. Activation of NF-κB by SDF-1α was slower than that by TNF-α in primary astrocytes (Figures 1a and 2a), suggesting involvement of one or more intermediates. This possibility was confirmed by protein synthesis inhibition experiments in which NF-κB DNA binding activity was entirely blocked by treatment with CHX (Figure 2b). The notion that TNF-α mediated SDF-1α–induced IL-1 and RANTES expression in primary astrocytes was supported by the following observations: (1) TNF-α induced IL-1 and RANTES expression in primary astrocytes (data not shown); (2) induction of TNF-α (within 30 minutes) was earlier than that of IL-1 (after 1 hour) and RANTES (after 2 hours) (Figure 3a), and anti–TNF-α antibodies inhibited induction of IL-1 and RANTES (data not shown); and (3) CHX blocked IL-1 and RANTES expression but minimally affected TNF-α expression (Figure 4a). The ability of CHX or anti–TNF-α antibodies to partially suppress TNF-α expression is probably due to inhibition of autocrine induction of TNF-α.

Selective induction of TNFR2 on $CD8^+$ T cells by CXCR4 activation was observed during HIV infection or upon treatment of cells with SDF-1α or HIV gp120 (48). Interaction between macrophage mbTNF-α and TNFR2 on $CD8^+$ T-cells resulted in apoptosis of $TNFR2^+$ cells (48). The selective induction of TNFR2 expression along with TNF-α by SDF-1α in primary astrocytes suggested that autocrine effects via TNF-α/TNFR2 might mediate SDF-1α–induced signaling events and biological activities in astrocytes.

TNF-α mediates SDF-1α–induced NF-κB activation and cell death. SDF-1α treatment resulted in astrocyte death, and this effect was comparable to that of TNF-α and H_2O_2 (Figure 5). Anti–TNF-α antibodies significantly suppressed cytotoxic effects of SDF-1α on astrocytes (Figure 5), indicating that TNF-α was a major mediator of SDF-1α–induced cell death. The partial effect of neutralizing TNF-α may be related to the stoichiometry of cytokines and antibodies in the culture, or induction of other TNF-α family members by SDF-1α. In previous reports, blocking mbTNF-α on macrophages or TNFR2 on $CD8^+$ T cells suppressed SDF-1α–induced apoptosis of $CD8^+$ T cells (48). In that system, apoptosis of $CD8^+$ T cells could result from direct effects of TNF-α engagement with TNFR2 or TNF-α/TNFR2-mediated adhesion between macrophages and $CD8^+$ T cells, thereby enhancing other proapoptotic mechanisms. Our data revealed that soluble TNF-α was directly cytotoxic to astrocytes. Further experiments are being undertaken to confirm the possibility that TNFR2 induction by SDF-1α could facilitate TNF-α effects on astrocytes.

Erk1/2 MAPK activation and roles in SDF-1α–induced TNF-α induction and NF-κB activation. SDF-1α activates Erk1/2 MAPK in several cell types, including astrocytes (14–16). Activation of Erk1/2 MAPK is sensitive to a chemical inhibitor, PD98059, which specifically inhibits MEK1 and MEK2. Kinetic analysis of Erk1/2 activation in astrocytes revealed biphasic Erk1/2 phosphorylation in SDF-1α–treated cells (Figure 6a). We proposed that SDF-1α–induced biphasic phosphorylation of Erk1/2 was mediated by two different mechanisms. Early Erk1/2 activation by SDF-1α was direct via MEK1/2 signaling, without new protein synthesis or Gαi participation (Figures 5d and 6a). Late Erk1/2 phosphorylation was shown to be signaled by TNF-α, since anti–TNF-α antibodies and blockade of TNF-α induction by CHX pretreatment each suppressed Erk1/2 phosphorylation (Figure 6c and data not shown).

The TNF-α promoter contains MAPK-dependent and -independent regulatory elements that are used in a cell-type-specific fashion (46). Consistent with these prior reports, PD98059 suppressed SDF-1α–induced TNF-α mRNA accumulation (Figure 7a), protein expression (data not shown), and TNF-α promoter reporter activity (Figure 7b), revealing that early Erk1/2 MAPK activation by SDF-1α is involved in TNF-α transcription in primary astrocytes. As expected, PD98059 suppressed all effects of SDF-1α that were attributed to TNF-α (induction of IL-1 and chemokines; stimulation of astrocyte cell death). These observations support the proposed sig-

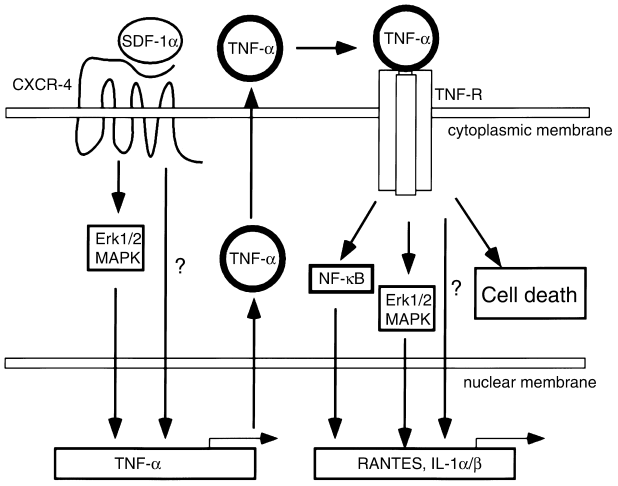

Figure 8
Schematic representation of the signaling pathways activated by SDF-1α in primary astrocytes. SDF-1α binding to CXCR4 elicits intracellular signaling including the Erk1/2 MAPK cascade, which initiates activation of TNF-α transcription. TNF-α protein is then synthesized and secreted to the extracellular space (or medium), where it binds to its receptors. TNF-α engagement with its receptors initiates signals leading to cell death and activation of NF-κB and Erk1/2 MAPK. Activated NF-κB and Erk1/2 MAPK in turn translocate to the nucleus and activate IL-1 and RANTES expression.

naling cascade in which multiple Gαi-independent effects of SDF-1α are mediated by TNF-α (Figure 8).

Physiologic relevance of SDF-1α–induced TNF-α in HIV infection of the CNS. TNF-α expression in the CNS is maintained at a very low level under physiologic conditions, presumably to maintain CNS homeostasis (49). TNF-α receptors are present on all neural cell types, rendering them responsive to TNF-α.

CXCR4 is a coreceptor for HIV infection, and accordingly, SDF-1α and its various analogues inhibit CXCR4-mediated HIV-1 infection in vitro. Previously, we showed that TNF-α suppressed CXCR4 expression in astrocytes (14), indicating a possible role of TNF-α in limiting HIV infection of glial cells. It is appropriate to consider whether downregulation of CXCR4 expression by TNF-α would affect SDF-1α–induced NF-κB activation, IL-1 and RANTES induction, and astrocyte death. We propose that that effect will be quite limited because of kinetic considerations: SDF-1α induces downregulation of CXCR4 mRNA expression through TNF-α after SDF-1α stimulation for 4 hours (data not shown). By this time point, TNF-α signaling through TNFR2 is already complete. These observations, translated to the chronic process that characterizes CNS infection with HIV, underline the complex potential functions of SDF-1α in this disorder and suggest that SDF-1α could mediate several pathogenic events. First, SDF-1α induction of TNF-α by astrocytes provides a local source of soluble cytotoxic factors in the CNS. TNF-α, with other soluble factors secreted by infected or activated cells, could induce neuronal cell death, contributing to the pathogenesis of HAD (25, 27, 50). Second, NF-κB activation through TNF-α by SDF-1α could promote reactivation of latent HIV in microglia and macrophages (51). Third, SDF-1α induction of RANTES and other chemokines (Figure 3a and data not shown) via TNF-α could promote recruitment of HIV-infected $CCR5^+$ cells to the CNS. Finally, SDF-1α and TNF-α could promote and accelerate local inflammation and damage by paracrine and autocrine mechanisms. SDF-1α can be detected in brains from normal controls and is modestly upregulated in the brains of patients dying with HAD (19). Taken together, present knowledge of SDF-1α shows it to represent a double-edged sword in HIV infection by blocking CXCR4-dependent HIV entry into cells while promoting HIV neuropathogenesis in many other respects.

Acknowledgments

This research was supported by NIH (RO1 NS-32151; PO1 CA-62220) and by the Williams Family Fund for Multiple Sclerosis Research.

We thank George R. Stark for supplying κB-luciferase construct and Dmitry V. Kuprash and Sergei A. Nedospasov for providing murine TNF-α promoter construct.

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