SDF-1α induces chemotaxis and enhances Sonic hedgehog-induced proliferation of cerebellar granule cells

Robyn S. Klein1,*,‡, Joshua B. Rubin2,*, Hilary D. Gibson1, Elliot N. DeHaan1, Xavier Alvarez-Hernandez4, Rosalind A. Segal2,3 and Andrew D. Luster1

1Center for Immunology and Inflammatory Diseases, Division Rheumatology, Allergy and Immunology, Massachusetts General Hospital, Harvard Medical School, Charlestown, MA 02129, USA
2Department of Pediatric Oncology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA 02115, USA
3Department of Neurobiology, Harvard Medical School, Boston, MA 02115, USA
4Department of Pathology, New England Regional Primate Research Center, Harvard Medical School, Southboro, MA 01772, USA

*These two authors contributed equally
‡Author for correspondence (e-mail: rklein@partners.org)

Accepted 1 March 2001

SUMMARY

The chemokine SDF-1α (CXC12) and its receptor CXCR4 have been shown to play a role in the development of normal cerebellar cytoarchitecture. We report here that SDF-1α both induces chemotactic responses in granule precursor cells and enhances granule cell proliferative responses to Sonic hedgehog. Chemotactic and proliferative responses to SDF-1α are greater in granule cells obtained from cerebella of animals in the first postnatal week, coinciding with the observed in vivo peak in cerebellar CXCR4 expression. SDF-1α activation of neuronal CXCR4 differs from activation of CXCR4 in leukocytes in that SDF-1α-induced calcium flux is activity dependent, requiring predepolarization with KCl or pretreatment with glutamate. However, as is the case in leukocytes, neuronal responses to SDF-1α are all abolished by pretreatment of granule cells with pertussis toxin, suggesting they occur through Gαi activation. In conclusion, SDF-1α plays a role in two important processes of granule cell maturation – proliferation and migration – assisting in the achievement of appropriate cell number and position in the cerebellar cortex.

Key words: Chemokine, SDF-1α, Sonic hedgehog, Granule cell, Cerebellum, Mouse

INTRODUCTION

The factors that control cell number and position in the developing central nervous system (CNS) are complex and incompletely understood, but can be readily examined in the cerebellum. During the first two postnatal weeks in the mouse cerebellum, granule precursor cells proliferate in a specialized zone known as the external granule cell layer (EGL). Postmitotic granule cells then migrate to form the internal granule cell layer (IGL) composed of mature granule neurons (Miale and Sidman, 1961; Altman, 1972; Burgoyne and Cabray-Deakin, 1988; Hatten, 1993; Altman and Bayer, 1996). Sonic hedgehog (SHH), fibroblast growth factor (FGF), epidermal growth factor (EGF) and insulin-like growth factor (IGF)-1 have all been shown to promote granule cell proliferation (Gao et al., 1991; Tao et al., 1996; Ye et al., 1996; Lin and Bulleit, 1997; Dahmane and Ruiz-i-Altaba, 1999; Traiffort et al., 1999; Wallace, 1999; Wechsler-Reya and Scott, 1999; Kenney and Rowitch, 2000). SHH, which is produced by Purkinje neurons, is the single most potent in vitro mitogen, capable of inducing 100-fold granule cell proliferation, but only at high concentrations (Dahmane and Ruiz-i-Altaba, 1999; Wallace, 1999; Wechsler-Reya and Scott, 1999). Lower concentrations of SHH (nanomolar) induce a 2- to 4-fold increase in granule cell proliferation in vitro (Dahmane and Ruiz-i-Altaba, 1999; Wechsler-Reya and Scott, 1999). In vivo, SHH may be present at these lower concentrations and thus may act in concert with other factors to promote substantial granule cell proliferation. Inhibition of SHH responses are well known to result from increases in cAMP and protein kinase A (PKA) activity (Hynes et al., 1995; Concordet et al., 1996; Epstein et al., 1996; Ungar and Moon, 1996), but enhancers of SHH actions have not yet been identified.

Factors that localize granule cell precursors to the proliferative environment of the EGL would be expected to enhance their proliferation. The chemokine, stromal-cell-derived factor (SDF)-1α has been identified as one such factor, as targeted deletion of the gene encoding SDF-1α or its receptor, CXCR4, resulted in the premature migration of granule cell precursors out of the EGL (Ma et al., 1998; Zou et al., 1998). SDF-1α expression in the pia mater during embryonic development and its receptor CXCR4 is expressed in the EGL during the same period (McGrath et al., 1999). Together these data suggest that SDF-1α and CXCR4 play a role in retaining granule cells in the EGL.
Chemokines are a superfamily of over 40 structurally homologous chemotactic cytokines that are involved in the trafficking of leukocytes to areas of inflammation (Luster, 1998). Chemokines induce cell migration and activation by binding to a subfamily of seven transmembrane-spanning receptors shown to be coupled to pertussis toxin sensitive Gαi proteins (Premack and Schall, 1996). Most chemokine receptors bind more than one chemokine ligand and many chemokines bind more than one chemokine receptor, however, SDF-1α binds only CXCR4 and is its only ligand (Rossi and Zlotnik, 2000). CXCR4 is expressed on all leukocytes and more recently has been shown to be present on subpopulations of cortical neurons (Lavi et al., 1997; Westmoreland et al., 1998; Zhang et al., 1998; Banisadr et al., 2000). While the role of CXCR4 and SDF-1α in leukocyte trafficking and activation has been extensively studied, very little is known about their function in neuronal physiology. We and others have shown that human fetal cortical neurons express functional CXCR4 in vitro (Sanders et al., 1998; Klein et al., 1999). SDF-1α activates neuronal CXCR4 and induces increases in intracellular calcium. As in leukocytes, neuronal responses to SDF-1α are pertussis toxin sensitive, indicating coupling of neuronal CXCR4 to Gαi (Klein et al., 1999). The expression of CXCR4 and the ability of SDF-1α to induce calcium transients has been observed in rat cerebellar granule cells (Limatola et al., 2000). SDF-1α has also been shown to induce chemotaxis of rat neuronal progenitor cells and to affect apoptotic responses to various stimuli in cultured rat neurons (Meucci et al., 1998; Kaul and Lipton, 1999; Zheng et al., 1999; Lazarini et al., 2000).

SDF-1α has multiple effects on hematopoietic cells, including localization, augmentation of proliferation and promotion of survival (Nagasawa et al., 1994; Nagasawa et al., 1996; Bleul et al., 1998; Bradstock et al., 2000; Lataillade et al., 2000; Nanki and Lipsky, 2000). We hypothesized that SDF-1α could play similar roles in both granule cell proliferation and in the maintenance of the EGL. We demonstrate here that SDF-1α is a chemoattractant for cerebellar granule precursor cells and acts synergistically with SHH to promote their proliferation. The interaction between SDF-1α and SHH occurs via a pertussis toxin-sensitive pathway, suggesting it occurs through Gαi. CXCR4 expression in the neonatal cerebellum peaks during the first postnatal week, coinciding with peak SDF-1α-induced chemotactic and proliferative responses. These studies support a model of cerebellar development in which SDF-1α is essential for the maintenance of the EGL and the establishment of granule cell number.

**MATERIALS AND METHODS**

**Granule cell preparation**

BALB/c mice breeding pairs or pregnant rats were obtained from Charles River Laboratories (Wilmington, MA). Pups were sacrificed at various postnatal days by decapitation. Cerella were placed in calcium- and magnesium-free Hanks’ balanced salt solution, (HBSS) and meninges were removed under 10x magnification using fine forceps. Cerella were washed twice in HBSS, and then treated with 1 mg/ml trypsin with 125 U/ml DNase (Sigma, St. Louis, MO) for 10-20 minutes, depending on the age of the animals from which they were obtained. Enzymatic digestion was quenched with DMEM/10% FCS and the tissue was pelleted in a clinical centrifuge. Pellets were washed in HBSS, dissociated by trituration through a P-200 pipette tip and dissociated cerebellar cells were layered over a step gradient of Percoll (Sigma) (2 ml of 60% Percoll in HBSS, 2 ml of 35% Percoll in HBSS, and then 2 ml of cell suspension in HBSS). The gradient was spun in a clinical centrifuge at 2370 g for 20 minutes. The gradient was then separated in thirds and the middle third, which contained the granule cells, was plated in DMEM with 10% FCS on a tissue culture dish pretreated with poly-D-lysine (PDL) (50 μg/ml) for 1 hour to remove adherent glial cells. In some experiments, cells were treated with 200 ng/ml pertussis toxin during this adherence step. Nonadherent cells were then washed in HBSS, counted and either plated on PDL-coated coverslips at 2.5x10^6 cells/ml in serum-free DMEM (supplemented with N2 growth medium (Gibco, Grand Island, NY) and 20 mM KCl) or resuspended in the same medium at 2.5x10^6 cells/ml for use in chemotaxis assays.

**Northern blot analysis**

Whole litters of mouse pups were sacrificed at postnatal days 3, 5, 7, 9 and 12 and cerebella were rapidly removed and snap-frozen on dry ice. RNA was obtained from pooled cerebella using lysis in guanidium hydrochloride followed by pelleting through a CsCl2 gradient as previously described (Khan et al., 2000). 20 μg total RNA was electrophoresed on a 1.2% agarose formaldehyde gel and then capillary transferred to a GeneScreen membrane (NEN Life Science Products, Boston, MA). Prehybridization and probe preparations were carried out according to previously published procedures (Khan et al., 2000). A 584 bp coding fragment of the murine CXCR4 cDNA cloned into the BamHI site of T7T3 vector (Incyte Genomics, Inc., St. Louis, MO) was used for probe synthesis after confirmatory sequence was obtained. A 180 bp coding fragment of murine SDF-1α gene was obtained by PCR of total murine thymic RNA and cloned into the BamHI site of Bluescript (ks) (Promega Life Sciences, Madison, WI). 32P-labeled CXCR4 and SDF-1α probes were generated by random primed DNA synthesis of BamHI fragments according to the manufacturer’s instructions (Boehringer Mannheim, Mannheim, Germany). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (kindly provided by M. Pyröstowsky) was used as a control for RNA loading. Signal was quantified using a phosphoimager (Molecular Imager System, Bio-Rad, Hercules, CA) and values for each sample were normalized based on the GAPDH signal.

**In situ hybridization and immunohistochemical analyses**

**Tissue preparation**

Brains from postnatal days 4 and 8 BALB/c mice were removed, postfixed in 4% paraformaldehyde for 24 hours and cryoprotected in 30% sucrose. Serial sagittal sections (15 μm) of cerebella were obtained using a Reichart Jung Cryostat.

**Probe preparation**

The orientation of CXCR4 and SDF-1α probes was determined by direct sequencing. Sense and antisense digoxigenin-labeled riboprobes were synthesized according to the manufacturer’s instructions (Boehringer Mannheim, Mannheim, Germany).

**In situ hybridization**

Formamide-fixed tissue sections were digested with 20 μg/ml proteinase K for 10 minutes at room temperature. Sections were refixed in 4% paraformaldehyde and washed in PBS. In situ hybridization was conducted for 20 hours at 65°C using DIG-labeled cRNA probes in hybridization buffer (50% formamide, 5x SSC, 100 μg/ml yeast tRNA, 100 μg/ml heparin, 1x Denhardt’s, 0.1% Tween 20, 0.1% CHAPS, 5 mM EDTA). The sections were washed with 0.2x SSC, 0.1% Tween 20 at 65°C and then treated with blocking reagent (20% sheep serum in buffer) and then anti-DIG antibody followed by antibody detection according to the manufacturer’s protocol (Boehringer Mannheim, Mannheim, Germany).
Immunohistochemistry
Tissue sections were permeabilized with 0.1% Triton X-100 (Sigma) and nonspecific antibody binding was blocked with 5% normal goat serum (Biofluids, Rockville, MD) for 1 hour at room temperature. Polyclonal antibodies specific for CXCR4 and SDF-1α were applied at 1 μg/ml in PBS containing 5% normal goat serum and 0.1% Triton X-100 overnight at 4°C. Sections were washed free of unbound antibody by repeated immersion in PBS containing 5% normal goat serum and 0.1% Triton X-100. Primary antibodies were detected with secondary goat anti-rabbit antibody conjugated to Cy3. Nuclei were counterstained with DAPI (Sigma) for 1 minute at room temperature. Sections were then washed with 20 mM Tris, and mounted using Immunomount (Shandon, Pittsburgh, PA).

Immunocytochemistry and confocal microscopy
Granule cells were prepared and seeded onto coverslips as described above and cultured for 1-2 days. Cells were fixed in 4% paraformaldehyde for 1 hour, washed with sterile PBS and then immunostained. Cells were treated for 30 minutes with a blocking buffer consisting of 10% normal goat serum and 5% mouse serum in sterile PBS. Cultures were assessed for purity using a monoclonal antibody to calbindin (a marker for Purkinje cells). Cultures were found to contain ~3-4% Purkinje cells (~96% granule cells). Double and single-label immunofluorescence was performed using polyclonal antibodies to CXCR4 (1 μg/ml; kindly provided by Jose Gonzalo, Millennium Pharmaceuticals Inc., Cambridge, MA; Gonzalez et al., 2000) and monoclonal antibody to calbindin (1:200; Sigma) plus topro-3 (Molecular Probes, Eugene, OR) staining to identify cell nuclei. Primary antibodies were detected using secondary anti-rabbit or mouse antibodies conjugated with Texas Red (1:200; Sigma) or FITC (1:200; Sigma), respectively. Control coverslips were treated with blocking buffer (containing non-immune serum) and then secondary antibodies alone. Confocal microscopy was performed using a Leica TCS SP laser scanning microscope, fitted with a 100× Leica objective (PL APO, 1.4 NA), and using the Leica image software. Images were collected at 512×512 pixel resolution. The stained cells were optically sectioned in the z-axis and the images in the different channels (photo-multiplier tubes) were collected simultaneously. The step size in the z-axis was varied from 0.2 to 0.5 μm to obtain 30-50 slices per imaged field. The images were transferred to a Macintosh G3 computer and NIH Image v1.61 software was used to render the images.

Chemotaxis assay
The in vitro migration of granule cells in response to human SDF-1α (Peprotech Inc., Rocky Hill, NJ) was assessed using a pol-D-lysine and nonspecific antibody binding was blocked with 5% normal goat serum (Biofluids, Rockville, MD) for 1 hour at room temperature. Polyclonal antibodies specific for CXCR4 and SDF-1α were applied at 1 μg/ml in PBS containing 5% normal goat serum and 0.1% Triton X-100 overnight at 4°C. Sections were washed free of unbound antibody by repeated immersion in PBS containing 5% normal goat serum and 0.1% Triton X-100. Primary antibodies were detected with secondary goat anti-rabbit antibody conjugated to Cy3. Nuclei were counterstained with DAPI (Sigma) for 1 minute at room temperature. Sections were then washed with 20 mM Tris, and mounted using Immunomount (Shandon, Pittsburgh, PA).

Proliferation assay
Primary cultures of mouse cerebellum were established as previously described (Segal et al., 1995). Briefly, cerebella of P6 BALB/c mice were removed and placed in HBSS with 6 mg/ml glucose and 15 mM Heps pH 7.4. Meninges were removed under 10X magnification and tissue was digested with 1 mg/ml trypsin with 125 U/ml DNase (Sigma), 0.15 mM EDTA and 15 mM Heps pH 7.4 for 20 minutes at 37°C. Digestion was stopped by the addition of an equal volume of 20% fetal calf serum in DMEM. Tissue was pelleted in a clinical centrifuge by spinning for 4 minutes at 740 g. Cells were washed twice by resuspending in HBSS and spinning as above. The final cell suspension was passed through a 100 μm cell strainer. Cells were counted and diluted to a final concentration of 2×10⁵ cells/ml with DMEM/F12 supplemented with N2 growth medium, 6 mg/ml glucose and 20 mM KCl. Cells were plated in the presence of all additives such as siHu (0.14 μg/ml or 7 nM; Curis Inc., Cambridge, MA), human SDF-1α (1000 ng/ml; Peprotech Inc.), pertussis toxin (25 ng/ml; Sigma), or forskolin (10 μM; Sigma) onto 96-well culture plates (Falcon) for approximately 20 hours before the addition of 5 μCi/well of [³H]thymidine (1 mCi/ml, NEN Inc., Boston, MA). After 4 hours of labeling, cells were lysed with 1% Triton X-100 for 10 minutes at room temperature. Subsequently, trichloroacetic acid (TCA) was added to 10% and incorporated thymidine was precipitated for 1 hour on ice. Precipitates were collected by vacuum filtration and filters were washed extensively with ice-cold TCA and dried with 100% ethanol. Dried filters were solubilized in Scint-Safe liquid scintillant (NEN Inc., Boston, MA) and incorporated counts were determined with a standard liquid scintillation counter. Experiments were performed in triplicate and data are expressed as fold-induction over control ± s.e.m. Data were analyzed for statistical significance between groups using Student’s t-test.

Calcium flux analysis
Peripheral blood mononuclear cells
Human peripheral blood mononuclear cells (PBMCs) were obtained from whole peripheral blood of healthy donors by passing blood through a Ficoll-Hypaque (Sigma) density gradient as previously described (Garcia-Zepeda et al., 1996). PBMCs were washed in HBSS and loaded with 5 μM fura-2 AM (Molecular Probes, Eugene, OR) for 60 minutes in a dark chamber at 37°C at 5×10⁶ cells/ml in RPMI/1% heat-inactivated FCS. Loaded cells were washed twice and resuspended in calcium flux buffer as previously described (Garcia-Zepeda et al., 1996). Two milliliters of cells were placed in a continuously stirring cuvette at 37°C in a dual-wavelength excitation source fluorimeter (Photon Technology Inc, South Brunswick, NJ). Changes of cytosolic free calcium were determined after addition of SDF-1α (Peprotech Inc.) by monitoring the excitation fluorescence intensity emitted at 510 nm in response to sequential excitation at 340 nm and 380 nm. The data are presented as the relative ratio of fluorescence at 340 and 380 nm.

Granule cells
Granule cells were prepared as described above, and seeded onto 110 μm thick coverslips and cultured as above. Cells were washed with PBS and loaded with 5 μM fura-2 AM for one hour in a dark chamber at 37°C. Cells were then washed with PBS and kept at 37°C under serum-free medium until analyzed for calcium flux responses (up to 1 hour). For calcium flux analysis, coverslips were placed under buffer at 37°C and examined under an inverted microscope connected to a spectrophluorimeter. Groups of 10-20 neurons were analyzed for their response to stimulation with 100 ng/ml SDF-1α after pretreatment with 20 mM KCl or 100 μM glutamate. Additional experiments were performed after pretreatment of neurons with 200 ng/ml pertussis toxin or 10 μg/ml polyclonal antibodies to CXCR4.

SDF-1α in cerebellar granule cell proliferation 1973
Calcium flux tracings were analyzed for the maximum increase in intracellular calcium according to the formula $[\text{Ca}]_i = 224 \text{ nM} \times \frac{(R - R_{\text{min}})}{(R_{\text{max}} - R)}$, assuming a $K_d$ of 224 nM, as previously described (Rothenberg et al., 1996), where $R_{\text{max}}$ is the amount of calcium increase after treatment with the nonspecific calcium ionophore ionomycin (5 μg/ml) and $R_{\text{min}}$ is the level of calcium following calcium chelation with Tris-EDTA. Calcium concentrations are expressed as the mean level ± s.e.m.

RESULTS

SDF-1α and CXCR4 mRNA are expressed in the postnatal murine cerebellum and CXCR4 is expressed by granule cells in vitro

In embryonic animals SDF-1α and CXCR4 are expressed in the pia mater and EGL, respectively. We hypothesized that continued expression of SDF-1α and CXCR4 could function to localize granule cells to the EGL through chemotaxis. We therefore examined the kinetics and location of SDF-1α and CXCR4 mRNA and protein expression during postnatal development by northern blot, in situ hybridization and immunohistochemical analyses. Northern blot analysis was performed on RNA isolated from pooled cerebella from whole litter of mouse pups taken at P3, P5, P7, P9 and P12 (Fig. 1A) and levels of CXCR4 and SDF-1α expression were quantitated (Fig. 1B). CXCR4 was expressed at all days examined but was progressively downregulated by approximately 70% during the second postnatal week (P7-P12). SDF-1α was also expressed at all days examined, without significant variation between ages.

In order to localize the expression of SDF-1α and CXCR4 mRNA and protein within the developing cerebellum, in situ hybridization and immunohistochemical analyses were performed on cerebellar tissue sections from P4 and P8 mouse pups. CXCR4 mRNA and protein were found within the EGL and IGL in both P4 (Fig. 2A,D) and P8 (data not shown) mouse cerebellum. There was no expression of CXCR4 in the pia. The expression of SDF-1α mRNA and protein in cerebellar sections was consistently localized to the pia mater in both P4 (Fig. 2B,E) and P8 (data not shown) animals. Small amounts of diffuse staining for SDF-1α mRNA and protein were observed throughout the cerebellum. This may represent expression by glial elements, as astrocytes have been shown to express SDF-1α (Bajetto et al., 1999). Tissue sections probed with control sense RNA transcripts for either CXCR4 (Fig. 2C) or SDF-1α (data not shown) did not detect any specific staining. The localization of SDF-1α to the pia mater overlying the EGL suggests that SDF-1α could act to prevent CXCR4 expressing granule cells from migrating away from the EGL during cerebellar development.

In order to determine uniformity of CXCR4 expression in vitro, we stained enriched populations of granule cells with anti-CXCR4 polyclonal antibodies. Replicate cultures were also double stained for calbindin, a marker for Purkinje neurons, to assess purity in addition to CXCR4 expression. All granule cells expressed CXCR4 with punctate staining observed over the cell body and length of the axon (Fig. 2D). Calbindin-positive cells (approx. 4%) were negative for CXCR4 (data not shown). The cellular distribution of CXCR4 is similar to previously published results for human and macaque fetal neurons (Klein et al., 1999). Control experiments in which cells were treated with non-immune serum demonstrated that CXCR4 staining was specific (Fig. 2E).

SDF-1α induces chemotaxis of granule cells

SDF-1α-induced chemotaxis has been reported for various leukocyte populations and for rodent neural cell types. Given the high level of expression of SDF-1α observed in the pia mater overlying the CXCR4-positive EGL, we tested the ability of SDF-1α to induce chemotaxis in purified populations of granule cells in vitro. Utilizing a modified Boyden chamber microchemotaxis assay, we detected specific migration of granule cells toward SDF-1α. Dose response studies showed a biphasic curve that is characteristic for chemoattractant-induced migration observed in leukocytes with a peak at 1000 ng/ml of the chemoattractant (Fig. 3A). Granule cells did not demonstrate a significant chemotactic response in control
experiments using the chemokine MIP-1α, whose receptors, CCR1 and CCR5, are not present on granule cells (Fig. 3A). The chemotactic response to SDF-1α was abolished by pretreatment of cells with pertussis toxin or with a polyclonal antibody to CXCR4 (Fig. 3B). In addition, chemokinesis controls, in which chemokine was added to both the upper and lower wells of the chemotaxis chamber were negative, demonstrating that SDF-1α induces directed cell migration rather than random movement (Fig. 3B). The effect of pertussis toxin on SDF-1α-induced chemotaxis of granule cells is consistent with coupling of neuronal CXCR4 to Gαi, as it is in leukocytes.

The expression of CXCR4 mRNA was observed to peak in the cerebellum during the first postnatal week (Fig. 1). Thus, we compared the SDF-1α-induced chemotactic responses of granule cells obtained from cerebellium during the first and second postnatal weeks. We were unable to use mouse cerebella for these experiments, as P4 mice cerebellum were too small to generate the cell numbers required for statistical accuracy. We therefore performed age comparisons in granule cell preparations using rat pups taken during the first and second postnatal weeks (P3-4 vs. P7-8). Experiments utilizing rat pups as the source of cerebellar tissue for granule cell preparations produced the same dose response curves as those observed using murine tissue, with peak responses at the dose of 1000 ng/ml (data not shown). Granule cells obtained from cerebella of animals taken at P3-4, however, demonstrated significantly greater chemotactic responses to SDF-1α at this peak dose than granule cells obtained from cerebella of animals taken at P7-8 (Fig. 3C). These results are consistent with a higher level of expression of CXCR4 mRNA observed at this developmental timepoint.

**SDF-1α and SHH are synergistic for granule cell proliferation**

CXCR4 mRNA expression and the SDF-1α-induced chemotactic response of granule cells are greatest in tissue and cells examined during the first postnatal week, the time of maximal granule cell proliferation (Miale and Sidman, 1961; Lodin and Srager, 1970). We questioned whether SDF-1α might also play a direct role in the establishment of granule cell number during cerebellar development. Several granule cell mitogens have been identified including EGF, FGF and SHH (Gao et al., 1991; Tao et al., 1996; Ye et al., 1996; Lin and Bulleit, 1997; Dahmane and Ruiz-i-Altaba, 1999; Traiffort et al., 1999; Wallace, 1999; Wechsler-Reya and Scott, 1999). SHH is the most potent of these (Dahmane and Ruiz-i-Altaba, 1999; Wechsler-Reya and Scott, 1999). SHH activity is known to be antagonized by protein kinase A, presumably in the setting of increased cAMP (Jiang and Struhl, 1995; Epstein et al., 1996; Hammerschmidt et al., 1996; Noveen et al., 1996; Hammerschmidt and McMahon, 1998). Based on our chemotaxis data indicating neuronal CXCR4 coupling to Gαi, we reasoned that SDF-1α might decrease intracellular cAMP and enhance SHH-induced proliferation. Primary cultures of mouse cerebellum derived from P6 mice were treated for 24 hours with SHH at 0.14 μg/ml (7 nM) in the presence or absence of 1000 ng/ml SDF and or pertussis toxin or forskolin, and proliferation was measured by tritiated thymidine incorporation. Consistent with previous studies,
Chemotactic responses of granule cells to SDF-1α (Fig. 3A) or MIP-1α (Fig. 3B) were added to the bottom wells of a modified Boyden chamber. The chemotactic response of purified granule cells was determined by counting migrating cells per high-powered field (Cells/HPF ± s.e.m.) in five fields from replicate wells. Data shown is combined from 5 separate experiments using cells derived from P8-P10 mice where each data point was performed in triplicate. Chemotactic responses of granule cells to SDF-1α are significantly above background (0 concentration) and significantly greater than chemotactic responses to MIP-1α at every concentration (P<0.05). Chemotactic responses to MIP-1α are not significantly greater than background (negative control). (B) Granule cells derived from P8 mice were pretreated with 200 ng/ml pertussis toxin for 1 hour, 10 μg/ml polyclonal antibodies to CXCR4 for 15 minutes, or medium alone before being placed in a modified Boyden chemotaxis chamber. For some wells, 1000 ng/ml SDF-1α was added to both the top wells with cells and the bottom wells to test for SDF-1α mediated random migration (chemokinesis). The chemotactic response of purified granule cells was determined by counting migrating cells per high-powered field (Cells/HPF) in five fields from replicate wells and results are expressed ± s.e.m. All cells were examined for chemotactic responses to 1000 ng/ml SDF-1α, which was deemed to produce maximal chemotactic responses in dose response experiments (see A). Data shown is combined from 2 separate experiments using cells derived from P8 mice where each data point was performed in triplicate. (*P<0.005).

(C) The chemotactic response of purified granule cells derived from P3-4 vs. P7-8 rats to 0 and 1000 ng/ml SDF-1α was determined by counting migrating cells per high-powered field (Cells/HPF ± s.e.m.) in five fields from replicate wells. Data shown is combined from 2 separate experiments where each data point was performed in triplicate. (***P<0.05).

CXCR4 and SHH during a period of maximal granule cell proliferation suggests that this is a relevant in vivo relationship.

**SDF-1α induces calcium flux in depolarized granule cells**

In hematopoietic cells, SDF-1α signaling through CXCR4 induces transient increases in intracellular calcium, which have been demonstrated to be essential for biological responses, such as chemotaxis (Aiuti et al., 1997; Dutt et al., 1998). Leukocyte responses to SDF-1α are robust, transient and do not require treatment with depolarizing agents (Fig. 5A, tracings 1 vs. 2). We have previously demonstrated that both human and macaque cortical neurons express functional CXCR4 that mediates an increase in intracellular calcium in response to SDF-1α (Klein et al., 1999). Murine granule cells also flux calcium in response to treatment with SDF-1α but, as we have observed with primate cortical neurons, require depredopolarization with 20 mM KCl in order to demonstrate this response (Fig. 5B, tracings 1 vs. 2). Depolarization of granule cells produces its own calcium transient, but anti-CXCR4 neutralizing antibodies abolish only the calcium flux response to SDF-1α, indicating that this response is mediated by CXCR4 (Fig. 5B, tracing 2 vs. 5C, tracing 1). Granule cell calcium flux responses to SDF-1α are pertussis toxin sensitive (Fig. 5B, tracing 2 vs. 5C, tracing 2), suggesting that this response of CXCR4 is also mediated by Gαi, as in leukocytes. Similar results were obtained with granule cells obtained from P2, P4, P6, P8 and P10 mice (data not shown).
Intracellular calcium concentrations were calculated as previously described (Klein et al., 1999), using ionomycin to activate all calcium channels and determine maximum intracellular calcium concentrations. SDF-1α induced an approximately fourfold increase in granule cell Ca$^{2+}$ concentrations from a baseline of 42.5±12.2 nM to a post-treatment value of 152.0±21.0 nM.

The requirement for predepolarization for neuronal CXCR4 activation suggests that neuronal CXCR4 signaling may be coupled to neuronal activity. We performed additional experiments to determine whether glutamate, a known excitatory neurotransmitter active on granule cells (Altman and Bayer, 1996), could enhance SDF-1α-induced calcium flux responses in granule cells. Pretreatment of granule cells with 100 μM glutamate enhanced SDF-1α-induced calcium flux (Fig. 5D). This suggests that glutamate can modulate granule cell CXCR4 responses.

**DISCUSSION**

We have found that SDF-1α has two essential roles during cerebellar development: chemoattraction of granule cells resulting in their localization to the EGL and the enhancement of SHH-induced proliferation, resulting in the appropriate expansion of granule cells within the EGL.

**Granule cell chemotaxis and maintenance of the EGL**

A role for SDF-1α and its receptor CXCR4 in the formation and maintenance of the EGL was first suggested by targeted gene deletion of each of these genes. Normally, most granule cell precursor proliferation and migration occurs postnatally (Miale and Sidman, 1961; Lodin and Strager, 1970; Altman, 1972; Burgoyne and Cabray-Deakin, 1988; Hatten, 1993; Altman and Bayer, 1996; Doughty et al., 1998; Raetzman and Siegel, 1999). Deletion of SDF-1α or CXCR4 resulted in premature migration of granule cell precursors away from the proliferative environment of the EGL, with small numbers of granule cell precursors found ectopically, outside EGL, by E18 (Ma et al., 1998; Zou et al., 1998). The mechanisms responsible for these effects is not known. Based on SDF-1α’s chemoattractant effects on leukocytes and some neural cell types (Aiuti et al., 1997; Tanabe et al., 1997; Lazarini et al., 2000), we hypothesized that chemoattraction of granule cells to a pial source of SDF-1α might serve to promote their localization to the EGL. This would be similar to the effect of SDF-1 on lymphocyte localization in secondary lymphoid organs. At these sites, subsets of B-lymphocytes that respond to SDF-1α localize to specific microenvironments where they proliferate and differentiate (Bleul et al., 1998). We report here that granule cells display dose-dependent chemotaxis to SDF-1α, which peaks at a concentration similar to the peak chemotactic dose observed for B-lymphocytes (Bleul et al., 1998). In addition, chemotactic responses were observed to be greater in cells obtained from animals at P3-4 compared with those from animals at P7-8, coinciding with the peak expression of CXCR4 as demonstrated by northern blot analysis. The localization of SDF-1α to the pia and CXCR4 to granule cells of the EGL suggests that, in vivo, SDF-1α attracts granule cells towards the pia mater. This supports the hypothesis that maintenance of granule cell residence in the EGL is a function of SDF-1α chemoattraction and provides a mechanism for the observation that granule cells migrate prematurely out of the EGL in animals lacking SDF-1α or CXCR4 (Ma et al., 1998; Zou et al., 1998).

Granule cell migration from the EGL peaks between P8 and P12 in vivo. This period coincides with the observed decline in the expression of CXCR4 and is consistent with SDF-1α playing an inhibitory role in granule cell emigration from the EGL. Other factors essential for granule cell migration, such as BDNF, display an opposite pattern of expression, increasing during the period of peak granule cell migration out of the EGL (Maisonpierre et al., 1990). Thus, the age-dependent decrease in expression of CXCR4 and increasing migratory effects of BDNF may both contribute to the movement of granule cells out of the EGL.

**SDF-1α enhances SHH-Induced granule cell proliferation**

Previous experiments to explore possible interactions between SHH and the other known mitogens for granule cells have failed to demonstrate any additive or synergistic effects (Wechsler-Reya and Scott, 1999). We report here that SDF-1α directly enhances the proliferative effects of SHH without possessing any mitogenic activity of its own. We demonstrate that SDF-1α increases the proliferative effects of nanomolar SHH by approximately 50%; increasing a 2.5-fold mitogenic
effect to a nearly 4-fold effect. Importantly this concentration of SHH may better approximate physiologic concentrations. Recent studies have shown that SDF-1α, acting together with other cytokines can induce the proliferation of CD4+ T lymphocytes and pluripotent hematopoietic stem cells (Lataillade et al., 2000; Nanki and Lipsky, 2000). Thus proliferative responses to SDF-1α of both immune and neural cell types can require receptor cross talk.

SDF-1α signaling in cerebellar granule cells

In some regards, SDF-1α signaling in cerebellar granule cells appears to resemble SDF-1α signaling in leukocytes. Calcium flux and chemotaxis are mediated through CXCR4 activation and can be blocked by pertussis toxin. However, granule cell SDF-1α responses differ from leukocyte responses in their requirement for membrane depolarization. No calcium flux in response to SDF-1α is observed in granule cells without their prior treatment with KCl. The KCl effect is likely to reflect a need for membrane depolarization as glutamate can substitute for KCl. Similar observations have been made for cortical neuron responses to SDF-1α (Klein et al., 1999). Notably, granule cell chemotaxis and proliferation assays are both performed with high potassium conditions (20 mM; see Materials and Methods). Activity dependent signaling during CNS development has been linked to trophic interactions between neurons and their targets as well as to regulation of apoptosis (Ghosh and Greenberg, 1995; Katz and Shatz, 1996).

**Fig. 5.** Neuronal calcium flux responses to SDF-1α. PBMC preparations or replicate granule cell cultures derived from P8 mice were loaded with 5 μM fura-2 and analyzed using a cuvette- (leukocytes) or microscope- (neurons) based calcium flux apparatus. (A) Leukocyte responses to SDF-1α are not altered by pretreatment with 20 mM KCl. (B) Granule cell neurons display calcium flux responses to 100 ng/ml of SDF-1α only after predepolarization with 20 mM KCl (tracings 1 vs. 2). (C) Although all cells produce calcium flux responses during predepolarization, pretreatment with polyclonal antibodies to CXCR4 (tracing 1) or pertussis toxin (tracing 2) abolishes neuronal responses to SDF-1α. (D) Pretreatment of granule cell neurons with glutamate (100 μM) also produces maximal responses to 100 ng/ml SDF-1α. Leukocyte populations of 5×10⁶ cells/ml were analysed and groups of 10-20 neurons were visualized and analyzed. Data are presented as the relative ratio of fluorescence at emission frequency of 510 nm and excitation frequencies of 340 and 380 nm and are representative of 2 separate experiments with leukocyte preparations and 4 separate experiments with neuronal preparations.
In the developing cerebellum, correctly located granule cells may be depolarized by glutamate derived from neighboring granule cells. This would allow for selective expansion of those granule cells that had achieved the correct position and proliferation during cerebellar development. Depicted in the model is a granule cell in the EGL of the neonatal cerebellum where its position enables it to respond to two separate factors: SHH and SDF-1α. SHH, produced by Purkinje cells, is known to stimulate granule cell proliferation by releasing the inhibition of smoothened (SMO) that is exerted by the SHH receptor, patched (PTC). SHH effects can be blocked by PKA or forskolin. We propose that pial derived SDF-1α, through the activation of Giα, reduces cAMP and PKA activity and thereby enhances SHH-induced proliferation. In addition, SDF-1α also promotes the localization of granule cells to the EGL through a chemotactic effect that is directed towards the pia. SDF-1α thus contributes to granule cell proliferation both directly, by augmenting SHH effects, and indirectly by maintaining granule cell position within the proliferative environment of the EGL. EGL, external granule cell layer; ML, molecular layer; IGL, internal granule cell layer; SDF-1α, stromal derived factor-1α; SHH, Sonic hedgehog; PTC, patched; SMO, smoothened; PKA, protein kinase A.

Conclusions

In hematopoietic tissues, chemokines play multiple roles in the movement, proliferation and differentiation of leukocytes. We show here that the chemokine SDF-1α plays a central role in cerebellar development by influencing both the movement and proliferation of cerebellar granule cells. The persistence of CXCR4 expression in the IGL suggests that this receptor may also play important roles in neuronal functioning beyond development. In this regard there are now data to suggest that chemokines can modulate neuronal signaling (Meucci et al., 1998; Zheng et al., 1999; Limatola et al., 2000), protect neurons from excitotoxicity (Bruno et al., 2000) and promote neuronal survival during inflammatory states (Kaul and Lipton, 1999; Meucci et al., 2000; Zujovic et al., 2000).

We would like to thank Drs Jose-Angel Gonzalo and Jose-Carlos Gutierrez-Ramos for kindly providing the anti-CXCR4 polyclonal antibodies; Dr Andrew Lackner for help with the confocal imaging experiments; Curis Inc. for the kind gift of Sonic hedgehog protein; and Dongin Yuk and Dr David Rowitch for their help with the in situ hybridization experiments. This work was supported by NIH Grants DA00522 (to R. S. K.), HD 01393 (to J. B. R.), NS 37757 (to R. A. S.), and AI46999 (to A. D. L.).

REFERENCES


Banisadr, G., Dicou, E., Berbar, T., Rostene, W., Lombet, A. and Haour,


