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CXCR7 agonists inhibit the function of CXCL12 by down-regulation of CXCR4

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ABSTRACT

The CXCL12/CXCR4 axis is involved in many cellular responses for host homeostasis, and malfunction of this signaling pathway is associated with a variety of diseases. It is now known that CXCL12 also binds to another newly identified chemokine receptor, CXCR7, which does not couple with a G-protein. CXCR7 can form homodimers, or heterodimers with CXCR4, and is believed to sequester the chemokine CXCL12, although the CXCL12/CXCR7 axis activates MAP kinases through β-arrestin. Therefore, it has not been well defined how CXCR7 activation affects CXCL12-induced cellular events. To elucidate the function of CXCR7, we prepared CXCR7 agonist Compound 1. Compound 1 is a selective and potent CXCR7 agonist that clearly has the activity to recruit β-arrestin toward CXCR7. It also activates MAP kinases Akt and ERK. Using this compound, we confirmed that the CXCR7 agonist, but not an antagonistic antibody, did inhibit CXCL12 induced HUVEC tube formation, suggesting that activation of CXCR7 ameliorates CXCL12 induced cellular events, probably by affecting on CXCR4 function. We show that β-arrestin recruitment to CXCR4 is reduced by over-expression of CXCR7 and activation of CXCR7 by agonist treatment reduces the protein level of CXCR4. Based on our results, together with reported information, we propose that CXCR7, when up-regulated upon inflammation, can act as a negative regulator of CXCR4 by heterodimerizing with CXCR4, inducing its internalization and degradation. This mechanism suggests that CXCR7 agonists can have a therapeutic effect on CXCL12 causing diseases by countering the effects of CXCL12.

1. Introduction

CXCL12, also called SDF-1 (stromal cell-derived factor-1), is a chemokine known as a critical factor in several diseases including cancer or autoimmune diseases. Accumulating evidence has been implicating CXCL12 in tumor cell metastasis and proliferation [1,2]. In the case of Rheumatoid Arthritis (RA), the expression of CXCL12 is upregulated in the synovial tissue of RA patients compared to that of osteoarthritis patients, and CXCL12 may act to induce leukocyte accumulation, stimulate chondrocytes to release matrix metalloproteinase 9, and enhance angiogenesis in the synovium [3–5]. CXCL12 used to be believed to bind only to receptor CXCR4, but recently CXCR7 has been identified as another receptor for CXCL12 [6,7]. CXCR7 binds with high affinity to CXCL12 and also CXCL11 (ITAC; interferon-inducible T cell α chemoattractant). Unlike classical chemokine receptors, CXCR7 signals through β-arrestin in response to agonists without detectable activation of G-proteins [8,9]. A variety of functions of CXCL12 has been demonstrated as a ligand for CXCR4, but the role of CXCR7 is largely unknown yet. Several reports have suggested that CXCR7 associates with CXCR4 and affects its internalization, or that CXCR7 scavenges CXCL12 resulting in the modulation of CXCR4 activity [10–12]however, the precise mechanism still remains unclear.

Chemical compounds that specifically bind to CXCR7 showed efficacy in several mice models of cancer or autoimmune diseases such as collagen-induced arthritis (CIA) and experimental autoimmune encephalomyelitis (EAE) [7,5,13]. Although these compounds had been originally thought to be CXCR7 antagonists, several studies have shown that they have agonistic activity in terms of CXCR7 dependent β-arrestin recruitment [14]. It has not yet been established how CXCR7 agonists ameliorate the clinical scores of various mouse disease models. In our study, we show that upon binding to CXCR7, CXCR7 agonists reduced the expression level of CXCR4 which resulted in reduction of the cell's sensitivity against CXCL12. As a result, CXCR7 agonists negatively regulate CXCL12–CXCR4 induced cellular events such as angiogenesis.

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2. Materials and methods

2.1. Materials

Tango™ CXCR7-bla U2OS cells, Tango™ CXCR4-bla U2OS cells and LiveBLAzer™ FRET B/G substrate were obtained from Invitrogen (Carlsbad, CA). pORF9-hCXCR7 expression vector was purchased from Invivogen (San Diego, CA). Human CXCL12/SDF-1α recombinant protein, anti-CXCR7 antibody (clone 11G8) and mouse IgG1 isotype control were obtained from R&D Systems, Inc. (Minneapolis, MN). Antibodies for Akt (rabbit polyclonal), pThr308 Akt (rabbit, clone 244F9) and β-actin (rabbit, clone 13E5) were obtained from Cell Signaling Technology Inc. (Beverly, MA). Rabbit polyclonal anti-CXCR4 antibody was purchased from Abcam plc. (Cambridge, UK).

2.2. Beta-lactamase reporter assay (Tango™)

The Tango™ U2OS cell lines (Invitrogen, Carlsbad, CA) were maintained as described [15]. When CXCR7 gene was transfected, the cells were plated at 3×10^5 cells/well in a 6-well-plate and incubated overnight at 37 °C with 5% CO_2. The cells were then transfected with 2 μg of receptor expression plasmids (treated with Ase1 and Ssp1 to cut the ampicillin resistant region beforehand) using FuGENE®6 Transfection Reagent (Promega Corporation, Madison, WI) as directed by the manufacturer's protocol. The details of β-lactamase reporter assay have been described elsewhere [16]. Specifically, the cell lines were exposed to the compound for 30 min prior to treatment with CXCL12 for 5 h at 37 °C with 5% CO_2. The fluorescence emission values at 460 nm and 535 nm were obtained using an Envision plate reader (PerkinElmer Inc., Waltham, MA).

2.3. In vitro tube formation assay

HUVECs were cultured for 4 days in endothelial basal medium (EBM-2) containing growth factors (EGM-2 bullet kit; Lonza, Basel, Switzerland). The medium was then changed to growth factor-free EBM-2 to remove angiogenesis-inducing activities. Growth factor-reduced Matrigel (BD Biosciences, San Jose, CA) was thawed at 4 °C over night, and 120 μl of Matrigel were added to each well of a 48-well plate and incubated for 30 min at 37 °C to polymerize. After 24 h-starvation, the HUVECs were incubated with or without the compound for 15 min and then cultured further on the Matrigel, with recombinant human CXCL12 (100 ng/ml, R&D Systems) added to the wells for 20 h. Cells were photographed using a BIOREVO BZ-9000 microscope equipped with a CCD camera (Keyence Corp., Osaka, Japan). The length of tube-like structures in the images was measured and the relative tube length was calculated as follows: the average length of the tubes per field with stimulation and/or inhibitor divided by the average length of the tubes without stimulation in each experiment.

2.4. Gene transfection

pORF9-hCXCR7 vector was transfected into HEK293FT cells by using FuGENE®6 Transfection Reagent (Promega Corporation) according to the manufacturer's instruction. After 2 days, cells were treated with CXCL12 or Compound 1 for following processes.

2.5. Quantitative RT-PCR

RNA was reverse transcribed using oligo-dT primers. Real time PCR was performed using KAPA SYBR® FAST qPCR Kits (Kapa Biosystems Inc., Woburn, MA). Gene-specific primers for human GAPDH, CXCR4 and CXCR7 were obtained from Takara Bio Inc. (Otsu, Japan).

2.6. Western blotting

Cells were lysed in ice-cold Cell Lysis Buffer (Cell Signaling Technology, Inc.) containing a cocktail of protease inhibitors (Nacalai Tesque Inc., Kyoto, Japan). Proteins were denatured by heating to 100 °C for 5 min in SDS sample buffer, loaded onto and separated by 4–20% gradient SDS polyacrylamide gels (Bio-Rad Laboratories Inc., Hercules, CA), and then transferred electronically to a polyvinylidene fluoride (PVDF) membrane. The membrane was blocked with Block Ace (DS Pharma Biomedical Co., Ltd., Osaka, Japan) for 1 h and then was incubated overnight with the following dilution of primary antibodies: polyclonal anti-Akt (1:1000), monoclonal anti-p-Akt (Thr308) (1:1000), polyclonal anti-CXCR4 (1:500) and monoclonal anti-β-actin (1:1000). The membranes were incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibody at 1:5000 dilution for 1 h at room temperature, and after washes, visualized for immunoreactivity using an Enhanced Chemiluminescence (ECL) System (GE Healthcare UK Ltd., Amersham Place, UK).

3. Results

3.1. The compound specific for CXCR7 activated signal downstream of CXCR7

CXCR7 has only been identified as a chemokine receptor for CXCL12 relatively recently [6,7]and its biology is still largely unknown. We therefore tried to examine how its interaction with CXCL12 and possibly CXCR4 affects cellular events. Compound 1, and its analogs (Compound 2 and 3), are reported to be CXCR7-specific binding compounds, with potency in the low nanomolar range (WO2007/059108, Fig. 1A). To validate the biological activity of the generated compound, we performed SelectScreen® profiling in the Tango™ CXCR7-bla U2OS expression system (Invitrogen, San Diego, CA). These cells express CXCR7 modified to contain a TEV protease site that is linked to an integrated Gal4-VP16 transcription factor. Binding of CXCL12 to CXCR7 and consequent recruitment of β-arrestin leads to cleavage of Gal4-VP16 by the TEV protease tagged with β-arrestin, resulting in detectable β-lactamase activity. Compound 1 strongly induced β-arrestin recruitment to CXCR7 in this system in a dose dependent manner (Fig. 1B and Supplement 1). On the other hand, it failed to inhibit CXCL12-induced β-arrestin recruitment to CXCR7 and to recruit β-arrestin to CXCR4 (data not shown, Fig. 1B). As CXCL12 binding to CXCR7 was reported to activate Akt [17], the effect of Compound 1 on Akt phosphorylation was investigated. As a result, Compound 1 activated the phosphorylation of Akt in HEK293 cells (Fig. 1C). We therefore propose that the compound generated for targeting CXCR7 is a chemical agonist.

3.2. β-arrestin recruitment induced by CXCR7 agonists is required for the inhibition of angiogenesis

As blocking CXCL12 function has been reported to suppress angiogenesis [5], we performed tube formation assay on HUVECs to determine the effect of CXCR7 agonists. HUVECs were incubated with the compound for 15 min and then stimulated with CXCL12 for 20 h, after which tube lengths were measured. Compound 1 showed inhibitory effect on tube formation with high potency (IC50; 0.96 nM, Fig. 2A). Therefore, we suggest that the CXCR7 agonist suppresses CXCL12-induced angiogenesis. Since both CXCL12 and the CXCR7 agonist clearly recruited β-arrestin to CXCR7 (Fig. 1B and Suppl. Fig. S2), we next asked whether β-arrestin recruitment to CXCR7 is necessary for the inhibitory effect on CXCL12

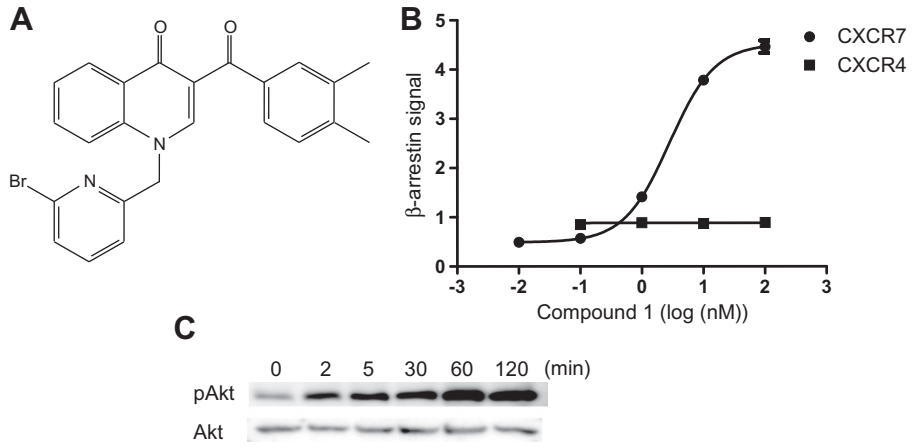

Fig. 1. Generation of Compound 1, an agonist that induces β-arrestin recruitment to CXCR7 and Akt activation. (A) The chemical structure of the CXCR7 agonist, Compound 1. Compound 1 was synthesized internally based on the published patent information from ChemoCentryx. (B) Compound 1 binds to CXCR7 and recruits β-arrestin to CXCR7. Tango™ CXCR7 (filled circles) and CXCR4 (filled squares) were exposed to increasing concentrations of Compound 1. *N* = 3, mean ± SEM. (C) Compound 1 activates Akt in HEK293 cells. HEK293 cells with overexpression of CXCR7 were exposed to Compound 1 for the indicated time and phosphorylated Akt was investigated with Western blotting.

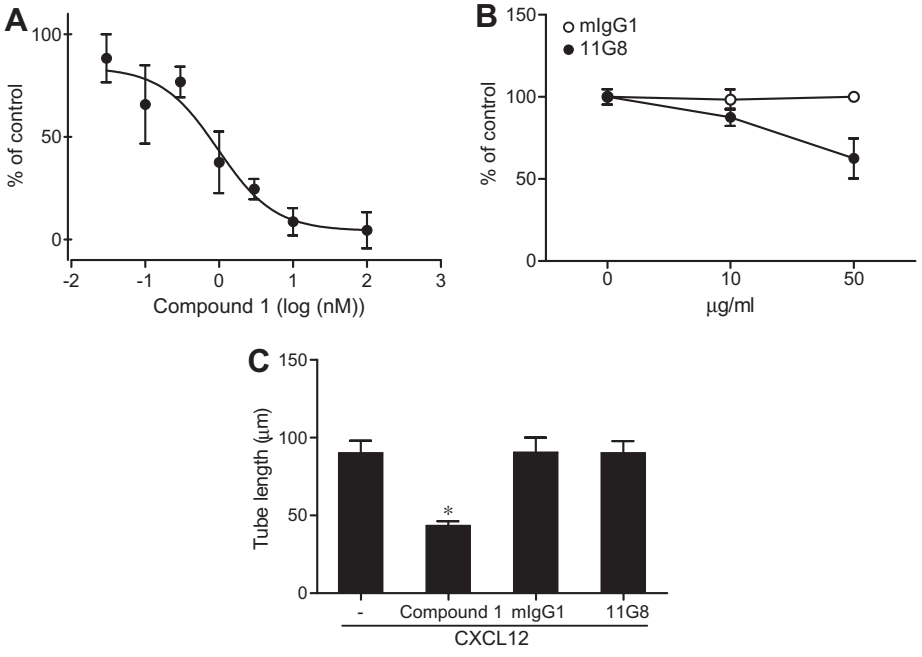

Fig. 2. CXCR7 agonist, but not antagonistic antibody, inhibited CXCL12 induced HUVEC tube formation. (A) Compound 1 inhibited CXCL12-induced angiogenesis. HUVECs were treated with increasing concentrations of Compound 1 for 15 min and seeded on the Matrigel with 100 ng/ml of CXCL12 for 20 h. Data were normalized as % of the tube length of HUVECs cultured without compound (CXCL12 only, 100%). The tube length of control (culture with PBS) was set to 0%. N = 6, mean ± SEM. (B) CXCR7 antibody blocks CXCL12-induced β-arrestin recruitment. The Tango™ CXCR7 cell line was exposed to 100 ng/ml of CXCL12 and anti-CXCR7 antibody or its isotype control (empty circles; mouse IgG, filled circles; anti-CXCR7, clone 11G8). Data are shown as % of control (CXCL12 only). N = 3, mean ± SEM. (C) CXCR7 antibody does not inhibit CXCL12-induced HUVEC tube formation. HUVECs were treated with anti-CXCR7 antibody (11G8) or its isotype control (mIgG1) and seeded on the Matrigel with 100 ng/ml of CXCL12 for 18 h and tube lengths were measured. *N* = 6, mean ± SEM. Compound 1 was used as a positive control. $^{*}P < 0.05$ compared to all the other groups.

signaling, or blocking the CXCL12 binding to CXCR7 is enough for the inhibitory effect. To clarify this question, anti-CXCR7 antibody was tested in the tube formation assay. The anti-CXCR7 antibody (clone 11G8), which has antagonistic activity in the CXCL12-induced β-arrestin recruitment to CXCR7 (Fig. 2B), showed no effect on the CXCL12-induced tube formation in HUVECs (Fig. 2C), demonstrating that the β-arrestin recruitment to CXCR7 is required for the inhibition of angiogenesis. Therefore, it is confirmed that β-arrestin recruitment to CXCR7 induced by the agonist is indispensable for the suppression of CXCL12-induced cellular events.

3.3. CXCR7 is a negative regulator of CXCR4

CXCL12 can bind not only CXCR7 but also CXCR4 and induce β-arrestin recruitment to CXCR4, whereas Compound 1 does not recruit β-arrestin to CXCR4 (Fig. 1B). These results prompted us to hypothesize that CXCR7 negatively regulates CXCR4, and CXCR7 agonists work indirectly as inhibitors of the CXCL12-CXCR4 signal relay. To determine whether CXCR7 suppresses signaling from CXCL12 binding to CXCR4, CXCR7 was overexpressed in CXCR4-bla U2OS cells to observe the effect on β-arrestin recruitment to CXCR4.

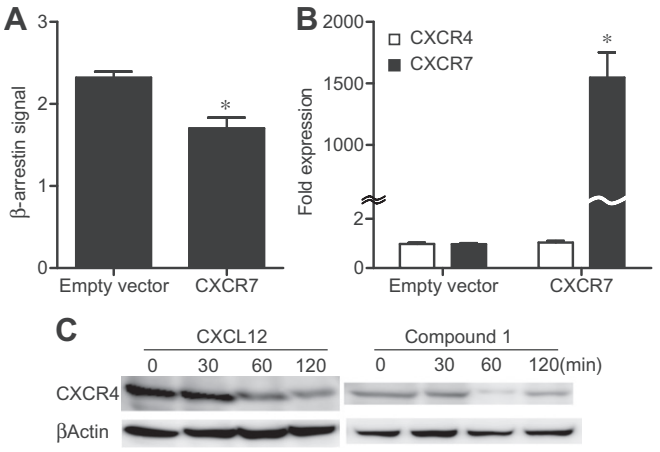

Fig. 3. Stimulation of CXCR7 reduces CXCR4 signaling. (A) The Tango™ CXCR4-bla cell line was transfected with CXCR7 or the empty vector and exposed to 100 ng/ml of CXCL12 for 5 h. $N = 6$, mean ± SEM. $^{*}P < 0.01$ vs. control (empty vector). (B) The mRNA expression level of CXCR4 and CXCR7 after CXCR7 gene transfection into Tango™ CXCR4-bla cells was measured by qRT-PCR. Empty columns show CXCR4 expression and filled columns show CXCR7 expression. Data are fold expression compared to the empty vector-transfected cells. $N = 6$, mean ± SEM. $^{*}P < 0.01$ vs. control (empty vector). (C) CXCR7over-expressed HEK293 cells were treated with CXCL12 or Compound 1 for the indicated time and CXCR4 expression was examined with Western blotting.

Upon CXCR7 overexpression, CXCL12-induced β-arrestin recruitment to CXCR4 was significantly reduced (Fig. 3A), implying that CXCR7 has an inhibiting effect on CXCR4 functions. When CXCR7 was overexpressed, the expression level of CXCR4 mRNA did not change in CXCR4-bla U2OS cells even when an extremely high level of mRNA expression of CXCR7 was induced (Fig. 3B). Since CXCR7 has been reported to be able to affect the expression of CXCR4, it is hypothesized that signal from CXCR7, but not just expression of CXCR7, affects the expression of CXCR4. Therefore the effect of Compound 1 on the expression of CXCR4 was examined. HEK293 cells were transfected with CXCR7-expression plasmid and treated with Compound 1 for 60 min. The protein expression level of CXCR4 was remarkably decreased by Compound 1 treatment and the similar effect was induced by CXCL12 (Fig. 3C). This result indicates that activation of CXCR7 reduces the protein amount of CXCR4.

4. Discussion

The current study provides evidence that CXCR7 agonism reduces the amount of CXCR4 protein and that inhibits CXCL12-induced cellular events. Since the finding that CXCR7 is a receptor for CXCL12 in 2005 [6], many studies on CXCR7 have been published. However, although expression on malignant cells and effects on angiogenesis have been proven, mechanisms of action for this receptor remained unclear. Chemical compounds originally developed by ChemoCentryx as "CXCR7 inhibitors" showed efficacy in models of tumor suppression and arthritis [5,7]. These studies suggest CXCR7 inhibitors may be an interesting intervention point for treating a variety of human diseases. Several consecutive studies, however, have revealed that CCX733, originally synthesized as a CXCR7 antagonist, or its derivatives recruit β-arrestin to CXCR7, suggesting that these compounds work as CXCR7 agonists. Furthermore, whereas CXCR4, another receptor for CXCL12, is expressed ubiquitously, CXCR7 is expressed on a limited number of cell types and the expression is transiently induced by certain stimuli like inflammation. Why the agonists of the transiently-upregulated receptor can reduce the effects of CXCL12 remains to be unclear. CCX733 is highly selective for CXCR7 and does not bind CXCR4, suggesting that a mechanism to indirectly inhibit the CXCL12-CXCR4 axis by CXCR7 agonists exists. In answer of this question, Naumann et al. reported that CXCR7 is a scavenger for CXCL12 and negatively regulates CXCL12 functions [12]. However, since CXCL12 might be released continuously under inflammatory conditions, only scavenging CXCR7 may not be enough to suppress the effect of CXCL12. We therefore hypothesized that there should be an additional mechanism negatively regulating CXCL12 function by CXCR7 agonists. It is documented that the expression of CXCR7 is upregulated by inflammatory cytokines such as IL-1β [5], and that CXCR7 forms heterodimers with CXCR4 and the interaction may regulate inter-receptor relationship [11]. Here we confirmed that CXCR7 itself modulates the function and amount of CXCR4.

CXCR7 does not couple with G-proteins, but interacts with β-arrestin as CXCR4 also does. Our results demonstrated that the CXCL12-induced β-arrestin recruitment to CXCR4 is inhibited by increasing CXCR7 expression, suggesting that CXCR7 affects signaling downstream of CXCR4. Others proved that β-arrestin is involved in receptor internalization [18,19], so it has been suggested that signaling through β-arrestin from activated CXCR7 plays important roles in the receptor recruitment. Indeed, our results showed that the increase of CXCR7 expression by transfection without any agonist stimulation did not influence the CXCR4 expression on mRNA level. CXCR7 agonists transduce the signal from CXCR7 and promote internalization of CXCR4, which forms heterodimers with CXCR7 [11]. It is reported that most of CXCR4 is degraded after internalization, whereas CXCR7 comes back to the cell surface [12]. CXCR4 contains a degradation motif (SSLKILSKGK) in the carboxyl terminus and ubiquitination on the lysine residues [20] triggers its degradation, whereas ubiquitination of CXCR7 is responsible for the correct trafficking of CXCR7 from and to the plasma membrane [21]. By overexpressing CXCR7 in HEK293 cells, which originally express CXCR7 at a low level, we observed that the CXCR7 agonists markedly reduced the protein expression of CXCR4. This observation leads us to propose that CXCR7 actively promotes CXCR4 degradation.

The effects of CXCL12-CXCR4 signal have been well studied, and CXCR4 antagonists are of high clinical interest in the context of mobilization of hematopoietic stem cells and cancer biology, as well as inflammatory diseases. However, CXCL12-CXCR4 signal is also critical for host homeostasis such as normal angiogenesis. As mentioned before, the expression level of CXCR7 is low under normal conditions, and it is up-regulated in tumor cells or under inflammatory conditions. Modulating the effect of CXCL12 by CXCR7 agonists could thus be a therapeutic option for treatment of CXCL12 involving diseases.

Competing interest statement

We have the following interest. Ayako Uto-Konomi, Julia Wirtz, Yayoi Sato, Ai Takano and Shinobu Suzuki are employed by Nippon Boehringer Ingelheim Co., Ltd. and Bryan McKibben is employed by Boehringer-Ingelheim Pharmaceutical, Inc. There are no patents, products in development or marketed products to declare. Other authors have declared that no competing interests exist.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.01.032.

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