Functional expression of CXCR4 in somatotrophs: CXCL12 activates GH gene, GH production and secretion, and cellular proliferation

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Abstract

The interaction of chemokine (C–X–C motif) ligand 12 (CXCL12) and its receptor CXCR4 may play an important role in the regulation of anterior pituitary function. In this study, we investigated the expression of CXCL12 and CXCR4 and their role in normal rat pituitary and GH–producing GH3 tumor cell line. RT-PCR analysis and immunohistochemistry revealed that CXCR4 was expressed in normal rat anterior pituitary and GH3 tumor cells. Double immunofluorescent staining showed the complete colocalization of CXCR4 with GH in rat pituitary, indicating that CXCR4 is specifically expressed in rat somatotrophs. Using rat primary pituitary cell cultures and GH releasing hormone receptor expressing stable GH3 cells (GH3-GHRHR), we evaluated the function of CXCL12 compared with GHRH.

CXCL12 stimulated *GH* gene activation in both primary rat anterior pituitary cells and GH3-GHRHR cells. CXCL12 also stimulated GH secretion from primary rat pituitary cells in a dose-dependant manner. BrdU incorporation was increased in response to CXCL12 addition in GH3 cell culture, indicating CXCL12-induced cell proliferation. CXCL12-dependent phosphorylation of ERK1/2 was also confirmed by western blot analysis, supporting the evidence that MAPK is an intracellular mediator of CXCL12/CXCR4 interaction in GH3 cell proliferation. In conclusion, these results indicate that CXCL12/CXCR4 interaction plays an important role in GH production, secretion, and the proliferation of somatotrophs.

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Introduction

The neural and endocrine systems are sophisticatedly connected through small molecules such as neurotransmitters, neuropeptides, and cytokines including chemokines. Chemokines, or 'chemotactic cytokines,' are small secreted proteins (6–10 kDa) that have the ability to dictate the migration and activation of leukocytes (Bajetto et al. 2001). Recently, several reports have suggested that some chemokines and their receptors are expressed in the brain as well as inflammatory sites and might function as neurotransmitters or neuromodulators in the central nervous system (Callewaere et al. 2007, Rostene & Buckingham 2007). Chemokine (C–X–C motif) ligand 12 (CXCL12/CXCL12) was originally cloned from a murine bone marrow stromal cell line (Tashiro et al. 1993), and its amino acid sequences are highly conserved during evolution, implying that this substance might play an important biological role. CXCL12/CXCR4 interaction has been shown to be involved in the embryologic development of the vascular, cardiac, hematopoietic, and central nervous systems (Ma et al. 1998, Tachibana et al. 1998, Stumm et al. 2003), and is associated with tumor cell proliferation or migration (Hall & Korach 2003, Bajetto et al. 2006, 2007).

After CXCL12 binding sites were discovered in the adult rat pituitary gland by autoradiographic assay using I[131] labeled CXCL12 (Banisadr et al. 2000), a group demonstrated that CXCL12 stimulated cell proliferation and growth hormone (GH) secretion in the rat pituitary adenoma-derived cell line GH4C1 (Florio et al. 2006, Massa et al. 2006), suggesting that interaction between CXCL12 and CXCR4 regulates the function of the anterior pituitary gland. Furthermore, it has recently been reported that CXCL12 and CXCR4 are expressed in the posterior pituitary neurons and their interaction is important for regulating arginine vasopressin (AVP) secretion (Callewaere et al. 2006). In this study, we demonstrated the colocalization of CXCR4 with rat pituitary somatotroph and investigated the role of CXCL12/CXCR4 interaction on the secretion and production of GH and cell proliferation in both normal rat pituitary and rat pituitary somatotroph tumor GH3 cells.

Materials and Methods

RNA isolation and RT-PCR for Cxcl12 *and* Cxcr4

Expression of *Cxcl12* mRNA and *Cxcr4* mRNA was analyzed by RT-PCR using total RNA isolated from rat pituitary tissues and GH3 cells. RNA was extracted using TRIZOL reagent (Invitrogen Corp.) as described by the manufacturer's instructions. 20 μg RNA were treated with RNase-free DNase-I (QIAGEN Korea Ltd) for 10 min at 25 °C. Random hexamers were used to synthesize single-stranded cDNA using 2 μg DNase-I treated RNA in a 20 μl reaction volume containing 50 mM of Tris–HCl, pH 8·3, 8 mM MgCl$_2$, 50 mM NaCl, 1 mM dithiothreitol, 1 mM dNTPs, 22 U RNase inhibitor, and 10 U moloney murine leukemia virus (MMLV) RT (Promega) for 60 min at 37 °C. A portion (1/40) of cDNA solution was treated with 2·5 U Taq DNA polymerase (Promega Corp.) for amplification of CXCL12, CXCR4, and GAPDH as a control. Cycle conditions were: 2 min hot start at 96 °C, followed by 35 cycles of 1 min at 94 °C, 45 s at 55 °C, followed by 1 min at 72 °C, and extension at 72 °C for 15 min. An aliquot (20%) of each PCR product was resolved by electrophoresis on 1·5% agarose gels and DNA products were visualized with ethidium bromide. Oligonucleotides used for PCR amplification were: CXCL12, forward 5′-CAT GGA CGC CAA GGT CGT-3′ and reverse 5′-TCC AGG TAC TCT TGG ATC CA-3′; CXCR4, forward 5′-CAA GCA AGG ATG TGA GTT CG-3′ and reverse 5′-GCT GGA GTG AAA ACT TGA GG-3′; GAPDH (390 bp), forward 5′-CCA AAG TTG TCA TGG ATG AC-3′ and reverse 5′-CCC TTC ATT GAC CTC AAC TA-3′.

Immunohistochemistry and immunofluorescence

Immunohistochemical study for the expression of CXCL12 and CXCR4 was performed using paraffin-embedded pituitary glands of Sprague–Dawley rats. 5 μm sections of the pituitary were prepared. After deparaffinization and hydration of the slides, peroxidase quenching was performed with 3% hydrogen peroxide in PBS for 10 min. After preincubation with serum blocking solution (Zymed Laboratories Inc., San Francisco, CA, USA) containing 5% horse serum, specimens were incubated with mouse monoclonal anti CXCR4 (5 μg/ml, sc-12764, Santa Cruz Biotechnology Inc., Burlingame, CA, USA) or anti-CXCL12 antibody (10 μg/ml, R&D System Inc., Minneapolis, MN, USA), which are compatible to human and rat species for 3 h at room temperature. After washing the slides with tris-buffered saline/0·025% Tween, biotinylated horse anti-mouse immunoglobulin (5 μg/ml, Vector Laboratories, Inc., Burlingame, CA, USA) and streptavidin-peroxidase conjugate (Vector Laboratories) were added sequently. DAB (diaminobenzidene; DAKO Corp., Carpinteria, CA, USA) was used as a chromogen. We also tested other anti-CXCR4 antibodies originated from goat (N-terminus specific, sc-6279, Santa Cruz) that can detect CXCR4 of the human, rat, and mouse.

Immunoglobulin G originated from mouse and goat was used as negative controls against anti-CXCR4 antibody. We used two different tissue controls including the posterior lobe for positive control and adrenocorticotrophin (ACTH)-secreting human pituitary tumor for negative control. CXCR4 was detected in the posterior lobe (Fig. 1C), whereas this was not detected in ACTH secreting pituitary tumor tissues (data not shown).

Double immunofluorescent staining was performed to colocalize CXCR4 with pituitary hormones including GH and prolactin (PRL). After preincubation with serum blocking solution (10% donkey serum), rabbit polyclonal anti-human GH (1:50; Zymed Laboratories) and goat polyclonal anti-PRL (2 μg/ml 1:100; Santa Cruz Biotechnology) were used to detect somatotrophs and lactotrophs, respectively. Anti-CXCR4 antibody was used as described above. After incubation for 3 h with the primary antibodies, staining was performed using fluorescent labeled secondary antibodies (1:100, Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) including fluorescein isothiocyanate (FITC) donkey anti-mouse immunoglobulin, FITC donkey anti-goat immunoglobulin, Texas Red donkey anti-rabbit immunoglobulin, and Texas Red donkey anti-goat immunoglobulin. To minimize autofluorescence, slides were incubated in 70% ethanol supplemented with 0·1% sudan black B for 15 min and washed vigorously in running water. Fluorescent mounting media (Vector Laboratories) containing 4′,6′ diamidino-2 phenylindol was used for nuclei-counterstaining and mounting.

To detect the expression of CXCR4 or GHRHR in GH3-GHRHR cells, cells were plated on fibronectin-coated cover slips and washed with PBS twice, then fixed with 4% paraformaldehyde in PBS (pH 7·4) for 5 min. The slides were incubated with rabbit anti-human GHRH (hGHRH) receptor (1:1000; provided by Dr Bruce Gaylinn, University of Virginia, Charlottesville, VA, USA) or mouse monoclonal anti-CXCR4 at room temperature for 1 h. After washing, staining was performed using biotinylated secondary antibodies (Vector Laboratories), and streptavidin-FITC (1:100; Vector Laboratories). Images were obtained using a Zeiss microscope (Axioskop, Carl Zeiss Inc., Oberkochen, Germany).

Establishment of stable GH3 cells expressing GHRHR

GH3 cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and grown in Dulbecco's Modified Eagle Medium (DMEM)/Ham's F-12 containing 10% FBS. All media were supplemented with 100 U/ml penicillin and 100 mg/ml streptomycin, and maintained at 37 °C with 5% CO$_2$. Stable GH3-GHRHR were selected. Briefly, GH3 cells at 70% confluency were transfected with pCDNA3 carrying hGHRHR cDNA (provided by Kelly E Mayo, Northwestern University, Chicago, IL, USA) using low-serum Opti-MEM (Invitrogen Corp.) and lipofectamine 2000 (Invitrogen Corp.) for 4–5 h. The transfected cells were washed and incubated in DMEM/Ham's F-12 medium
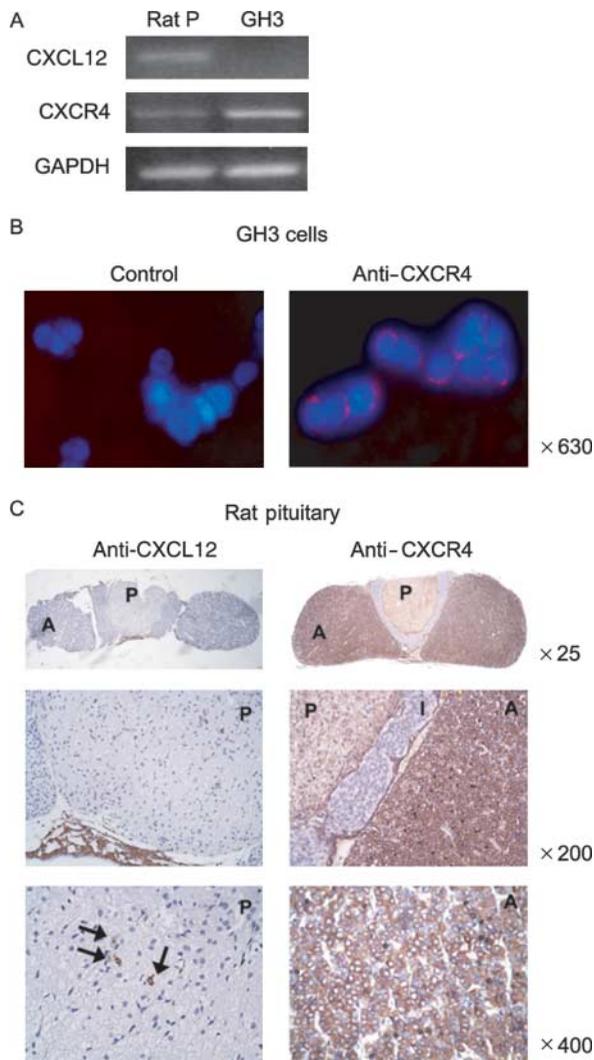
Figure 1 Expression of CXCL12 and CXCR4 in rat pituitary and GH3 cells. A) RT-PCR analyses of *Cxcl12* and *Cxcr4* mRNA in rat pituitary and GH3 cells were performed. Amplification of GAPDH mRNA was used as a control. Rat P, pituitary glands of Sprague–Dawley rats; GH3, GH producing GH3 pituitary adenoma cells. B) Immunofluorescent assay using anti-CXCR4 antibody (stained with red) was performed on GH3 cells. Control indicates GH3 cells incubated with mouse IgG for primary antibody (left). Nuclei were counterstained with DAPI. Magnification, $630\times$. C) Immunohisto-chemical analyses of CXCL12 and CXCR4 in the pituitary of Sprague–Dawley rats. The left column shows the distribution of CXCL12-positive cells and the right column shows the localization of CXCR4-expressing cells. CXCL12 immunostaining was only observed in the posterior lobe of the pituitary (arrows in the bottom panel of left column) and remnants of neural tissues (stained in dark brown in the second picture of left column). A strong CXCR4 immunostaining is observed in rat pituitary tissue, particularly in the anterior pituitary cells. No CXCL12- or CXCR4-positive cells were detected in the intermediate lobe of pituitary glands. A, anterior pituitary; P, posterior pituitary; I, intermediate lobe of rat pituitary. Magnification, top panels, $25\times$; middle panels, $200\times$; bottom panels, $400\times$.

(GIBCO BRL) containing 300 µg/ml of Geneticin (G418, Life Technologies). GH3-GHRHR cells that grew into monoclones were selected from the media and transferred to other plates. Each selected monoclone was harvested and the expression of GHRHR was confirmed by RT-PCR, western blot analysis, and immunofluorescent staining.

Western blot analysis

GH3-GHRHR cells in 10 cm plates were lysed in radio-immunoprecipitation assay (RIPA) buffer (20 mM Tris, 2 mM EDTA, 150 mM NaCl, 0·5% Triton X-100, 5% phosphatase inhibitors, 0·5% protease inhibitors) and protein content was measured using Coomassie (Bradford, UK) Protein Assay Kit (Pierce, Rockford, IL, USA). Equal amounts of protein (10 µg) were heat denatured in 2X sample buffer (2% SDS, 62·5 mM of Tris, pH 6·8, 0·01% bromphenol blue, 1·43 mM mercaptoethanol, and 0·1% glycerol), separated on 10% SDS-polyacrylamide gel, transferred onto polyvinylidene difluoride (PVDF) mem-brane (Bio-Rad), and blotted with the appropriate antibodies: anti-hGHRH receptor antibody, p44/42 MAPK (ERK1/2) antibody, and phosphor-p44/42 MAPK antibody (Cell Signaling Technology Inc). Detection of immunocomplexes was performed by enhanced chemiluminescence system (Cell Signaling Technology Inc., Danvers, MA, USA).

Luciferase assay

To determine whether CXCL12 is involved in GH-gene promoter activity, GH3-GHRHR cells were seeded in 12-well plates at a density of 5×10^5 cells/well, then plasmids containing the human GH promoter ($-610, +58$ from transcription start site; Lee & Jameson 2002) luciferase constructs were transfected using lipofectamine 2000 according to the manufacturer's instructions. The transfected cells were treated with SDF1β ($1·8\times10^{-8}$ M, Human/Feline CXCL12/SDF-1β, 96% identical with rat, R&D Systems, Inc.), hGHRH (1–29; 10^{-9} M, Sigma–Aldrich Inc.), and somatostatin (10^{-7} M, Sigma–Aldrich) in serum-free medium for 16 h, then lysed for assay of luciferase activities (Luciferase Assay System, Promega). Relative light units were determined using MicroLumat LB96PEG&G luminometer (Berthold Technologies, Bad Wildbad, Germany). Luciferase activity was normalized as described previously (Ishikawa *et al*. 2004).

Real-time PCR

To assess the level of gene expression, real-time RT-PCR was carried out on the experimental samples using ABI 7300 Sequence Detection System (Perkin–Elmer Applied Bio-systems, Foster City, CA, USA). The synthesized cDNAs, obtained by the method described above, were further amplified in triplicate by PCR using SYBR Green I as fluorescent dye and $1\times$PCR Master Mix (Takara Bio Inc., Tokyo, Japan) containing 300 nmol of forward and reverse primers in a final

volume of 25 µl. *GAPDH* served as a reference gene used for normalization of *GH* mRNA level. The gene-specific primers used for *GH* amplification were: forward 5′- GCT GCG TTC TGC TTC TCA G-3′ and reverse 5′- CCG AGG TAC CAA ACA TCA G-3′. PCR cycling conditions were: initial denaturation and enzyme activation at 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 15 s, and extension at 72 °C for 1 min. Calculation of relative expression levels of the target mRNAs was conducted based on the cycle threshold (CT) method (Higuchi *et al.* 1993). The CT for each sample was calculated using ABI 7300 Real-time PCR detection system software with an automatic fluorescence threshold setting.

Primary rat pituitary cell cultures and measurement of GH

Anterior pituitary glands from male Sprague–Dawley rats (250–300 g, 8 weeks old, Orient Bio Inc., Kyoungki, Korea) were obtained after careful removal of the posterior pituitary described previously (Colin & Jameson 1998) and dispersed by enzymatic digestion for 15 min in 0·125% trypsin (Worthington Biochemical Corp., Lakewood, NJ, USA)/ PBS solution containing no calcium and magnesium ions with shaking. Ten U/ml of DNAse-I (Sigma–Aldrich) were added to the solution and incubated for 2 min. Cells were treated with 0·125% collagenase type IV (Sigma–Aldrich) for 5 min and filtered and washed with DMEM (Hyclone, UT, USA), then plated in 12-well culture plates in a mixture of DMEM (Hyclone) supplemented with 10% FBS, 100 U/ml of penicillin, and 100 mg/ml of streptomycin for 48 h.

Rat anterior pituitary cells were maintained in serum-free DMEM for 1 h before the experiment. Then, serum-free DMEM containing SDF1β (18 nM), GHRH (1 nM), or both CXCL12 and GHRH was replaced, aliquots were collected at 30, 60, and 120 min, and stored at −70 °C. The levels of GH in culture medium were measured using a rat GH RIA kit (LINCO Research, Inc., St Charles, MO, USA).

Animal experiment and protocol were approved by Institutional Animal Care and Use Committees of Yonsei University, College of Medicine.

BrdU incorporation analysis of GH3 cells

GH3-GHRHR cells were plated at 5×10^4/well in 96-well plates, serum starved for 48 h before being treated with SDF1β for 16 h, and in the last 2 h, cells were incorporated with BrdU. Cell proliferation was measured by BrdU incorporation assay according to the manufacturer's protocol (Chemicon International, Temecula, CA, USA).

Statistical analyses

Data are presented as the mean ± S.D. The statistical significance of differences was analyzed by Student's *t*-test for comparison between control and experiment groups. $P < 0.05$ was considered statistically significant. Statistical analyses were performed using SPSS for Windows software (version 12.0; SPSS, Chicago, IL, USA).

Results

Expression of CXCL12 and CXCR4 in rat pituitary and GH3 cells

To evaluate the putative role of the CXCL12/CXCR4 system in the function of pituitary, expression of chemokine receptor CXCR4 and its natural ligand CXCL12 was studied in the pituitary gland of Sprague–Dawley rats and GH3 cells. RT-PCR analysis showed that *Cxcr4* mRNA was detected in both rat pituitary and GH3 cells whereas *Cxcl12* mRNA was expressed only in rat pituitary tissues that contained neurons of the posterior pituitary. Immunofluorescent staining showed that CXCR4 was localized along the membrane of GH3 cells (Fig. 1B), while CXCL12 was not detected (data not shown). Immunohistochemical staining of rat pituitary glands revealed that a number of cells were stained with anti-CXCL12 and anti-CXCR4 antibodies in the posterior pituitary whereas CXCL12 positive cells were not observed in the anterior and intermediate lobe. A large number of cells in the anterior and posterior lobe were stained with anti-CXCR4 antibody, whereas the intermediate lobe was not stained at all. The intensity of positivity in the anterior pituitary is stronger compared with that in the posterior pituitary (Fig. 1C). Using different antibody and control IgGs originating from mouse and goat, the same results were obtained (data not shown).

Colocalization of CXCR4 with GH in rat pituitary

Because a large number of anterior pituitary cells were positive for CXCR4 by immunohistochemical staining, we investigated whether they were somatotroph or lactotroph. Double immunofluorescent staining revealed that CXCR4 expression was strictly colocalized with GH whereas PRL was not colocalized at all (Fig. 2). This finding suggests that CXCR4 is specifically expressed in the somatotroph of the anterior pituitary.

Activation of GH gene by Cxcl12 in GH3-GHRHR and primary rat pituitary cells

To investigate the role of CXCR4/CXCL12 interaction on *GH* gene activation in primary rat pituitary cell cultures and GH3 in comparison with GHRH and somatostatin, which are major regulators of production and secretion of the *GH* in the somatotroph, we examined the activity of the *GH* promoter by luciferase assay and the expression of GH mRNA by real-time PCR. Because GH3 cells do not possess receptors for GHRH (Zeytin *et al.* 1984), stably transfected GH3 cells with hGHRHR (GH3-GHRHR) cells were established. RT-PCR and western blot analysis were performed to assess hGHRHR expression in 4 clones
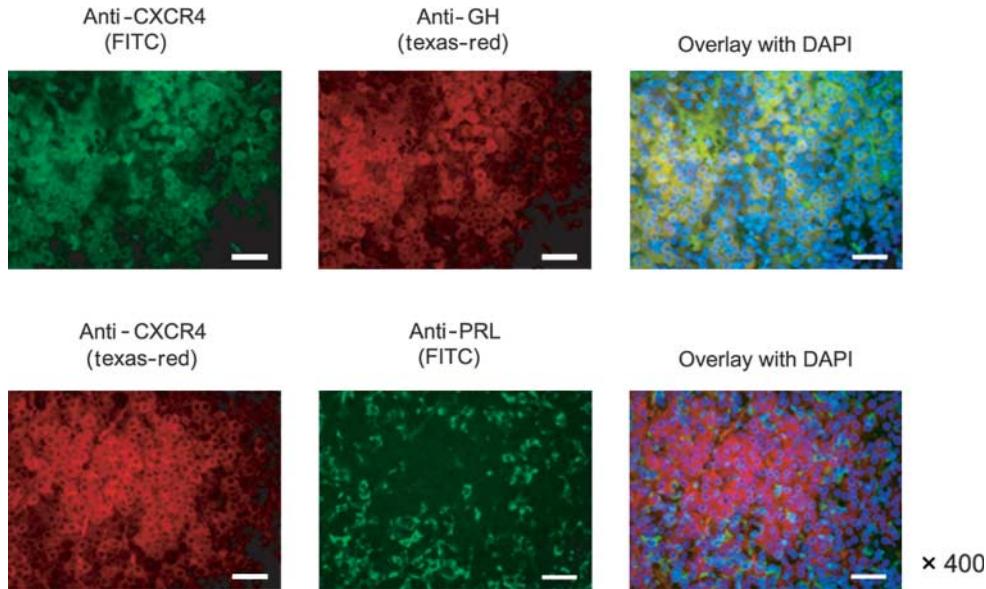
Figure 2 Colocalization of CXCR4 with GH in rat anterior pituitary. Double immunofluorescent staining of CXCR4 with GH or PRL was performed in the rat anterior pituitary. CXCR4 positivity (FITC) strictly colocalizes with GH-positive somatotroph (Texas-red) in the anterior pituitary (upper panel). CXCR4-positive cells (Texas Red) were not colocalized with PRL positive lactotroph (FITC). Magnification, upper and lower panels, 400×; scale bar, 50 μm.

obtained. An hGHRH receptor-specific band of ∼52 kDa was detected in GH3-GHRHR cells and an additional band of higher molecular weight was also observed, presumably corresponding to a different form of glycosylated hGHRHR (Fig. 3A; Lee *et al.* 2001). Immunofluorescent staining showed that expression of hGHRH receptor was readily detected in membranes of GH3-GHRHR cells (clone 4; Fig. 3B). Experiments were then performed using clone 4.

The activity of *GH* promoter in GH3–hGHRHR cells was measured by luciferase assay to determine whether CXCR4/CXCL12 signaling stimulates the *GH* promoter. SDF1β (18 nM) and GHRH (1 nM) increased luciferase activity (about 85 and 20% increments respectively; Fig. 4A). Interestingly, addition of both ligands increased luciferase activity slightly. Luciferase activity was declined in somato-statin-treated cells compared with the control group, implying an inhibitory effect of somatostatin on *GH* transcription. These findings indicate that SDF1β and GHRH increase *GH* promoter activity in GH3-GHRHR cells.

Real-time PCR analysis was performed to evaluate the effect of SDF1β on *GH* mRNA expression in GH3-GHRHR cells and primary rat anterior pituitary cells in culture. SDF1β stimulated *GH* mRNA expression in both GH3-GHRHR cells (2-fold) and primary rat anterior pituitary cells (3-fold; Fig. 4B and C). GHRH addition also stimulated *GH* mRNA expression (1·5-fold) in both cells. The activated levels (1·5–2·0-fold) of *GH* mRNA by the addition of both ligands were similar to those by GHRH addition.
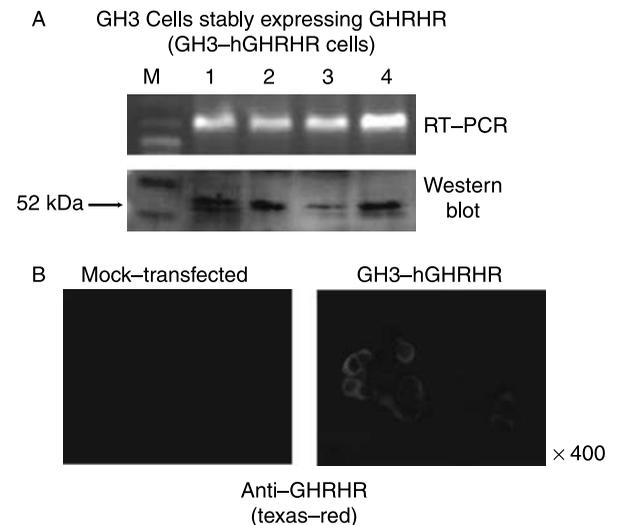

Figure 3 Expression of GHRHR in GH3-GHRHR cells. A) RT-PCR (upper panel) and western blot analysis (lower panel) of the hGHRH receptor in stably transfected GH3 cells with hGHRHR (GH3-GHRHR), and *hGHRH* receptor mRNA was detected at ∼300 bps. Lanes 1–4 indicate the different clones of GH3-GHRHR cells. B) Immunofluorescent localization of the hGHRH receptor. Immuno-fluorescent staining of GH3-hGHRHR cells (clone 4) was performed using rabbit anti-hGHRHR receptor antibody (stained with red). Left, Mock-transfected GH3 cells; right, GH3-GHRHR cells (clone 4). Magnification, 400×.

Effect of CXCL12 on GH secretion in primary rat anterior pituitary cells

To investigate whether CXCL12 stimulates GH secretion in primary rat anterior pituitary cells, measurement of GH in culture media was performed after treatment of SDF1β or GHRH (Fig. 5). The posterior pituitary was carefully removed before culturing to minimize the effect of endogenous CXCL12 secreted from the posterior pituitary.
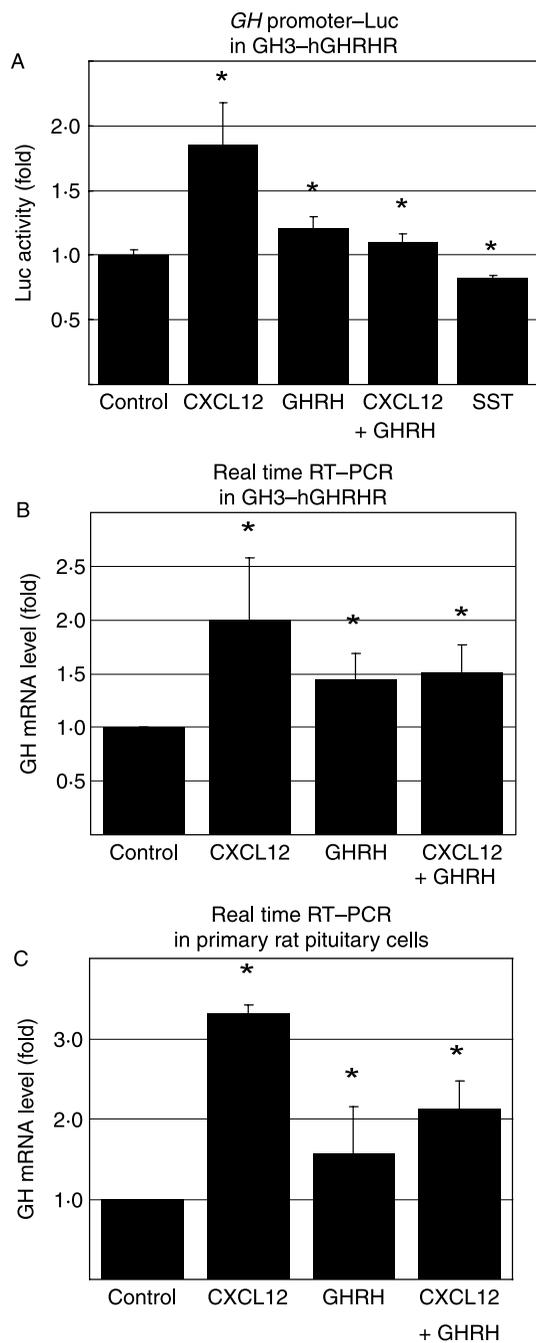
SDF1β significantly stimulated the secretion of GH at 30 and 60 min up to 2·5–3·5-fold compared with the control. GH was also increased after GHRH alone (2-fold) or co-treatment (3·0-fold) of SDF1β and GHRH.

CXCL12 stimulation of GH3 cell proliferation through ERK1/2 pathway

Proliferation of GH3-GHRHR cells was examined by BrdU incorporation assay after treatment of SDF1β, GHRH, or somatostatin for 16 h (Fig. 6A). GH3-GHRHR cells were maintained in serum-free medium to avoid a possible proliferative effect of various growth factors. As previously reported GHRH has a mitogenic effect on GH3 cells expressing GHRH receptors (Lee *et al*. 2001), SDF1β and GHRH significantly stimulated the proliferation of GH3-GHRHR cells by 30% compared with the control whereas somatostatin reduced cell proliferation slightly.

To identify the possible intracellular signaling pathways involved in proliferation of GH3 cells, western blot analysis was performed to evaluate the effect of SDF1β on phosphorylation of ERK1/2. Phosphorylation of ERK1/2 occurred in GH3 cells 5 min after addition of SDF1β. This result is similar to that of a previous report using GH4C1 cells (Massa *et al*. 2006).

Discussion

Chemokines are proinflammatory chemoattractant cytokines that play fundamental roles in the immune system by trafficking and regulating different types of cells related to inflammation and development. Among more than 50 different chemokines, CXCL12 and its exclusive receptor CXCR4 have been intensively studied in the fields of neurology and oncology. Interactions between CXCL12 and CXCR4 have various effects on cell proliferation, anti-apoptosis, tumor vascularization, and cell migration (metastasis; Kryczek *et al*. 2007). In addition, CXCL12 is reported to be a key molecule involved in developing the central nervous

Figure 4 Effect of CXCL12 on activation of *GH* promoter and *GH* mRNA. A) Transcriptional activities of GH promoter in GH3-GHRHR cells stimulated by various substances were evaluated using luciferase reporter system. GH3-GHRHR cells were transiently transfected with plasmids containing GH promoter-luciferase constructs. Cells (5×10^5 cells/well) were treated with SDF1β (18 nM), GHRH (1 nM), and somatostatin (100 nM) in serum-free medium for 16 hr, lysed, and relative luciferase activities (RLA) were calculated. *$P < 0.05$ as compared with control. B, C) Relative *GH* mRNA expression in GH3-GHRHR cells (B) or rat anterior pituitary cells (C) following SDF1β and GHRH administration. Real-time RT-PCR was performed at 1 h after treatment with SDF1β (18 nM) or GHRH (1 nM). GAPDH served as a reference gene used for normalization of GH mRNA level. *$P < 0.05$ as compared with control. Results (A–C) were averaged from three independent experiments and plotted as mean ± S.D. for triplicate wells.

GH secretion from
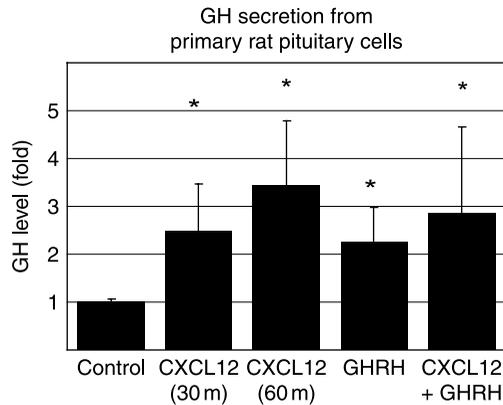primary rat pituitary cells

GH3–GHRHR cells

A

Figure 5 Effect of CXCL12 on GH secretion in rat anterior pituitary cells. The level of GH secreted from rat anterior pituitary cells treated with SDF1β (18 nM), GHRH (1 nM), or both was measured at 60 min by rat GH RIA. GH level was also measured at 30 min after treatment of SDF1β. Results were averaged from 3 independent experiments and plotted as mean±S.D. for triplicate wells. *P<0·05 as compared with control.

system and modulating neuronal functions in the hippocampus, cerebellum, and hypothalamus (Lazarini *et al.* 2003).

However, few studies to date have investigated the possible role of CXCL12/CXCR4 signal in the pituitary. Since low densities of CXCL12 binding sites were detected in rat pituitary gland (Banisadr *et al.* 2000), Florio *et al.* only reported that SDF1α induces cell proliferation and GH secretion in the rat pituitary adenoma-derived cell line GH4C1 (Florio *et al.* 2006, Massa *et al.* 2006). The GH4C1 cell line, established from GH3 cells that produce PRL and large amounts of GH, expresses low basal levels of GH but higher PRL levels (Tashjian *et al.* 1968, 1970). According to the evidence that GH4C1 cell responses to IGF1 were opposite to those of GH3 cells or primary rat pituitary cells (Castillo & Aranda 1997), GH3 cells may be more similar to normal somatotrophs in characteristics compared with GH4C1 cells. Our experiments were conducted using primary rat pituitary cells and GH3 cells, which are known as valuable sources for studies of hormonal secretory physiology and signal transduction pathways (Lee *et al.* 2001).

Expression of CXCL12 and CXCR4 was determined in the pituitary of Sprague–Dawley rats and GH3 pituitary tumor cell lines in our study. In contrast to the finding that rat pituitary cells produced *Cxcl12* and *Cxcr4* mRNA, only 7-transmembrane receptor CXCR4 was localized along membranes of GH3 cells. Immunostaining showed that CXCL12- and CXCR4-positive cells were observed in the posterior pituitary glands, which contained neurons projected from hypothalamic nuclei, and was consistent with a previous report (Callewaere *et al.* 2006). However, in our results, the anterior pituitary was strongly stained with anti-CXCR4 antibodies. The anterior pituitary consists of various types of endocrine cells. Among them, somatotrophs and lactotrophs are 2 major cell components. However, it is notable that only somatotrophs possess receptors for CXCL12, confirmed by
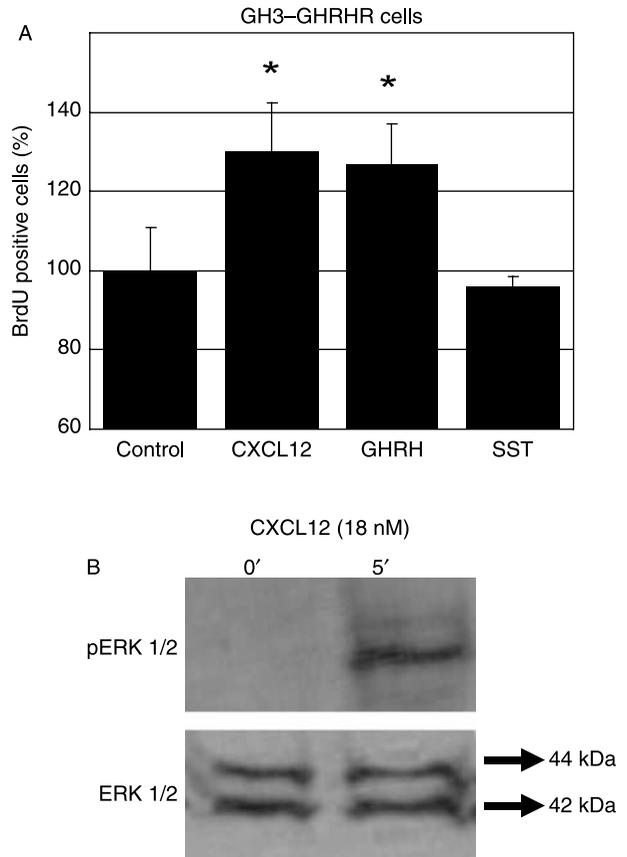
CXCL12 (18 nM)

B

Figure 6 Effect of CXCL12 on cell proliferation of GH3-GHRHR cells. A) Proliferation of GH3-GHRHR cells was assessed by BrdU incorporation assay. BrdU-positive cells were detected by ELISA kit and represented as a relative percentage compared with the control group. SDF1β (18 nM) and GHRH (1 nM) increased cell proliferation by 30 and 27% respectively, whereas somatostatin reduced cell proliferation slightly. Results were averaged from three independent experiments and plotted as mean±S.D. for triplicate wells. *P<0·05 when compared with control. B) Activation of ERK1/2 by CXCL12 in GH3-GHRHR cells. Western blot analysis was performed to examine phosphorylation of ERK1/2 using a specific anti-phosphorylated ERK 1/2 antibody after CXCL12 treatment for 5 min. Upper panel, phosphor-ERK1/2; lower panel, total form of ERK 1/2.

double immunofluorescent staining. This result was demonstrated for the first time in the rat pituitary gland. This result suggests that CXCR4 could play a role in normal physiological function of the somatotroph.

In our study, SDF1β increased transcription of *GH* mRNA by activating GH promoter in GH3 cells expressing GHRHR. There are two splice forms of CXCL12, designated as SDF1α and SDF1β, which are identical in the first 89 amino acid (AA) sequences, but SDF1β has 4 additional AA at the C-terminus (Tashiro *et al.* 1993, Shirozu *et al.* 1995). The first 17AA, which are important to the binding ability of CXCL12 to CXCR4 (Loetscher *et al.* 1998,

Doranz *et al.* 1999), are identical in both isoforms while SDF1α is more sensitive to proteolysis by serum CD26/ dipeptidyl peptidase than SDF1β (De La Luz Sierra *et al.* 2004), implying the differences in functions or regulatory roles *in vivo*. Furthermore, SDF1β is extensively expressed throughout the rat brain, especially in cerebral endothelial cells, while SDF1α is localized in specific neuronal structures such as cingulate (Stumm *et al.* 2002). In the present experiments, we chose SDF1β, which showed about a 2-fold potency, compared with SDF1α (Crump *et al.* 1997), and are generally distributed over entire brain area.

We observed that the activation of *GH* promoter or *GH* mRNA expression by SDF1β in GH3–GHRHR cells was higher than by GHRH. This difference might be due to the relatively low concentration of GHRH (1 nM) compared with the relatively higher concentration of SDF1β (18 nM) although we used 1 nM GHRH before (Lee *et al.* 2001). Barbieri *et al.* (2007) showed that similar levels of GH increases were achieved using 12·5 nM of SDF1α or 300 nM of GHRH in GH4C1 cells. It is intriguing to predict the actual concentrations of chemokine CXCL12 and hormone GHRH in the microenvironment of the pituitary *in vivo*.

We examined the effects of SDF1β on synthesis and secretion of GH in primary rat anterior pituitary cells for the first time. Although anterior pituitary glands consist of heterogeneous groups of endocrine cells, only somatotrophs in the anterior pituitary expressed the receptor CXCR4, according to double immunofluorescent studies (Fig. 2). After the administration of SDF1β, transcription of *GH* mRNA and release of GH were significantly increased compared with those induced by GHRH. Higher GH secretion was maintained up to 2 h by time-dependent manner after treatment (data at 120 min not shown). Interestingly, no synergistic or additive effects on GH secretion or *GH* mRNA transcription were observed after administration of both SDF1β and GHRH, suggesting that CXCL12/CXCR4 signaling and GHRH-mediated pathway may possess the same intracellular mediators or interfere with each other. After GHRH binds to Gs protein-coupled GHRHR in the membrane of somatotrophs, activated adenylyl cyclase produces intracellular cAMP, followed by secretion of GH. Although SDF1β induces G inhibitory protein-linked reduction in concentrations of cAMP in cytoplasm (Zheng *et al.* 1999, Peng *et al.* 2004, Florio *et al.* 2006), increased intracellular calcium levels stimulated by SDF1β may be related to GH release. Further study is needed to clarify this mechanism.

We demonstrated the mitogenic effect of SDF1β in GH3 cells using BrdU incorporation assay. GH3 cell proliferation was significantly increased after treatment with SDF1β, which induced phosphorylation of ERK1/2. Ca^{++}–independent activation of ERK1/2 and Ca^{++}–dependent stimulation of cytosolic tyrosine kinase, Pyk2, were reported to be important SDF1α-related intracellular signaling pathways that mediate growth of pituitary GH4C1 cells (Florio *et al.* 2006, Massa *et al.* 2006). Furthermore, CXCL12 increases the percentage of cells in the S phase and inhibits tumor cell apoptosis by activation of NF-κB (Helbig *et al.* 2003, Kryczek *et al.* 2007). Based on

evidence that various kinds of tumor cell proliferations are associated with CXCL12 (Scotton *et al.* 2002, Barbero *et al.* 2003, Hall & Korach 2003), development of the pituitary tumor may be related to CXCL12-mediated intracellular pathways. Pituitary tumors are known to derive from the transformed single pituicyte in which initiating mutations of candidate genes such as *Ras* or *Gsp* have caused a gain of proliferative functions, followed by clonal expansion stimulated by promoting factors such as locally produced growth factors and cytokines (Faglia & Spada 2001, Asa & Ezzat 2002).

It has been demonstrated that neurons from hypothalamic nuclei and posterior pituitary produce CXCL12, which could modulate AVP secretion induced by autocrine mechanism (Callewaere *et al.* 2006, 2007). From our immunohisto-chemical analyses showing that anterior pituitary glands did not express CXCL12 but CXCR4, the function of anterior pituitary cells might be controlled by external CXCL12 originating from hypothalamic neurons or systemic circulation via blood vessels (Barbieri *et al.* 2007). Disruption of these putative pathways related to CXCL12/CXCR4 interactions might play a role in the development of pituitary adenoma. Further studies are required to prove this hypothesis.

In conclusion, we investigated the expression of CXCR4 and CXCL12 and their physiological roles in normal rat pituitary cells and GH-producing GH3 tumor cells. CXCL12/CXCR4 interaction plays an important role in the synthesis and release of GH and cell proliferation. These results may suggest that CXCL12/CXCR4 signaling could be a promising target for the treatment of GH-producing pituitary tumors, and should be considered in future studies.

Declaration of interest

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