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Differential Expression of CXCL12 and CXCR4 During Human Fetal Neural Progenitor Cell Differentiation

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Abstract

Stromal cell-derived factor 1 alpha (SDF-1 α , CXCL12) and its receptor CXCR4 play an important role in the central nervous system (CNS) development and adulthood by mediating cell migration, enhancing precursor cell proliferation, assisting in neuronal circuit formation, and possibly regulating migration during repair. The expression pattern of CXCR4 and CXCL12 during neurogenesis has not been thoroughly elucidated. In this study, we investigated the expression of CXCL12 and CXCR4 during neural progenitor cells (NPC) differentiation by microarray analysis and reverse transcriptase-polymerase chain reaction (RT-PCR) using human fetal NPC as a model system. The production of CXCL12 was measured by enzyme-linked immunosorbent assay (ELISA). CXCR4 expression was determined by fluorescence-activated cell sorting (FACS) analysis, immunocytochemical staining, and CXCR4-mediated inhibition of cyclic AMP (cAMP) accumulation. Our data demonstrated that CXCR4 expression is significantly upregulated when NPC are differentiated into neuronal precursors, whereas CXCL12 is upregulated when differentiated into astrocytes. We also provide evidence that CXCR4 localization changes as neurons mature. In neuronal precursors, CXCR4 is localized in both neuronal processes and the cell body, whereas in mature neurons, it is primarily expressed on axons and dendrites. This differential expression of CXCR4 and CXCL12 may be important for the temporal regulation of neuronal migration and circuit formation during development and possibly in adult neurogenesis and repair.

Keywords

CXCL12; CXCR4; neurogenesis; neural progenitor cells; neural precursor; astrocytes

Introduction

The chemokine stromal cell-derived factor 1 alpha (SDF-1 α , CXCL12) and its only receptor CXCR4 play an important role in both the developing brain and adult CNS. Both in vivo and in vitro studies suggest that CXCL12 and CXCR4 are involved in neurogenesis processes such as neural progenitor/stem cell (NPC) migration, proliferation, and differentiation. In vivo studies have shown that CXCL12 or CXCR4 knockout mice have malformations in the cerebellum, hippocampal dentate gyrus (DG), and neocortex resulting from improper migration and proliferation of NPC and neuronal precursor cells (Ma et al. 1998; Zou et al. 1998; Lu et al. 2002; Stumm et al. 2003). In vitro evidence has shown that NPC, expressing CXCR4, migrate toward CXCL12 (Klein et al. 2001; Peng et al. 2004). Besides acting as a

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chemotactant for progenitor and precursor cells, the work by Klein et al. (2001) indicated that CXCL12 acts synergistically with sonic hedgehog (SHH) to increase proliferation of granule cell precursors. Thus, CXCL12 signaling through CXCR4 influences both the proper migration and correct number of precursor and progenitor cells.

To understand the role of CXCL12 and CXCR4 interaction during CNS development, defining the cell type and expression pattern for CXCL12 and CXCR4 in the brain is critical. During development of the DG and neocortex, CXCR4 is expressed by migrating NPC and neuronal precursors, and CXCL12 is expressed by cells lining the migratory path and endpoint of migration (McGrath et al. 1999; Lu et al. 2002; Stumm et al. 2003). Previous studies have indicated that astrocytes, neurons, and NPC express CXCL12, but astrocytes seem to be the major cell type of neural lineage expressing high levels of CXCL12 (Zheng et al. 1999; Langford et al. 2002; Rostasy et al. 2003; Peng et al. 2006). The spatial and cellular expression pattern of CXCL12 and CXCR4 in the brain is consistent with their role in regulating the migration and possibly, as is the case with cerebellar granule cells, the proliferation of neuronal and progenitor cells. However, the expression pattern of CXCL12 and CXCR4 during neurogenesis process has not been thoroughly investigated. We hypothesized that CXCL12 and CXCR4 are differentially expressed when NPC differentiate to neurons and astrocytes.

To test this hypothesis, we used a human fetal brain-derived multipotent cell culture model system to examine differences in CXCL12 production in NPC, astrocytes, and neurons and to address the expression pattern of CXCL12 and CXCR4 during NPC differentiation. We demonstrated that neuronal differentiation of NPC cultures increases CXCR4 expression, whereas astrocyte differentiation increases CXCL12 expression. This suggests that newly differentiated astrocytes may play a role in regulating the migration and proliferation of progenitor and neuronal precursor cells.

Methods and materials

Human fetal neural progenitor cell isolation, culture, and differentiation

Human fetal brain tissue (12–16 weeks postconception) was obtained from elective abortions carried out in full compliance with the University of Nebraska Medical Center and NIH ethical guidelines. Human fetal NPC were isolated and cultured as described previously (Peng et al. 2004). The phenotypes of human NPC were examined by immunocytochemical staining. Over 90% of cells expressed the neural stem cell marker, nestin. Less than 3% of cells expressed the astrocyte marker, glial fibrillary acidic protein (GFAP); less than 6% of cells expressed the neuronal marker, β -III-tubulin. For neuronal differentiation (N-diff), dissociated NPC were plated on poly-D-lysine-coated cell culture dishes or coverslips (Sigma-Aldrich, St. Louis, MO) with serum-free neurobasal medium (Gibco, Carlsbad, CA) supplemented with B27 (Gibco). For astrocyte differentiation (A-diff), dissociated cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 (Gibco) and 10% fetal bovine serum (FBS). Cells were grown in differentiation media for 1–10 days before analysis.

RNA extraction and TaqMan real-time RT-PCR

Total RNA was extracted from NPC, N-diff, and A-diff cultures with TRIzol Reagent (Invitrogen, Carlsbad, CA) and RNeasy Mini Kit (Qiagen, Valencia, CA). The real-time reverse transcriptase-polymerase chain reaction (RT-PCR) was performed using the one-step quantitative TaqMan real-time RT-PCR system (Applied Biosystems, Foster City, CA). The primers used were human CXCL12 (SDF-1 α , ID no., Hs00171022-m1), human CXCR4 (ID no., Hs00607978_s1), and human GAPDH (ID NO., 4130884E); all primers were purchased from Applied Biosystems. The levels of CXCL12 and CXCR4 were determined and standardized by comparison to glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Microarray analysis

Total RNA from N-diff, A-diff, and NPC cultures were used to make tagged complementary DNA (cDNA), which was hybridized to Affymetrix (Santa Clara, CA) human genome U133 plus 2.0 gene chips. The hybridized gene chips were incubated with a fluorescent probe and scanned with an Axon 4000A scanner. The data was analyzed and intensities measured using GCOS software (Affymetrix).

CXCL12 ELISA

The concentration of secreted CXCL12 in differentiated cultures was determined by ELISA as described previously (Peng et al. 2006). NPC were cultured in poly-D-lysine coated 48-well plates in differentiation media. The supernatant was collected at 1, 4, 7, and 10 days. Samples were added in triplicate to a 96-well plate coated with mouse anti-CXCL12 antibody (R&D Systems, Minneapolis, MN), incubated overnight at 4°C followed by incubation with biotinylated goat anti-CXCL12 (R&D Systems) and horse-radish peroxidase (HRP)-conjugated streptavidin (R&D Systems). The reaction product was detected as previously described.

Flow cytometry

NPC were dissociated and differentiated cells were detached by 10-min incubation with accutase (Upstate, Lake Placid, NY). After three washes with phosphate-buffered saline (PBS), cells were blocked with PBS with 3% bovine serum albumin (BSA) for 30 min and stained with anti-CXCR4-PE (1:5; BD Pharmagen, San Diego, CA) for 30 min at 4°C. After another three washes, cells were fixed with 4% paraformaldehyde. Expression was determined by immunofluorescence flow cytometry and analyzed with CellQuest software.

Immunocytochemistry

Immunocytochemistry for detecting the phenotype of NPC or the differentiated cultures was performed as described previously (Peng et al. 2004). Briefly, cells were fixed with methanol/acetone (1:1) and then washed with PBS. Cells were then incubated with antibodies for nestin (1:100; Sigma-Aldrich), β -III-tubulin (1:400; Sigma-Aldrich), microtubule-associated protein-2 (MAP-2, 1:100; Chemicon), neurofilament (1:200; Chemicon), or GFAP (1:1000; Dako, Carpinteria, CA) overnight. This was followed by incubation with goat anti-mouse and anti-rabbit IgG Alexa Fluor 488 or 594, and donkey anti-rabbit IgG Alexa Fluor 647 (1:200; Molecular Probes, Eugene, OR). For CXCR4 staining, cells were fixed in 4% paraformaldehyde for 30 min and methanol for 2 min followed by immunostaining. Hoechst 33342 (1:5,000) was used for nuclear staining. Double and triple immunostaining was examined by an E800 Eclipse microscope (Nikon, Japan). The slides were also examined with a Bio-Rad MRC1024ES laser scanning confocal microscope (Hercules, CA).

cAMP assay

The assay for cAMP accumulation was performed as described previously with minor modification (Zheng et al. 1999). Briefly, human NPC were plated in 24-well plates and cultured for 2 days in neurosphere initiation medium (NPIM). N-diff or A-diff cells were cultured for 7 days in differentiation media. Cells were loaded with 5 μ Ci [3 H]-adenine (NEN Life Science Product, Boston, MA) at 37°C for 90–120 min and treated with 30 μ M forskolin (Sigma) with or without different concentrations of CXCL12. Intracellular [3 H]cAMP was extracted overnight with ice-cold 5% trichloroacetic acid (TCA) containing 1 mM unlabeled cAMP (an internal control). [3 H]cAMP was separated from the tritiated nucleotides by sequential ion-exchange chromatography over Dowex and Alumina columns (Sigma-Aldrich). The adenosine triphosphate (ATP) and cAMP fractions were collected, and the radioactivity

was determined by liquid scintillation spectroscopy. Values were expressed as the percentage conversion of [^3H]ATP to [^3H]cAMP.

Statistical tests

All results were expressed as means $\pm$ standard deviation of the mean (SD) or standard error of the mean (SEM). All experiments were done in triplicate or quadruplicate. Data was evaluated statistically by analysis of variance (ANOVA). Significance was considered to be p less than 0.05. To account for any donor specific differences, all experiments were performed with a minimum of three donors.

Results

Human NPC culture and differentiation

Human NPC were cultured in serum-free media containing epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), and leukemia inhibitory factor (LIF), in which nestin positive (nestin⁺) progenitor cells were maintained as 3D of undifferentiated, actively proliferating cells (Peng et al. 2004). These cells were differentiated into a majority of β -tubulin III positive neuronal population by changing to serum-free neurobasal media supplemented with B27 (NB27). Alternatively, they were differentiated into a majority of GFAP⁺ astrocyte population by changing the media to a serum-containing media (DMEM/F12 with 10% FBS). The phenotypes of NPC, A-diff, and N-diff cultures were verified by immunocytochemistry (Fig. 1). The majority of NPC expressed nestin (>90%), a neural stem cell marker (Fig. 1a). A-diff cultures had 56% GFAP positive cells and 25% β -tubulin III positive cells. N-diff cultures had 52% β -tubulin III positive cells and 25% GFAP positive cells. The phenotype of the cultures were also verified by immunofluorescent flow cytometry: N-diff cultures contained 42% β -tubulin III positive cells and 2% GFAP positive cells, whereas 19.4% of the cells were positive for β -tubulin III and 56.8% were positive for GFAP in A-diff cultures (data not shown). Because the majority of cells in A-diff cultures are GFAP⁺ and the majority of cells in N-diff cultures are β -tubulin⁺, this culture system provides an excellent human NPC model system to examine differences in molecular factors during different stages of neural cell development.

Differential expression pattern of CXCL12 and CXCR4 during NPC differentiation

The expression pattern of CXCR4 and CXCL12 during NPC differentiation was evaluated by microarray analysis. Microarray analysis suggested that NPC, N-diff, and A-diff all express CXCL12 and CXCR4, but they had a differential expression pattern during differentiation (Table 1). These results were verified by real-time quantitative RT-PCR (Fig. 1f, g). N-diff cultures had an 8.5-fold increase in CXCR4 messenger RNA (mRNA), and A-diff cultures had a 10.7-fold increase in CXCL12 mRNA as compared to undifferentiated NPC. These findings showed that CXCR4 expression is increased during neuronal differentiation and CXCL12 expression is increased during astrocyte differentiation.

Astrocyte differentiation increases CXCL12 production

To determine if the increase in mRNA during astrocyte differentiation correlated with an increase in protein production, the amount of CXCL12 secreted during A-diff and N-diff was measured by ELISA (Fig. 2e). NPC were differentiated, and supernatants were collected at 1, 4, 7, and 10 days. CXCL12 production by A-diff cultures continually increased during this time period (an increase from 0.20 to 0.69 ng/ml after 10 days), whereas there was no significant change in CXCL12 production during neuronal differentiation. This is consistent with the microarray assay and real time RT-PCR observations, indicating that astrocyte precursors produce more CXCL12 than NPC or neurons.

Neuronal differentiation increases CXCR4 expression and CXCL12-mediated signaling

NPC were differentiated for 7 days, and the expression of CXCR4 was determined by FACS analysis. NPC, N-diff, and A-diff cultures all contained high amounts of CXCR4 positive cells (57.48% in NPC, 87.10% in N-diff, and 61.46% in A-diff cultures), but the percentage of CXCR4 expressing cells was significantly increased during neuronal differentiation (Fig. 2a–d). The fluorescence intensity was also higher on N-diff cells (147.22 on N-diff and 117.25 on NPC) indicating a greater concentration of CXCR4 on positive cells. A-diff cultures showed a small population of cells that highly expressed CXCR4. The forward scatter for CXCR4 positive cells in these cultures indicated that these cells had a small cell body and low granular composition (data not shown), indicating that most of the CXCR4 positive cells in A-diff cultures might be neuronal precursors.

Binding of CXCL12 to CXCR4 activates inhibitory G protein (G_i), which inhibits adenylate cyclase leading to a decrease in cytosolic cAMP concentration (Zheng et al. 1999). To further verify the CXCR4 expression level during NPC differentiation, the responsiveness of N-diff, A-diff, and NPC cultures to CXCL12 was measured using a cAMP accumulation assay (Fig. 2f). Data showed N-diff cultures were more sensitive to CXCL12 signaling (the response curve is shifted to the left) than NPC and A-diff cultures. A-diff cultures showed the least sensitivity (the response curve is shifted to the right). This confirms more accurately a higher concentration of CXCR4 on neuronal and neuronal precursor cells than on NPC or astrocytes.

Cellular localization of CXCR4 changes as neurons mature

A previous study showed that the cellular localization of CXCR4 changes during the maturation of rat hippocampal neurons (Pujol et al. 2005). In this study, neuronal precursors differentiated in NB27 showed a broad expression of CXCR4 on both the cell body and neurites (Fig. 3d–f) as indicated by confocal imaging. The mature neurons, which were maintained in culture for 3 weeks, expressed the mature neuronal markers MAP-2 and neurofilament. CXCR4 was expressed mostly along axons and dendrites with very little localized to the cell body (Fig. 3g–i). This is consistent with the findings by Pujol et al. (2005) showing that CXCR4 distribution changes from cell body and dendrites to mostly on dendrites as neuron mature.

Discussion

The chemotactic properties of CXCL12 (SDF-1 α) and the function of its receptor CXCR4 are known to be critical to proper CNS development (Ma et al. 1998; Zou et al. 1998; Lu et al. 2002; Stumm et al. 2003; Peng et al. 2004). We studied the expression patterns of CXCL12 and CXCR4 in human NPC during differentiation and maturation. In this study, we report that CXCR4 expression was increased on NPC differentiated to the neuronal lineage (Fig. 2a–d). This increase in receptor expression correlated to an increase in functional sensitivity to CXCL12 stimulation as compared to NPC and astrocytes (Fig. 2f). Increased expression of CXCR4 may facilitate an increased chemotactic response of neuronal precursors to CXCL12. Conversely, CXCL12 expression (Fig. 1g) and production (Fig. 2e) was found to be increased when NPC were differentiated to the glial lineage. To our knowledge, these observations provide the first in vitro evidence of the differential expression of CXCR4 and CXCL12 during human NPC differentiation.

Both the amount and distribution of CXCR4 expression likely play a role in the CNS. Our work and a previous study by Pujol et al. (2005) provide evidence that the cellular localization of CXCR4 changes as neurons mature (Fig. 3). CXCR4 is distributed along the cell body and cell processes in immature neurons, whereas in mature neurons, CXCR4 is primarily localized to axons and dendrites. These differences in localization suggest the role of CXCR4 changes as neurons mature. In addition to facilitating migration, studies have shown that CXCL12

signaling through CXCR4 is involved in neuronal circuit formation (Xiang et al. 2002; Arakawa et al. 2003; Pujol et al. 2005). Whereas neural precursors and neurons highly express CXCR4, astrocytes are a primary source of CXCL12 in the brain (Peng et al. 2006). Thus, astrocytes may play an important role in regulating both migration and neuronal circuit formation. Moreover, as NPC differentiation is generally a sequential process, where NPC differentiate to neurons before astrocytes (Sauvageot and Stiles 2002), an upregulation of CXCL12 during astrocyte differentiation may be important for the temporal and spatial regulation of these processes.

Although CXCL12 is constitutively expressed in the CNS, its role during inflammatory processes is still unclear. We recently reported that CXCL12 is upregulated during HIV-1 mediated brain inflammation in both in vitro and in vivo models. Increased CXCL12 expression is mediated by macrophage astrocyte interactions and is primarily produced by activated astrocytes (Peng et al. 2006). The role of increased CXCL12 expression in HIV-1 infected brains has yet to be determined. However, studies have shown that CXCL12 is upregulated after stroke (Hill et al. 2004). Evidence suggests that the increase in CXCL12 at the ischemic site mediates migration of both endogenous and transplanted NPC to this site (Zhang et al. 2001; Arvidsson et al. 2002; Imitola et al. 2004; Kelly et al. 2004; Robin et al. 2006). Some migrating cells survive and differentiate into neurons and a few astrocytes, but it is not known whether NPC differentiate as they migrate or once they enter ischemic site (Zhang et al. 2001; Arvidsson et al. 2002; Kelly et al. 2004). Our study indicates that neuronal differentiation by NPC increases their responsiveness to CXCL12 signaling, suggesting possible increased chemotactic response of neuronal precursors to CXCL12. An elegant study by Kelly et al (2004) suggested that NPC differentiate once they get to the ischemic site. An upregulation of CXCR4 as NPC differentiate may be important for axonal pathfinding and integration of the new neurons. Thus, the regulation of CXCL12 and CXCR4 expression and the role of astrocytes as a source of CXCL12 remain an important area for further investigation.

CXCL12 and CXCR4 are constitutively expressed in the brain and are critical to proper development. CXCL12 is involved in the migration of neuronal precursors and progenitor cells and in axonal pathfinding and elongation. The evidence in this study suggests the expression of CXCL12 and CXCR4 changes during NPC differentiation. CXCR4 expression is increased after precursors begin to differentiate to neurons, whereas CXCL12 production is increased when precursors are differentiated to astrocytes. This observation could provide insights into the temporal control of neuronal migration and circuit formation and the migration of progenitor cells to sites of neurodegeneration and inflammation.

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Fig. 1.

CXCR4 and CXCL12 expression during NPC differentiation. NPC were cultured in poly-D-lysine-coated chamber slides for 24 h. Immunocytochemical staining showed most cells were nestin positive cells (a). NPC were induced to differentiate into neurons (*N-diff*) or astrocytes (*A-diff*) by culturing them in neurobasal media with B27 (*N-diff* media) or DMEM/F12 media with 10% FBS (*A-diff* media), respectively, for 7 days. The phenotypes of the differentiated cultures were verified by immunocytochemistry. Cells were stained with the neuronal precursor marker β -tubulin III (green) (b) and the astrocyte specific marker GFAP (red) (c). The total number of β -tubulin III (d) or GFAP (e) positive cells were counted and represented as a percent of the total cells $\pm$ SD ($n=3$). The relative CXCR4 (f) and CXCL12 (g) mRNA levels of NPC, *N-diff*, and *A-diff* cultures were determined by RT-PCR and standardized to GAPDH. Data is presented as fold increase in mRNA level as compared to undifferentiated NPC $\pm$ SD. Representative data for three separate experiments with NPC, *N-diff*, and *A-diff* from three separate donors. (* $p<0.05$ compared to *N-diff*; ** $p<0.05$ compared to NPC).

Fig. 2.

CXCR4 expression and CXCL12 signaling during NPC differentiation. NPC, neuronal differentiated (*N-diff*), and astrocyte differentiated (*A-diff*) cells were harvested and stained with anti-CXCR4-PE. The number of CXCR4 positive cells was determined by flow cytometry. Representative FACS data for undifferentiated NPC (**a**) *N-diff* (**b**) and *A-diff* (**c**) showing the number of CXCR4 positive cells. **d** The average percentage of CXCR4⁺ cells of four donors $\pm$ SEM. CXCL12 production during neuronal and astrocyte differentiation as determined by ELISA ($n=3$) (**e**). A cAMP accumulation assay (**f**) was performed to show the responsiveness of NPC, *N-diff*, and *A-diff* cultures to CXCL12-mediated inhibition of Forskolin-stimulated cAMP accumulation. Representative data for three separate experiments with NPC, *N-diff*, and *A-diff* from three separate donors (* $p<0.05$ compared to *N-diff*; ** $p<0.05$ compared to NPC)

Fig. 3.

Cellular localization of CXCR4. Confocal images of immature neurons and mature neurons or astrocytes showing the cellular localization of CXCR4. In **a–c**, mature mixed neuronal culture (cultured for 3 weeks) was stained with CXCR4 (**a**; green) and GFAP (**b**; red). Merged (**c**) image shows little colocalization of CXCR4 and GFAP indicating little expression of CXCR4 by astrocytes. In **d** and **e**, NPC were cultured in neuronal differentiation media (N-diff) for 7 days. N-diff cells were stained for CXCR4 (**d**; green) and β -tubulin III (**e**; red). CXCR4 is localized both along neurites and the cell body in immature neurons (**f**). In **g–i**, mature human neurons (cultured for 3 weeks) were stained for CXCR4 (**g**; green) and neurofilament (NF) (**h**; red). In mature neurons, CXCR4 is localized mostly along axons and dendrites with little expression around the cell body (**i**). Nuclear staining (blue) is Hoechst. Representative data for three separate experiments with NPC, N-diff, and neuronal culture from three separate donors

Table 1
CXCL12 (SDF-1) and CXCR4 expression determined by microarray

	NPC	N-diff	A-diff
CXCL12	161.8	384.4	1,544.4
CXCR4	1182.6	3986.2	1,194.6

Total RNA was harvested from undifferentiated NPC, N-diff, and A-diff cultures. cDNA was made and hybridized to Affymetrix[®] human genome U133 Plus 2.0 gene chips. Summary showing an increase in CXCR4 expression during N-diff and an increase in CXCL12 expression during A-diff as compared to NPCs. Representative data for three separate experiments with NPC from three separate donors. Data represents the intensity.