Overexpression of Stromal Cell–Derived Factor 1 and Its Receptor CXCR4 Induces Autocrine/Paracrine Cell Proliferation in Human Pituitary Adenomas

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Abstract Purpose: Hypothalamic or locally produced growth factors and cytokines control pituitary development, functioning, and cell division. We evaluated the expression of the chemokine stromal cell–derived factor 1 (SDF1) and its receptor CXCR4 in human pituitary adenomas and normal pituitary tissues and their role in cell proliferation.

Experimental Design: The expression of SDF1 and CXCR4 in 65 human pituitary adenomas and 4 human normal pituitaries was determined by reverse transcription-PCR, immunohistochemistry, and confocal immunofluorescence. The proliferative effect of SDF1 was evaluated in eight fibroblast-free human pituitary adenoma cell cultures.

Results: CXCR4 mRNA was expressed in 92% of growth hormone (GH)-secreting pituitary adenomas (GHoma) and 81% of nonfunctioning pituitary adenomas (NFPA), whereas SDF1 was identified in 63% and 78% of GHomas and NFPA, respectively. Immunostaining for CXCR4 and SDF1 showed a strong homogenous labeling in all tumoral cells in both GHomas and NFPA. In normal tissues, CXCR4 and SDF1 were expressed only in a subset of anterior pituitary cells, with a lower expression of SDF1 compared with its cognate receptor. CXCR4 and SDF1 were not confined to a specific cell population in the anterior pituitary but colocalized with discrete subpopulations of GH-, prolactin-, and adrenocorticotropic hormone–secreting cells. Conversely, most of the SDF1-containing cells expressed CXCR4. In six of eight pituitary adenoma primary cultures, SDF1 induced a statistically significant increase in DNA synthesis that was prevented by the treatment with the CXCR4 antagonist AMD3100 or somatostatin.

Conclusions: CXCR4 and SDF1 are overexpressed in human pituitary adenomas and CXCR4 activation may contribute to pituitary cell proliferation and, possibly, to adenoma development in humans.

Pituitary adenomas constitute up to 15% of primary intracranial tumors and, in spite of their benign phenotype, are associated with significant morbidity due to local mass-related effects and/or hormone hypersecretion (1). Pituitary adenomas are classified according to their hormonal secretion: prolactinomas (∼30% of pituitary tumors), growth hormone (GH)-secreting adenomas (GHoma; 15-20%), and adrenocorticotropic hormone (ACTH)-secreting tumors (<10%). Adenomas that release biologically inactive hormones, do not detectably produce hormones, or derive from “null cells” are defined as clinically “nonfunctioning pituitary adenomas” (NFPA) and account for ∼30% of the pituitary tumors.

The molecular pathogenesis of pituitary adenomas is still unclear: only few alterations have been definitively proven to be involved in tumorigenesis (2). Pituitary adenomas are often monoclonal in origin and the neoplastic initiation is related to proto-oncogene mutations [ras, protein kinase C, and α subunits of Gs (gsp)], or Gi (gi)], overexpression of activating genes [i.e., pituitary tumor transforming gene (pttg)], or loss of tumor suppressor genes [Rb, menin, p53, p27, and p16; ref. 2]. Nevertheless, the pathogenesis of these tumors is extremely heterogeneous because these mutations are present only in subsets of adenomas but missing in the majority. The “initiating” events cause a proliferative “gain of function” in single pituitary cells, subsequently induced to clonal expansion by tumor-promoting molecules. A multiplicity of promoting factors, including hypothalamic hormones, locally produced...
growth factors (epidermal growth factor, basic fibroblast growth factor, nerve growth factor, and transforming growth factor), and cytokines [interleukin (IL)-1, IL-2, and IL-6], can determine pituitary tumor progression (3). Chemokines, initially described as regulators of leukocyte trafficking and chemotaxis, are now known to influence several physiologic and pathologic processes (4), including different brain diseases (neuroinflammatory and neurodegenerative diseases, HIV-associated neuropathology, and brain tumors; ref. 5). In particular, the chemokine receptor CXCR4 and its ligand stromal cell-derived factor 1 (SDF1 or CXCL12) are expressed in neuronal, astroglial, and microglial cells in the adult brain (6, 7) and play a critical role in directing cell migration during embryonic brain development (8). SDF1 supports growth and survival of normal and malignant cells, including hematopoietic progenitors (9), B leukemia cells (10), breast and ovarian carcinoma (11–13), meningiomas (14), and glioblastoma multiforme (14, 15). In vitro, SDF1 is a growth factor for normal astrocytes and glioblastoma multiforme cell lines (17, 18) and stimulates chemotaxis, survival, and proliferation in glioblastoma multiforme primary cell cultures and xenografted tumors (16, 19). CXCR4 activation by SDF1 is also involved in tumoral invasion (20), metastasis (21), and neoangiogenesis (22).

To date, the role of SDF1/CXCR4 in pituitary functioning has been scantily investigated. SDF1 expression was identified in vasopressinergic neurons in the hypothalamus and in nerve terminals in posterior pituitary (23). In rat anterior pituitary, the expression of CXCR4 mRNA, as well as SDF1-binding sites, were reported (24, 25). Still, the cellular distribution of SDF1- and CXCR4-expressing cells in rodent pituitary is unclear and no data are presently available about their expression and physiologic role in human normal pituitary or their role in pituitary adenoma pathogenesis (26).

Recently, using GH4C1 rat pituitary adenoma cells, we described a possible role for SDF1 in the regulation of pituitary functioning (24). In these cells, SDF1 increased GH and prolactin (PRL) release and proliferation. We identified two intracellular pathways activated by SDF1 that independently contribute to cell proliferation: (a) a direct activation of extracellular signal-regulated kinase 1/2 and (b) a Ca2+-dependent activation of the tyrosine kinase Pyk2 and the K+ extracellular signal-regulated kinase 1/2 and (4), which were virtually absent in all the samples (data not shown). In only one GHoma, sections contained a fragment of normal pituitary (tumor-adjacent pituitary tissues taken at the time of surgery) and were used as control to compare SDF1 and CXCR4 expression in normal and neoplastic pituitary.

Four human normal pituitaries were collected from autopsies and analyzed by immunohistochemistry and confocal microscopy.

In situ hybridization. SDF1 and CXCR4 mRNAs were detected in frozen sections from three adult male rat pituitaries according to a standard protocol, with well-characterized riboprobes (30). 35S-labeled sense and antisense probes were transcribed from linearized vector constructs using [35S]UTP and [35S]CTP. Hybridized slides were coexposed with NTB emulsion (Eastman Kodak) and exposed for 28 d. Hybridization signals were digitally imaged under dark field illumination with a Zeiss Axioskop microscope (Carl Zeiss MicroImaging GmbH).

Primary cultures. Primary cell cultures were obtained from nine fresh fragments of pituitary adenoma (six NFpas, two GHomas, and one ACTHoma) by mechanical disruption under sterile conditions. Dispersed cells were treated with antifibroblast microbeads (Miltenyi Biotec) for the removal of fibroblasts. Single-cell suspensions were seeded (100,000 cells per well) in MEM with low glucose (1 g/L), containing d-valine (Metachem) to avoid fibroblast proliferation, supplemented with 10% FCS, penicillin/streptomycin, and 2.5 μg/mL amphotericin B (Euroclone; ref. 31).

In vitro measurement of hormone release. Hormone release from isolated pituitary adenoma cells was evaluated after 24 h of serum deprivation using the Immulite 2000 ACHT and GH chemiluminescent immunoassays (Diagnostic Product Corp.).

SDF1 release was quantified using the Quantikine immunoassay (R&D Systems) following the manufacturer’s instructions.

RNA isolation and RT-PCR. Total RNA from frozen pituitary tissues or isolated adenoma cells was obtained using acid-phenol extraction. DNase-treated RNA (5 μg) was reverse transcribed with avian myeloblastosis virus reverse transcriptase (GE Healthcare). The primers used for amplification (Tib MolBiol) were previously described (16).

PCR was done using the Taq PCR Core kit (Qiagen). Amplification profile was as follows: 94°C for 5 min, 30 cycles at 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min, followed by 7 min at 72°C. β-Actin transcription levels were assessed on all samples as internal control.

Immunohistochemistry and immunofluorescence. Immunohistochemistry for CXCR4 and SDF1 was done on paraffin-embedded sections stained with monoclonal (clone 12G5; BD Pharmingen) or polyclonal (Afinity BioReagents, Inc.) anti-CXCR4 antibodies and the polyclonal anti-SDF1 (Torrey Pines BioLabs) antibodies.

Sections were dewaxed in xylene, rehydrated through graded alcohols, heated in a microwave in 10 mmol/L sodium citrate buffer (pH 6.0) for epitope retrieval, and treated in PBS-0.3% Triton X-100 for 10 min. Sections, saturated with 10% normal goat serum in TBS, were incubated overnight at 4°C with a 1:100 dilution of CXCR4 or SDF1 antibody to identify the presence of folliculostellate cells (29), which were virtually absent in all the samples (data not shown). In only one GHoma, sections contained a fragment of normal pituitary (tumor-adjacent pituitary tissues taken at the time of surgery) and were used as control to compare SDF1 and CXCR4 expression in normal and neoplastic pituitary.

Excess SDF1 or CXCR4 blocking peptide (human CXCR4 neutralizing

Patients, tumors, and tissue preparation. Sixty-five consecutive, unselected surgical specimens of human pituitary adenoma (27 GHomas, 37 NFpas, and 1 ACTHoma) were obtained from the Neurosurgery Division, University of Genova.

One fragment of the tumor was immediately stored at -80°C for RT-PCR analysis (28) and the other was paraffin embedded for immunohistochemical experiments. The lack of normal pituitary contamination was assessed by staining with H&E and anti-S100 antibody to identify the presence of folliculostellate cells (29), which were virtually absent in all the samples (data not shown). In one GHoma, sections contained a fragment of normal pituitary (tumor-adjacent pituitary tissues taken at the time of surgery) and were used as control to compare SDF1 and CXCR4 expression in normal and neoplastic pituitary.

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Excess SDF1 or CXCR4 blocking peptide (human CXCR4 neutralizing
peptide; Affinity BioReagents) was used to assess the specificity of the staining. Antibody binding to antigens was neutralized by preabsorption with the blocking peptides using a solution with equal weight/volume units of CXC4 peptide or 5-fold (by weight) excess of SDF1 for 2 h.

For GH, ACTH, PRL, and S100 immunostaining, the BenchMark XT automated immunohistochemical apparatus and the antibodies produced by Ventana Medical Systems were used following the manufacturer’s instructions.

Immunofluorescence staining was done as described for immunohistochemistry using the polyclonal antibodies (1:100 dilution) α-SDF1 (Torrey Pines BioLabs), α-CXCR4 (Sigma), α-GH (Dako), and α-procollagen type I (SP1D8, Development Studies Hybridoma Bank, Iowa University, Iowa City, IA) and the monoclonal antibodies α-GH, α-PRl (Abcam), and α-ACTH (Dako). Sections were labeled with 1:100 fluorochrome-conjugated secondary antibodies Alexa Fluor 488 and Alexa Fluor 568 (Molecular Probes, Invitrogen) in the dark for 1 h. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole. Slides were then stained with Sudan black B (0.3% in 70% ethanol) to avoid tissue autofluorescence (32), washed in TBS, and mounted with Mowiol. Immunohistochemical and immunofluorescence slides were photographed using a DM2500 microscope (Leica Microsystems) equipped with a DFC350FX digital camera (Leica Microsystems).

In double immunofluorescence experiments, sections were simultaneously incubated with both primary antibodies overnight at 4°C. Colocalization was assessed by confocal laser scanning microscopy (Bio-Rad MRC 1024 ES) and the LaserPix software (Bio-Rad).

Colocalization counter. When indicated, AMD3100 and SST were added 10 min before SDF1 and CXCR4 localization. To confirm the expression of SDF1 and CXCR4 in tumoral pituitary cells, we did immunohistochemical analysis on paraffin-embedded sections of representative specimens of our series of adenomas, selected from tumors expressing both SDF1 and CXCR4 mRNA in RT-PCR analysis.

Results

Tumor characteristics. Fifty-six surgical specimens of human pituitary adenomas were studied: patient data are summarized in Supplementary Table S1 Part A.

Part B of Supplementary Table S1 reports the details of further six NFPA, two GHomas, and one ACTH-secreting tumor analyzed in vitro, whereas the data from four autopic normal hypophyses are reported in Supplementary Table S1 Part C.

CXCR4 and SDF1 mRNA expression in human pituitary adenomas. The expression of CXCR4 and SDF1 was evaluated in all the human pituitary adenomas (25 GHomas and 31 NFPA) by RT-PCR. As shown in Table 1, 23 of 25 (92%) GHomas expressed CXCR4 mRNA, and 15 of 25 (60%) expressed SDF1 mRNA. Among NFPA, 24 of 31 (77%) expressed CXCR4 and SDF1 mRNA (Table 1). In all samples, the expression of β-actin was analyzed as internal positive control for cDNA amplification, whereas the absence of contaminating genomic DNA was shown by the lack of amplification products using RNA without reverse transcription (data not shown). Only two GHomas (8%) expressed neither CXCR4 nor SDF1, all SDF1-expressing adenomas contained also CXCR4 mRNA, and eight GHomas expressed CXCR4 but not SDF1 transcripts. In NFPA, coexpression of SDF1 and CXCR4 was the most frequent phenotype observed (18 of 31, 58%). Interestingly, in three cases, we detected only SDF1 mRNA, and in other three, we detected only CXCR4 transcripts. Four NFPA (13%) did not express either molecule.

These results were confirmed in nine fibroblast-free adenoma primary cultures (six NFPA, two GHomas, and one ACTHoma; Table 1), thus excluding that CXCR4 and SDF1 were expressed by fibroblasts present in the surgical specimens.

Considering both postsurgical specimens and purified adenoma cells, the expression of CXCR4 was 92% of 27 GHomas and 81% of 37 NFPA, whereas the percentage of expression of SDF1 was 63% and 78%, respectively (Table 1). The only ACTHoma analyzed expressed both CXCR4 and SDF1 mRNA.

SDF1 and CXCR4 localization. To confirm the expression of SDF1 and CXCR4 in tumoral pituitary cells, we did immunohistochemical analysis on paraffin-embedded sections of representative specimens of our series of adenomas, selected from tumors expressing both SDF1 and CXCR4 mRNA in RT-PCR analysis.

As shown in Fig. 1, for two representative NFPA and two GHomas, both histotypes displayed a marked expression of SDF1 and CXCR4 with a diffuse signal in all the tumor cells. The specificity of the staining was assured, in one NFPA, using two different anti-CXCR4 antibodies (one polyclonal and one monoclonal; Fig. 1A, lane 1) that showed the same pattern of immunoreactivity. Similar results were obtained in immunofluorescence experiments done using a third anti-CXCR4 antibody (polyclonal) and the same SDF1 antibody used in immunohistochemistry. In these experiments, done on sections derived from the GH1 adenoma, also tested in vitro in cell proliferation experiments (see below), a diffuse expression of CXCR4, SDF1, and GH in all the tumor cells was again observed (see below).

Altogether, these results confirmed the RT-PCR data and showed that NFPA and GHoma tumor cells express SDF1 and CXCR4 proteins.

Negative controls of the tumors 1 to 4 depicted in Fig. 1, obtained omitting the primary antibody, in which no signal was detected are reported in Supplementary Fig. S1 (left). Moreover, the preincubation of the anti-SDF1 antibody with molar excess of SDF1 and the anti-CXCR4 antibody with molar excess of the specific blocking peptide completely abolished the immunohistochemical staining in slices derived from the GH1 adenoma analyzed in Fig. 1 (row 3), confirming the specificity of the observed effects (Supplementary Fig. S1, right).

To date, the expression and localization of CXCR4 and SDF1 in human normal pituitary is unclear. To evaluate the differences in expression between normal and tumoral tissue, we analyzed a GHoma specimen in which normal tissue adjacent to the tumor was included. The immunohistochemical results are depicted in Supplementary Fig. S2. Tumor tissue is characterized by a diffuse pattern of cell growth, uniform cellularity, round-shaped or ovoid nuclei, and defined eosinophil cytoplasm (Supplementary Fig. S2, H&E panel, left part, T), whereas the normal adenohypophysis is organized in acini, enclosing the endocrine cells (Supplementary Fig. S2, right part, N), supported by folliculostellate cells that are not detected in the tumoral tissue (Supplementary Fig. S2, S100 panel).

Supplementary Fig. S2 (middle) reports the expression of CXCR4 and SDF1, in both adenoma (left portion of the slide)
and normal (right portion) tissues, as well as the negative control. SDF1 immunostaining, in agreement with the data reported in Fig. 1, showed a diffuse, homogenous expression in all the adenoma cells, although it was dramatically lower in the normal tissue, where only few positive cells were detected. Similarly, CXCR4 immunostaining in the adenomatous tissues seems uniformly and intensely positive. However, in normal anterior pituitary, a significant number of positive cells were also detected, although the presence of discrete cells not expressing CXCR4 was identified. Supplementary Fig. S2 (bottom) shows the staining for GH, ACTH, and PRL, confirming the absence of GH-secreting cells within the adenoma and normal pituitary and ACTH and PRL expression only in discrete normal pituitary cells.

Expression of SDF1 and CXCR4 in normal pituitary. To verify the different pattern of CXCR4 and SDF1 expression in normal versus tumoral pituitary cells, we did immunohistochemical experiments in four autopic normal pituitary sections (see Supplementary Table S1C). Figure 2A details images derived from the hnPit4, which are representative of what we observed in all the others. Although the expression of CXCR4 was much stronger than that of its ligand, which was confined to a small region of the anterior lobe (see Fig. 2A, d and e). In particular, differently from what we observed in pituitary adenomas, SDF1 and CXCR4 were not homogenously distributed throughout the tissue, with large portions of the anterior pituitary completely negative for both the proteins, areas where only scattered positive cells and few zones with a higher expression (Fig. 2A, f-h and i-k, respectively; magnification in l and m). However, the expression of SDF1 was always lower than that of its receptor. To quantify the level of expression of CXCR4 and SDF1, we counted positive cells in randomly selected microscopic fields from all the pituitary sections. About 1,000 cells per slide were evaluated: 34% of normal pituitary cells expressed CXCR4 and 12% expressed SDF1.

To identify the cell type(s) that actually expresses CXCR4 and SDF1 in the anterior pituitary, we did double immunofluorescence experiments (CXCR4 and SDF1 versus GH, PRL, or ACTH) and confocal microscopy analysis. A different pattern of expression for SDF1 and CXCR4 was detected in normal and tumoral pituitary cells, with all the adenoma cells diffusely expressing both the chemokine and its receptor (Fig. 2B, left) and a more discrete pattern of expression observed in the normal tissue (Fig. 2B, right). In the normal hypophysis, the expression of SDF1 and its receptor is not confined to a specific cell population (i.e., GH-, PRL-, or ACTH-secreting cells) but most CXCR4 positivity was observed in subsets of the different subpopulations (Fig. 2B, right). However, as observed in immunohistochemical experiments, most normal pituitary areas in the anterior lobe did not express neither CXCR4 nor SDF1 (occasionally isolated CXCR4 and SDF1 cells were observed in islets of hormone-positive cells; see Fig. 2B, right), although areas containing higher percentage of CXCR4 and SDF1 immunoreactive cells were also observed. The scattered SDF1-positive cells showed a lower percentage of colocalization with hormone-secreting cells mainly confined in ACTH-expressing pituitary cells (Fig. 2B). Interestingly, all the SDF1-labeled cells were also CXCR4 positive, whereas a significant number of CXCR4-positive/SDF1-negative cells were detected (Supplementary Fig. S3). Staining pituitary sections with all the hormones together, few cells expressing CXCR4 were identified that express neither GH, PRL, nor ACTH (Supplementary Fig. S4). Importantly, no colocalization of either CXCR4 or SDF1 was observed in endothelial cells (CD34 staining) in normal hypophysis and pituitary adenomas, and no expression of CXCR4 in folliculostellate cells (S100 staining) was detected in normal pituitaries (Supplementary Fig. S5).

Finally, we evaluated the localization of CXCR4 and SDF1 mRNA expression in normal pituitary by means of in situ hybridization. In fact, although we used three different CXCR4 antibodies to ensure the specificity of the signal, it is important to verify the data observed measuring the mRNA content. Unfortunately, due to paraffin embedding, the autopic specimens were not suitable for mRNA analysis. Thus, because SDF1 and CXCR4 mRNAs were identified in rat tissues (6, 24), we did these experiments in rat samples. Representative images, reported in Fig. 3, largely confirmed the data obtained in humans.

In situ hybridization revealed numerous cells expressing CXCR4 mRNA in the anterior lobe (Fig. 3A, C, and E). At the border of anterior and intermediate lobe, CXCR4-expressing cells were particularly frequent, whereas only few hybridization signals for CXCR4 were seen within the intermediate lobe (Fig. 3A and D). Cells expressing SDF1 mRNA were clustered at the border of anterior and intermediate lobe (Fig. 3B and G) but occasionally occurred also in the intermediate and posterior lobe (Fig. 3B, G, and H). In contrast, very few cells in the anterior lobe express SDF1 mRNA (Fig. 3F).

SDF1 modulation of in vitro proliferation of primary cultures of pituitary adenoma cells. SDF1 effect on cell proliferation was studied in eight primary cultures of human pituitary adenomas [two GHomas (GH1 and GH12), one ACTHoma (ACTH1), and five NFPAs (NFP1A, NFP2A, NFP3A, NFP4A, and NFP6A); see Supplementary Table S1B]. Experiments were carried out after magnetic fibroblast deprivation and isolated cells were grown in d-valine containing medium to assure a minimal fibroblast contamination (31). Immunoemunofluorescence experiments done on isolated cells from one adenoma (GH12) showed that all the cells in culture, also expressing CXCR4 and SDF1, are derived from adenomatous tissue because they express GH but not procollagen type I (a marker for fibroblasts; Supplementary Fig. S6). In addition, in the secreting tumors (ACTH1, GH1,
and GH2), the level of hormone released was determined to confirm the in vitro maintenance of the in vivo biological features. ACTH (mean, 332 ± 87 pg/mL) and GH (mean, 32 ± 1 and 27 ± 2 ng/mL) were indeed detected in the culture medium from the respective adenoma cell culture tested.

The expression of CXCR4 and SDF1 was evaluated in each short-term culture by RT-PCR. CXCR4 mRNA was present in all the samples, confirming its expression after cell dispersion and the culturing in vitro, whereas SDF1 expression was detected in four of five NFPAs and in all the GH- and ACTH-secreting adenoma cells (Figs. 4 and 5, insets; Table 1).

The proliferative activity of SDF1 was assessed by [3H]thymidine uptake assay. Because after dispersion of surgical specimens we recovered a small cell number and the elimination of fibroblasts (see Materials and Methods), while allowing the establishment of highly pure primary cultures (Supplementary Fig. S6), further reduces the number of available adenoma cells, we were not able to do complete dose-response curves and only 12.5 and 25 nmol/L SDF1 concentrations were tested.

In five cases (NFPA1, NFPA3, ACTH1, GH1, and GH2), SDF1 induced a statistically significant increase in DNA synthesis at the concentration of 25 nmol/L (+31%, +22%, +21%, +17%, and +15% over the basal activity, respectively, \( P < 0.05 \)), whereas NFPA6 cells showed a greater increase (+60%) at 12.5 nmol/L SDF1 (\( P < 0.01 \)). SDF1 effects were already statistically significant at the concentration of 12.5 nmol/L in cells derived from GH2 and ACTH1, although a lack of further increase in DNA synthesis using higher SDF1 concentrations was observed. In contrast, NFPA2 cells displayed an increase in [3H]thymidine uptake after SDF1 (25 nmol/L) treatment (+23% over basal), although it did not reach a statistical significance, likely due to intraexperimental variance, and NFPA4 cells did not respond to the chemokine stimulation. The data from the individual eight adenomas are summarized in Figs. 4 and 5.

The specificity of SDF1 proliferative activity was determined in all the pituitary adenoma cell cultures using the selective CXCR4 antagonist AMD3100 (19). AMD3100 (50 μmol/L), added to cultures 10 min before SDF1, completely abolished SDF1-induced cell growth, without affecting basal DNA synthesis (Figs. 4 and 5). These results indicate that SDF1 induces cell proliferation through the activation of CXCR4. Interestingly, in two adenomas (NFPA4 and GH2), a pronounced reduction of basal DNA synthesis was observed after AMD3100 treatment (Figs. 4 and 5). Thus, it is possible that a basal secretion of SDF1 occurs in these samples causing an autocrine constitutive stimulation of DNA synthesis. Indeed, using a specific ELISA, in vitro SDF1 secretion was studied in three adenomas (GH1, NFPA4, and NFPA6). SDF1 mean basal values, measured after 24 h of serum deprivation in cell
supernatants, were 1,250 and 4,870 pg/mL (0.15-0.6 nmol/L) for GH1 and NFPA4, respectively, whereas for NFPA6 SDF1 secretion was below the level of detection of the ELISA. Interestingly, when we compared the proliferative response to SDF1 of these three tumors with their autonomous SDF1 production, we found a significant inverse correlation (coefficient of correlation, $R^2 = -0.84$). NFPA4, which secretes the highest quantity of SDF1, is insensitive to exogenous SDF1 stimulation. On the contrary, NFPA6 has the greatest increase of proliferation (+60% over basal; see Fig. 4) but it displayed neither SDF1 mRNA (Fig. 4, inset) nor SDF1 secretion.

Fig. 2. Differential expression of CXCR4 and SDF1 in normal and adenomatous tissues from human pituitary. A, a to e, immunohistochemistry of an autopic human normal pituitary at the border between the anterior and neural lobes. a, H&E staining; b, negative control (omitting primary antibody); c, staining for S100 labeling only the folliculostellate cells in the anterior pituitary; d, staining for CXCR4; e, staining for SDF1. Magnification, × 4. f to h, SDF1 immunostaining in human normal anterior pituitary. Panels represent areas close to neurohypophysis (f) where the highest number of positive cells is detected, and areas where scatter (g) or none (h) SDF1-expressing cells are detected. Magnification, ×20. i to k, CXCR4 immunostaining in human normal anterior pituitary. Panels represent areas close to neurohypophysis (i) where the highest number of positive cells is detected, and areas where scatter (j) or none (k) CXCR4-expressing cells are detected. Magnification, ×20. l and m, larger magnification (×40) of SDF1-positive (l) and CXCR4-positive (m) cells. B, left, immunofluorescence staining for CXCR4, SDF1, and GH in sections derived from a GH-secreting pituitary adenoma (named GH1 in Supplementary Table S1B). From the top micrograph: CXCR4-positive green fluorescent – stained cytoplasms merged with the 4',6-diamidino-2-phenylindole counterstained nuclei (blue); SDF1-positive green fluorescent – stained cytoplasms merged with the 4',6-diamidino-2-phenylindole counterstained nuclei (blue); GH-positive red fluorescent – stained cytoplasms merged with the 4',6-diamidino-2-phenylindole counterstained nuclei (blue). Bottom micrographs. left lane, negative control with secondary green fluorescent antibody; middle lane, negative control with secondary red fluorescent antibody; right lane, blue nuclear staining. Original magnification, ×20. Right, confocal analysis of the colocalization between CXCR4 or SDF1 and GH, PRL, or ACTH. Magnification, ×60. CXCR4-positive cells displayed a certain degree of colocalization with all the pituitary hormones tested. On the contrary, SDF1, beside a general lower expression, seemed to be mainly colocalized with ACTH-expressing cells.
To test whether SDF1 release from pituitary cells is regulated, we did a quantitative determination of SDF1 secretion in one NFPA-derived cell culture supernatant (NFPA5) in response to depolarizing stimuli (KCl, 20 and 40 mmol/L): this treatment significantly increased SDF1 release (2.5- and 2.2-fold versus basal, respectively; Supplementary Table S2).

SST controls hormone secretion and proliferation in normal and tumor pituitary cells. Thus, we tested the effects of SST (100 nmol/L) on the proliferative activity induced by SDF1 in two adenomas (ACTH1 and GH1). SST significantly affected [3H]thymidine uptake in both SDF1-stimulated cultures (Fig. 5). In ACTH1 cells, we observed a complete inhibition of the DNA synthesis activity induced by SDF1 (12.5 nmol/L) after SST treatment. The growth-inhibitory activity of SST was more evident in GH1 cells in which SDF1 (12.5 and 25 nmol/L) increase of DNA synthesis was reduced below the basal values.

Discussion

Besides hypothalamic peptides, growth factors and cytokines derived from the portal or the general circulation or locally produced regulate pituitary cell functioning. Different cytokines are expressed by and act on anterior pituitary cells, regulating hormone secretion (34–37), although a similar pattern of expression and activity for these molecules was observed in both normal and adenomatous pituitary cells (38).

More recently, chemokines acquired a relevant role in the physiology, pathology, and tumor transformation of different cell types, although, to date, few studies addressed their role in the regulation of normal and tumor pituitary cell functions (26). For example, prosecretory effects of GRO (member of the IL-8 family) in rat anterior pituitary cells were reported (39). IL-8 expression was detected in a small percentage of human pituitary adenomas (3 of 25; ref. 40) and its receptor CXCR2 was identified in both human pituitary adenomas and normal pituitaries (41) without a tumor-specific phenotype. On the contrary, the biological role of SDF1 and CXCR4 was never studied at pituitary level, although both molecules are coexpressed in normal rat pituitary (24, 25) and hypothalamus (7).

Here, we investigated the expression of both SDF1 and CXCR4 in a cohort of 65 human pituitary adenomas and 4 normal hypophyses and the potential autocrine/paracrine role of SDF1 in pituitary cell proliferation. We provide the first evidence of SDF1 and CXCR4 expression in normal and adenomatous human pituitary. Importantly, we show that overexpression of both ligand and receptor occurs in adenomas compared with normal pituitary cells, thus suggesting that this

Fig. 3. Expression of CXCR4 and SDF1 mRNA in rat pituitary. Frozen pituitary sections were hybridized with 35S-labeled probes for CXCR4 (A, C, D, and E) and SDF1 (B, F, G, and H). Signals were detected by nuclear emulsion and photographed under dark field illumination. A, C, and E, numerous cells expressing CXCR4 mRNA are found in the anterior lobe (AL) and posterior lobe (PL). A to D, few signals for CXCR4 are seen in the intermediate lobe (IL). B, G, and H, cells expressing a high SDF1 mRNA level are clustered at the border of anterior lobe and intermediate lobe and are found in the intermediate lobe and posterior lobe. A and B, note the overlap of SDF1 and CXCR4 at the border of anterior lobe and intermediate lobe. Scale bars, 1 mm (A) and 100 μm (C).
expression profile may contribute to the enhanced proliferative rate of adenoma cells.

CXCR4 mRNA was present in >85% of the adenoma analyzed, whereas SDF1 mRNA was detected in a slightly lower percentage of NFPAs and GHomas. Similar RT-PCR data were obtained analyzing either postsurgical specimens or fibroblast-free primary cell cultures, supporting the localization of this chemokine and its receptor in tumor cells. Furthermore, these results were confirmed by immunohistochemical and immunocytofluorescence analyses on histologic preparations and dispersed adenoma cells, in which a homogeneous immuno-reactivity for SDF1 and CXCR4 within cells of both GHomas and NFPAs was observed.

The concomitant expression of this receptor-ligand pair in the same cells was previously described as an autocrine/paracrine mechanism of cancer cell stimulation, resulting in clinical aggressive behavior and faster growth of several tumoral histotypes (15, 42). We previously showed an autocrine/paracrine mitogenic activity of SDF1 in cell lines and primary cultures of human glioblastoma multiforme coexpressing CXCR4 (16, 18). In the present study, most of pituitary adenomas showed a similar pattern of coexpression, suggesting

Fig. 4. Effects of SDF1 and AMD3100 on DNA synthesis in human NFPA primary cultures. DNA synthesis was evaluated by [3H]thymidine incorporation in five NFPA primary cultures (NFPA1, NFPA2, NFPA3, NFPA4, and NFPA6) treated with SDF1 (12.5 and 25 nmol/L, C) alone or following AMD3100 (50 μmol/L) pretreatment (O). Experiments were done in triplicate and results are expressed as percentage ± SE of the basal incorporation. Basal values were as follows (cpm/100,000 cells): NFPA1 = 668 ± 12, NFPA2 = 2.912 ± 123, NFPA3 = 2.275 ± 89; NFPA4 = 1,986 ± 189; NFPA6 = 1,320 ± 94. *, P < 0.05 versus basal value; †, P < 0.05 versus SDF1 stimulation. CXCR4 (lanes C) and SDF1 (lanes S) mRNA expression, analyzed by RT-PCR in each purified adenoma cell preparation under basal conditions, is depicted in the inset to each graph.
that, also at pituitary level, an autocrine/paracrine mechanism may support cell proliferation.

In addition, the high percentage of human pituitary adenomas expressing CXCR4 suggests that alterations of CXCR4 activity, possibly via a deregulated SDF1 secretion from the adenomatous cells themselves, the hypothalamus, or directly coming from the bloodstream (26), may contribute to the clonal expansion of transformed pituitary cells.

Altogether, these findings suggest that an altered control of pituitary cell proliferation via the activation of the SDF1/CXCR4 system may lead to pituitary tumorigenesis.

On the other hand, histochemical analysis of human normal pituitary showed a significantly lower expression of CXCR4 compared with the tumor tissue, being localized in about one third of cells, whereas the expression of SDF1 was confined in even fewer cells.

Fig. 5. Effects of SDF1, AMD3100, and SST on DNA synthesis in human GH- and ACTH-secreting pituitary adenoma primary cultures. Left, DNA synthesis was evaluated by [3H]thymidine incorporation in primary ACTH- and GH-secreting adenoma cell cultures (GH1, GH2, and ACTH1) and treated with SDF1 (12.5 and 25 nmol/L; ■) alone or following AMD3100 (50 μmol/L) pretreatment (○). Basal incorporation values were 736 ± 46, 1,722 ± 25, and 882 ± 32 cpm/100,000 cells for ACTH1, GH1, and GH2, respectively. *, P < 0.05 versus basal value. CXCR4 (lanes C) and SDF1 (lanes S) mRNA expression, analyzed by RT-PCR in each purified adenoma cell preparation under basal conditions, is depicted in the inset to each graph. Right, DNA synthesis was evaluated by [3H]thymidine in two primary pituitary adenoma cultures (ACTH1 and GH1) treated with SDF1 (12.5 nmol/L) in the presence or absence of SST (100 nmol/L). Experiments were done in triplicate and results are expressed as percentage ± SE of the basal incorporation. *, P < 0.05 versus basal value; †, P < 0.05 versus SDF1-stimulated cells.
This corroborating evidence suggests that in pituitary tumorigenesis, after independent initiation phases (primary genetic defect), an overexpression of this chemokine/receptor system could occur, being responsible for the promotion/progression steps. In fact, differently from normal tissues, a marked expression of both CXCR4 and SDF1 in all the adenomatous cells was observed. These results differ from those reported for CXCR2 (IL-8 receptor) that was similarly detected in all the cells of both normal and tumor pituitary (41). Thus, we propose that the overproduction of SDF1 in CXCR4-overexpressing adenoma cells may participate to their increased proliferation rate.

Moreover, the identification of CXCR4-expressing cells in human normal pituitary confirms that SDF1/CXCR4 axis is also involved in the regulation of normal pituitary functions and suggests that direct or indirect still unknown alterations of their expression may contribute to deregulated cell growth.

In line with this hypothesis, we directly evaluated the effects of SDF1/CXCR4 signaling on pituitary adenoma cell proliferation in vitro, showing that SDF1 increases DNA synthesis (+20-30%) in six of eight human pituitary adenoma. Although this stimulation rate is not extraordinary compared with other human cancer cells analyzed in vitro, it is extremely significant for primary cultures of human pituitary adenoma cells, considering their very low growth potential in vitro (31). In addition, the observation that the CXCR4 antagonist AMD3100 alone has slight effects on cell growth suggests that in basal conditions this receptor is not activated by endogenous SDF1 in vitro. Indeed, sustained SDF1 release in vitro was obtained by membrane depolarization, suggesting that the peptide stored in these cells is released under regulated external stimuli, as likely occurs in vivo. The quantitative comparison of the expression of CXCR4 and the analysis of the different SDF1 secretory potential between normal and tumor pituitary cells will be necessary to definitively assess the role of this chemokine system in pituitary adenoma development. However, the deregulation of SDF1 secretion, at least in subsets of pituitary adenomas, is shown by the tonic SDF1 release by NFPA4 cells that sustains “basal” [3H]thymidine uptake in vitro. In this tumor (as well as in GH2, in which, however, SDF1 secretion was not evaluated), a significant reduction of DNA synthesis, after AMD3100 alone treatment, was observed. Thus, at least in some adenomas, the cell proliferation may be sustained by a constitutive release of SDF1 causing an autocrine activation of CXCR4.

SST is the main physiologic inhibitor of hormone secretion and proliferation in normal pituitary and pituitary adenomas (43). SST significantly inhibited SDF1-induced cell proliferation, suggesting that SDF1-regulated DNA synthesis is under the same regulatory mechanisms as other pituitary growth factors. Interestingly, SST antiproliferative activity is mediated by a phosphotyrosine phosphatase-dependent inhibition of extracellular signal-regulated kinase 1/2 (44, 45), whereas its activation is responsible for SDF1-induced cell proliferation (17, 18, 24). Thus, we hypothesize that in pituitary adenoma cells the antagonism of SST and SDF1 on extracellular signal-regulated kinase 1/2 activity may account for the inhibition of cell proliferation. The interaction between SST and SDF1 activities may be relevant for further research of therapeutic approaches to these tumors.

An important achievement of this study was the identification, for the first time, of CXCR4- and SDF1-expressing cells in human normal pituitary. The distribution of these cells was not homogenous but localized in areas with a higher expression in proximity of the neural lobe and, moving distally, large negative areas. Moreover, whereas CXCR4 was detected in about one third of cells, SDF1-expressing cells were mainly localized in the area surrounding the posterior lobe and very few isolated cells are present in the other anterior lobe portion. CXCR4-positive cells within the human normal anterior pituitary were not confined to a specific pituitary cell population but colocalized with subsets of cells expressing GH, PRL, or ACTH, although few CXCR4-positive cells were identified that did express none of such hormones. Conversely, SDF1 expression was mainly colocalized with ACTH, in agreement with the presence of these cells mainly in the anterior pituitary cells in proximity of the neural lobe (46). No colocalization between CXCR4/SDF1 and S100 (folliculostellate cells) or CD34 (endothelial cells) was observed. The main conclusion drawn from these data is that pituitary adenoma cells overexpress SDF1 and CXCR4 compared with normal tissue. A remaining open question is whether pituitary adenomas are actually generated as a consequence of such phenotype or the overexpression of SDF1 and CXCR4 is induced by other epigenetic alterations and only provides a selective proliferation advantage favoring clonal expansion of transformed cells. In fact, most of the CXCR4-expressing normal pituitary cells do not express SDF1 and the autocrine stimulation of CXCR4 seems to represent a key characteristic of pituitary adenoma cells. Importantly, beside a role in tumor progression, the autocrine activation of CXCR4 may play also a relevant role in the maintenance of the tumors due to its positive effects on cell survival (mainly mediated via phosphatidylinositol 3-kinase/Akt pathway; ref. 18).

CXCR4 and SDF1 are also important factors in the biology of stem and/or progenitor cells (47, 48). Although stem cells have not yet identified in normal pituitary or pituitary adenomas, evidence of stem-like cells (side population) was recently reported (49). It was proposed that these cells may contribute to pituitary plasticity, as occur during pregnancy in which the PRL-secreting cell population largely increases (50). Further studies should address the role of SDF1- and CXCR4-expressing cells in such processes.

In conclusion, we propose that SDF1 may represent a novel growth factor for pituitary cells, acting via either endocrine or autocrine/paracrine pathways. Moreover, CXCR4 overexpression in a large percentage of human pituitary adenomas suggests that, in conditions of deregulation, this receptor system may contribute to pituitary adenoma development and/or progression. Indeed, differently from normal pituitary cells, beside CXCR4, most of the adenomas analyzed express (and likely secrete) also SDF1, supporting an autocrine/paracrine role for this chemokine. It is conceivable that the activation of this autocrine loop in cells with a gain of function in proliferative pathways may contribute to the clonal expansion of the mutated cells to foster the development or the maintenance of adenomas.

These results strengthen the idea to target chemokine networks involved in tumor growth, representing a novel therapeutic approach for pituitary adenomas.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.
References


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Overexpression of Stromal Cell–Derived Factor 1 and Its Receptor CXCR4 Induces Autocrine/Paracrine Cell Proliferation in Human Pituitary Adenomas

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