REVIEW

Role of stromal cell-derived factor 1 (SDF1/CXCL12) in regulating anterior pituitary function

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Abstract

Chemokines are key factors involved in the regulation of immune response, through the activation and control of leukocyte traffic, lymphopoiesis and immune surveillance. However, a large number of chemokines and their receptors are expressed in central nervous system (CNS) cells, either constitutively or induced by inflammatory stimuli, playing a role in many neuro-pathological processes. Stromal cell-derived factor 1 (SDF1) is a chemokine whose extra-immunological localization and functions have been extensively studied. SDF1 and its receptor CXCR4 were identified in both neurons and glia of many brain areas, including the hypothalamus, as well as at the pituitary level. Importantly, SDF1 and CXCR4 expression is increased in brain tumors in which their activity induced tumor cell proliferation and brain parenchyma invasion. Despite their localization, to date very few reports addressed the role of CXCR4 and SDF1 in the modulation of the hypothalamus/pituitary axis and their possible involvement in the development of pituitary adenomas. In this review, we discuss previous literature data on the role of chemokines in normal and adenomatous pituitary cells, focusing on recent data from our group showing that CXCR4 activation controls proliferation and both prolactin and GH release in the pituitary adenoma cell line GH4C1 through a complex network of intracellular signals. Thus, the SDF1/CXCR4 system together with other chemokinergic ligand–receptor pairs, may represent a novel regulatory pathway for pituitary function and, possibly, be involved in pituitary adenoma development. These lines of evidence suggest that the inhibition of chemokine receptors may represent a novel pharmacological target for the treatment of pituitary adenomas.

Journal of Molecular Endocrinology (2007) **38**, 383–389

Role of chemokines in neuroendocrine regulation

Stromal cell-derived factor 1 (SDF1), also called CXCL12, is a chemokine of the CXC subfamily originally characterized as a pre-B-cell stimulatory factor and cloned from bone marrow cell supernatants. SDF1 exists in three alternative splicing variants (α , β and γ), of which SDF1 α is the most abundant in the brain (Nagasawa *et al.* 1994). The sequence of this chemokine is highly conserved among the species, with only one amino acid difference between murine and human SDF1 α , suggesting that this molecule may play a significant biological role. SDF1 is a chemotactic factor for T cell, monocytes, pre-B-cells, dendritic cells, and hematopoietic progenitor cells and supports B-cell progenitor and CD34⁺ cell proliferation (Luster 1998, Christopherson & Hromas 2001). However, its expression is not restricted to immune and hematic cells, but SDF1 mRNA and protein were also identified in the central nervous system (CNS) in neuronal, astroglial, microglial, and endothelial cells (Bajetto *et al.* 2001a).

SDF1 exerts its effects via the binding to CXCR4, a receptor member of the G-protein-coupled receptor superfamily. Their interaction is reported to be unique, different from other chemokines that recognize multiple receptors. The disruption of *CXCR4* or *SDF1* genes in mice, causes a similar embryological lethal phenotype, characterized by deficient B-lympho- and myelo-poiesis, defects in vasculogenesis and abnormal development of the heart and of different areas of the CNS (Nagasawa *et al.* 1996, Zou *et al.* 1998). Similarly to SDF1, CXCR4 is expressed outside the immune system, including endothelial cells, embryonic germinal neuroepithelium and mature neurons, astrocytes and microglia (Oh *et al.* 2001). Furthermore, CXCR4 was reported to represent a co-receptor of CD4 for the entry of T-lymphotropic strains of the immunodeficiencyvirus (HIV-1); SDF1, competing for CXCR4, can inhibit the fusion and replication of HIV-1 in CD4⁺/CXCR4⁺ cells (Feng *et al.* 1996).

Interestingly, besides a specific distribution of both SDF1 and CXCR4 in different brain areas and neuronal subpopulations, a selective localization of SDF1

expressing neurons was detected in many hypothalamic nuclei, including the paraventricular nucleus (PVN), the lateral hypothalamus, the lateral pre-optic area and the median eminence (Banisadr *et al.* 2003, Guyon *et al.* 2005). Furthermore SDF1 was reported to inhibit arginine vasopressin (AVP) release from the magnocellular neurons of the supraoptic and the paraventricular hypothalamic nuclei in normal rat. In addition, AVP projections to the anterior pituitary co-express SDF1 and CXCR4 thus demonstrating that the SDF1/CXCR4 axis represents an autocrine system that modulates brain and neuroendocrine activity (Callewaere *et al.* 2006). Importantly, although the majority of the SDF1-labeled neurons belonged to the magnocellular subpopulation projecting to the neurohypophysis, SDF1 expressing neurons, even though less numerous (20% of the neurons when compared with 60% in the vasopressinergic neurons), were also observed in the parvocellular part of the PVN (Banisadr *et al.* 2003).

Chemokine modulation of normal pituitary function

It has been known for many years that different immune-derived molecules can control pituitary hormone release. In particular, it was reported that interleukin 1 β (IL-1 β), that is produced by hypothalamic neurons (Johnson *et al.* 2004) inhibit prolactin secretion from dispersed rat pituitary cells through the regulation of both cAMP production and agonist-induced Ca^{++} fluxes (Schettini *et al.* 1990). Indeed, the expression of its receptors was detected within the mouse adenohypophysis (Cunningham *et al.* 1992). Moreover, IL-1 β mRNA was identified in rat pituitary cells after a lipopolysaccharide (LPS) challenge, supporting the hypothesis that IL-1 β may be involved in paracrine or autocrine regulation of pituitary function during infectious challenge (Koenig *et al.* 1990). However, the role of IL-1 β in the brain-endocrine-immune axis is not yet completely defined. Similarly also interleukin 6 (IL-6) is released by normal rat pituitary cells (Spangelo *et al.* 1990a) and its receptor was also identified in normal pituitary cells in humans (Hanisch *et al.* 2000). The pituitary source of IL-6 was identified in the folliculostellate (FS) cells in normal tissue and a paracrine mechanism was proposed for the cytokine regulation of different pituitary hormones release, including adrenocorticotrophic hormone (ACTH) and prolactin (Woloski *et al.* 1985, Spangelo *et al.* 1989).

However, to date none of these studies supported a physiological role for these cytokines in pituitary function. Indeed, it was proposed that their functional effects are mainly dependent on endotoxin challenge and thus a response to infections (Spangelo *et al.* 1990b).

In the past years, a better defined regulation of the hypothalamic-hypophyseal axis by some pleiotropic

chemokines was identified in rat. In fact, the cytokine-induced neutrophil chemoattractant (CINC, the rat counterpart of the human growth-related oncogene, GRO) was reported to be expressed in the PVN, the posterior pituitary and the median eminence. In response to stressful stimuli (i.e. immobilization stress) CINC synthesis is highly induced in the PVN and the chemokine is released in the median eminence (Sakamoto *et al.* 1996) to reach CXCR2 (the GRO receptor)-expressing pituitary cells. The biological effects of CINC on pituitary cells were the stimulation of prolactin release (and slight stimulation of GH secretion) and the inhibition of luteinizing hormone (LH) and follicle stimulating hormone (FSH) secretion (Sawada *et al.* 1994). Importantly, CXCR2 expression was identified also in normal human pituitary cells, thus supporting the relevance of this regulatory mechanism (Tecimer *et al.* 2000). Furthermore, a sustained CINC secretion was also observed in a subpopulation of cultured pituitary cells (Koike *et al.* 1994) and by FS cells (Zhang *et al.* 1997). Thus, again an autocrine/paracrine intrapituitary regulation may occur via the CINC/GRO stimulation of pituitary cells expressing CXCR2. However, GRO is not the only chemokine that may be involved in the regulation of pituitary function. For example, another CXCR2 ligand, interleukin 8 (IL-8), was also identified in brain areas (PVN of the hypothalamus and hippocampus) involved in the hypothalamus-pituitary-adrenal axis. Importantly, its secretion is under the control of steroids through a feedback mechanism, thus supporting a physiological role for this chemokine in the pituitary regulation of ACTH secretion (Licinio *et al.* 1992).

However, the chemokineric regulation of the hypothalamus-pituitary system is still poorly studied, and we can hypothesize that more chemokine/receptor pairs are effectively involved in its regulation both in physiology and in pathology. For example, SDF1 is emerging as a major point of convergence between immune and nervous systems. In particular, binding studies showed that at the pituitary level, high affinity SDF1-binding sites were present (Banisadr *et al.* 2000), and CXCR4 mRNA was identified in rat anterior pituitary explants (Bajetto *et al.* 1999). Thus, the concomitant expression of SDF1 in hypothalamic neurons and CXCR4 at the pituitary level, may suggest that this chemokine could represent a novel hypothalamic factor possibly contributing to the regulation of anterior pituitary function.

Pituitary and hypothalamic chemokines in the development of pituitary adenomas

Pituitary adenomas constitute up to 15% of primary intracranial tumors and are associated with significant morbidity due to local mass-related effects and/or hormone hypersecretion. Pituitary adenomas are

classified according to their secretory pattern: prolactinomas represent about 30% of pituitary tumors, GH hypersecretive adenomas (GHoma) account for approximately 15%, and ACTH-secreting tumors <10%. Pituitary adenomas secreting biologically inactive hormones (α subunit of glycoprotein hormones, such as thyroid stimulating hormone (TSH), LH and FSH, or the entire gonadotropins) or derived from 'null cells' are defined as clinically 'non-functioning pituitary adenomas' (NFPAs) and represent about 30% of the total pituitary tumors. To date, most aspects of molecular pathogenesis of pituitary adenomas remain unclear since few alterations have been definitively demonstrated to be involved in their tumorigenesis (Asa & Ezzat 2002).

Genetic studies reported that pituitary adenomas have a monoclonal origin with the neoplastic initiation often related to oncogenic mutations in proteins involved in cell proliferation control. Among others, mutations were identified in the genes encoding ras, PKC, the α subunit of the GTP-binding proteins Gs, (*gsp*) or Gi (*gip*). Furthermore, in some cases the overexpression of activating genes (i.e. pituitary tumor transforming gene, *hpttg*) or loss of tumor suppressor genes (*Rb*, *menin*, *p53*, *p27* and *p16*) was identified (Spada & Lania 2002).

Nevertheless, the pathogenesis of these tumors is heterogeneous with these genetic alterations identified only in subsets of adenomas. The current hypothesis proposes that these initiating events may cause a 'gain of function' in the proliferative activity of single pituitary cells on which promoting factors cooperate to induce the clonal expansion (Asa & Ezzat 2002).

A multiplicity of promoting factors, such as hypothalamic hormones, locally produced growth factors (EGF, bFGF, FGF-4, NGF, TGF) and cytokines (IL-1, IL-2, IL-6), have been reported to determine pituitary tumor progression (Ray & Melmed 1997, Renner *et al.* 2004).

Chemokines are now recognized as mediators in several physiologic and pathologic processes, including the proliferation and invasiveness of cancer cells. However, very few studies addressed the potential role of any component of the chemokine family in regulating pituitary functions. In particular, the receptor for IL-8, CXCR2, was detected in high concentration in human pituitary adenomas (Tecimer *et al.* 2000), suggesting a possible participation of this chemokine in the expansion of some pituitary adenomas. Interestingly, although there is an extremely variable incidence rate among different studies, that is, 3 out of 25 according to Suliman *et al.* (1999) or 17 out of 17 according to Green *et al.* (1996), the expression of IL-8 was also identified in adenoma pituitary cells altogether with its receptor; it is thus possible to hypothesize an autocrine/paracrine pathway of activation of the tumor cells.

On the other hand, SDF1 receptor CXCR4 is the most widely expressed chemokine receptor in human

malignancies and its activation by SDF1 causes proliferation, migration, invasion, and metastatization of cancer cells and promotes tumoral neo-angiogenesis (Burger & Kipps 2006). In particular, the SDF1/CXCR4 system was reported to support the survival and growth of leukemia, breast carcinoma (Balkwill 2004), and glioblastoma multiforme (GBM) cells (Rempel *et al.* 2000, Bajetto *et al.* 2006a). *In vitro* studies showed that SDF1 is a growth factor for GBM cells increasing their proliferation and migration (Barbero *et al.* 2003). In addition, it has been reported that SDF1 stimulates chemotaxis, survival and proliferation in GBM and medulloblastoma primary cell cultures and xenografted tumors (Rubin *et al.* 2003).

To date, the role of SDF1/CXCR4 in the pituitary function and in the genesis of pituitary adenomas has scarcely been investigated. However, the constitutive expression of the ligand and its cognate receptor in hypothalamic neurons and pituitary cells respectively, together with the powerful role demonstrated for SDF1 as tumor proliferation factor, suggest the possibility that this chemokine, via the activation of pituitary CXCR4, may act as a promoting factor for pituitary adenoma development.

Biological effects of SDF1 in pituitary adenoma cells: the GH4C1 cell model

We used the rat pituitary adenoma cell line GH4C1 to analyze the potential role of the SDF1/CXCR4 network in the regulation of pituitary function and, possibly, pituitary tumorigenesis (Florio *et al.* 2006). These cells represent one of the most studied model to evaluate pituitary regulation *in vitro* (Westendorf & Schonbrunn 1982). GH4C1 cells display a regulated release of both GH and prolactin, and a proliferative response reproducing at best *in vitro* that observed in pituitary adenoma cells.

Importantly, we showed that, different from most of the tumor cell lines analyzed, these cells express CXCR4 but not SDF1. This feature is particularly important, since CXCR4 is known to go through a very rapid desensitization and thus, in the presence of an endogenous release of the chemokine in a closed system (as occurs in most *in vitro* studies), it could be very difficult to identify the effects of the exogenous peptide.

To assess a role for SDF1 in pituitary adenoma development, we tested, in this cell model, the effects of the chemokine in the regulation of the two key features of pituitary adenoma cells, hormone release, and proliferation.

We found that low nanomolar concentrations of SDF1 caused a significant increase in both prolactin and GH secretion, although the latter was much more pronounced (+35 and +110% respectively when

compared with the basal secretion of the two hormones; Table 1). This secretagogue activity was completely abolished by pretreatment with pertussis toxin or somatostatin (100 nM; Table 1). These data indicate that SDF1 activity requires the activation of a G-protein of the Gi/Go subfamily, and that its effects are responsive to inhibitory stimuli as observed in both normal pituitary and secreting adenomas. Interestingly, pertussis toxin was able to abolish both the stimulatory effects of SDF1 (Florio *et al.* 2006) and the inhibitory effects of somatostatin (Florio & Schettini 1996).

SDF1 was also a powerful mitogen for GH4C1 cells, with a statistically significant effect already evident at a concentration of 6.25 nM and reaching a maximal increase of DNA synthesis at a concentration of 12.5 nM (Table 1). Higher concentrations either did not further increase the cell proliferation rate or even induced a lower effect, likely due to the rapid desensitization of CXCR4 caused by high ligand concentrations, as well characterized in other cell systems (Barbero *et al.* 2003). Importantly, the proliferative effects were also completely abolished by pertussis toxin and significantly reduced by somatostatin pretreatment (Table 1). The observation that pertussis toxin reversed both the stimulatory effects of SDF1 and the inhibitory action of somatostatin, depends on the observation that CXCR4 and all the somatostatin receptors are coupled to a pertussis toxin-sensitive G-protein. However, the opposite biological effects observed can be understood by the different intracellular signaling activated by the respective receptors (for review see Florio & Schettini 1996 and Bajetto *et al.* 2001a). For example, an opposite regulation of ERK1/2 activity was reported for CXCR4 (activation; Bajetto *et al.* 2001b) and the somatostatin receptors (inhibition; Massa *et al.* 2004).

To evaluate the relevance of these effects for pituitary functioning, we compared them with those induced by the known physiological pituitary regulator GHRH. Interestingly, we observed that the maximal proliferation induced by GHRH, was of the same magnitude as that obtained by SDF1 (Table 1).

Table 1 Effect of SDF1 on both GH secretion and proliferation of GH4C1 cells: comparison with GHRH-induced effects and antagonism by pertussis toxin or somatostatin pretreatment

	GH secretion (% of basal)	DNA synthesis (% of basal)
SDF1 (12.5 nM)	+110	+98
SDF1 + pertussis toxin	+6	+12
SDF1 + somatostatin (100 nM)	+13	+38
GHRH (300 nM)	+126	+85

Data are expressed as percentage of respective basal values and are derived from Florio *et al.* (2006), except the somatostatin data that are unpublished observations from F Diana and T Florio.

Very recently, we reported some preliminary results showing the expression of SDF1 and CXCR4 in human pituitary adenoma postsurgical specimens (Bajetto *et al.* 2006b). When confirmed these data will surely contribute to the establishment of a role for this chemokine in pituitary tumor functioning.

Intracellular mechanisms involved in SDF1 regulation of GH4C1 pituitary adenoma cell proliferation and GH release

We used GH4C1 cells to evaluate the intracellular mechanisms activated by SDF1 to induce hormone release and cell proliferation (Florio *et al.* 2006). We analyzed a set of intracellular mediators that were previously involved in CXCR4 effects, namely the regulation of the intracellular Ca⁺⁺ concentration, the activation of ERK1/2 and of the cytosolic Ca⁺⁺-dependent tyrosine kinase, Pyk2, and the stimulation of the large conductance, Ca⁺⁺-activated K⁺ channels BK_{Ca}. Interestingly, all these systems were activated following SDF1 treatment of GH4C1 cells.

Thus, using selective inhibitors on these intracellular pathways, we tried to establish a molecular ordering between the SDF1-activated second messengers. We used the cell permeable Ca⁺⁺ chelator BAPTA-AM to abolish the intracellular Ca⁺⁺ rise induced by the chemokine, the MEK inhibitor PD98059 to revert the activation of ERK1/2, salicylate to counteract Pyk2 activation, and TEA to prevent BK_{Ca} channel activation. We found that the SDF1-dependent regulation of intracellular Ca⁺⁺ was independent of the activation of ERK1/2, Pyk2 or BK_{Ca} (i.e. it was not inhibited by PD98059, salicylate or TEA) and that ERK1/2 activation lay on an independent pathway (it was not affected by BAPTA-AM, salicylate or TEA). Conversely, Pyk2 activation was Ca⁺⁺ dependent (it was blocked by BAPTA-AM, but not by PD98059 or TEA) and BK_{Ca} channel activity was dependent on both the SDF1-induced intracellular Ca⁺⁺ rise (it was inhibited by BAPTA-AM) and Pyk2 activation (inhibition by salicylate).

Thus, in GH4C1 cells two independent pathways activated by SDF1 were identified: the first one is Ca⁺⁺-independent and causes the activation of ERK1/2, likely through the βγ subunit of Gi, as reported for many G-protein-coupled receptors, and the second one is Ca⁺⁺-dependent involving the sequential activation of Pyk2 and BK_{Ca}.

Using the same pharmacological approach, we tried to establish which of these pathways was involved in the control of GH release and which controls the cell proliferation induced by SDF1.

Interestingly, we found that the SDF1-induced GH release was a solely Ca⁺⁺-dependent process, since it

Figure 1 Schematic representation of the signal transduction pathways activated by SDF1 in the GH4C1 pituitary adenoma cell line. SDF1 treatment is a powerful regulator of both cell proliferation and hormone release (Florino *et al.* 2006). SDF1-induced proliferation signaling occurs through two signaling pathways; the first one is Ca^{++} -independent and involves the ERK1/2 MAP kinase cascade, likely through the interaction of the $\beta\gamma$ complex with the GTPase Ras that mediates the sequential phosphorylation of the kinases Raf, MEK1/2 and ERK1/2. The second one is Ca^{++} -dependent: the chemokine-induced Ca^{++} increase activates Pyk2, which, in turn, regulates the BK_{Ca} channel activation. Conversely, GH secretion is a purely Ca^{++} -dependent process: chemokine receptor causes the activation of PLC, which leads to IP3 formation and Ca^{++} release from internal stores. Abbreviations: α - β - γ subunits of G-proteins; MEK1/2, MAP kinase kinase; ERK1/2, extracellular signal-regulated kinase 1 and 2; Pyk2, cytosolic Ca^{++} -dependent tyrosine kinase 2; PLC, phospholipase C; IP3, inositol 1,4,5-trisphosphate; BK_{Ca} , large conductance Ca^{++} -activated K^{+} channels.

was not abolished by the inhibitors of ERK1/2, Pyk2 or BK_{Ca} , but only by the Ca^{++} chelator BAPTA-AM.

On the other hand, the regulation of GH4C1 cells proliferation induced by this chemokine was dependent on both the Ca^{++} -independent stimulation of ERK1/2 activity and the Ca^{++} -dependent activation of Pyk2 and BK_{Ca} . Importantly, each of the inhibitory compounds tested completely reverted the proliferative stimuli of SDF1, indicating that all these intracellular second messengers (Ca^{++} , Pyk2, BK_{Ca} and ERK1/2) are necessary for such an effect. The schematic representation of intracellular pathways activated by SDF1 in GH4C1 cells is depicted in Fig. 1.

Conclusions

In this review, we analyze the potential role of chemokines and, in particular, of SDF1 as a novel pituitary growth factor. Although the role of growth factors in pituitary adenoma development and of chemokines (and SDF1, in particular) in the control the proliferation of many different tumor histotypes is well recognized, a possible chemokine regulation of

pituitary function has never been addressed in a systematic way. We suggest that SDF1, coming from the systemic circulation or possibly released from hypothalamic neurons, may reach CXCR4 expressing cells to regulate pituitary function (Fig. 2).

Although previous work reported the localization of chemokine receptors (CXCR2 and 4) at the pituitary level their ligands (IL-8/GRO and SDF1) in hypothalamic neurons, their possible role in pituitary adenoma development was never studied. Recently, we showed that SDF1 is a powerful mitogen and

Figure 2 Possible mechanisms by which SDF1 can control the pituitary function and, possibly, stimulate pituitary adenoma cell proliferation. SDF1 can control pituitary cells function acting at different levels: (1) SDF1, released by hypothalamic neurons, may act as a novel hypophysiostrophic factor. This regulation may occur either by influencing the neuronal cells to produce hypothalamic releasing hormones, that control the anterior pituitary cells functions, or by being released by hypothalamic neurons and reaching via the pituitary portal system CXCR4-expressing pituitary cells (since most of the SDF1 releasing neurons project to the posterior hypophysis, dotted arrow indicates a less likely occurrence of such pathway of stimulation); (2) SDF1, coming from the systemic circulation can reach the pituitary, enters into the capillary net of the anterior lobe of the pituitary gland and interacts with pituitary cells that express CXCR4; (3) moreover, during pituitary adenoma development, SDF1 could be produced by anterior pituitary cells and, through an autocrine/paracrine process may stimulate adenohypophyseal tumor-initiated cells to proliferate and to hypersecrete specific hormones. This pathway may represent a promoting factor for pituitary adenoma development.

secretagogue for the pituitary adenoma cell line GH4C1, and characterized the intracellular mechanisms involved in these effects. In particular, we demonstrated that a complex interplay of a multiplicity of second messengers was responsible for such regulation of pituitary adenoma cells. This observation may imply that the adenoma development could somehow be favored by the activation of an autocrine/paracrine stimulation of cell proliferation and hormone secretion induced by the adenomatous cells expressing the chemokine/receptor pair (Fig. 2). Importantly, similar data were also observed for the other chemokine IL-8, suggesting that the chemokine-nergic system may represent a novel pleiotropic regulator of pituitary function. Although the expression of SDF1 and CXCR4 was also identified in normal rat pituitary, the relevance of this chemokine in normal tissue represents an issue that needs to be addressed in the near future, especially as far as the evaluation of the level of expression in normal versus tumor tissue and whether normal and/or tumor pituitary cells co-express both the chemokine and its receptor, as observed for IL-8. Notwithstanding, these data are opening a very important new research area in the field of pituitary regulation that may lead to the identification of important new pharmacological targets (i.e. antagonists of CXCR4, such as the compound AMD3100 or newer derivatives Yan *et al.* 2006) for the therapy of pituitary adenomas.

Acknowledgements

This work was supported by an Italian Association for Cancer Research grant to T.F. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Received in final form 8 November 2006

Accepted 4 December 2006

Made available online as an Accepted Preprint 28 December 2006