To the Editor:

Fibroblast-like synoviocytes (FLS) proliferate in the synovial tissue of patients with rheumatoid arthritis (RA), and contribute to chronic inflammation and the destruction of articular cartilage due to the production of a variety of cytokines, chemokines, and matrix metalloproteinases (MMPs) (1). The expression of CC chemokine ligand 18 (CCL18) was shown to be increased in the RA synovium (2, 3); however, the pathogenic role of CCL18 remains unclear. In this study, we detected the expression of a recently identified receptor for CCL18, phosphatidylinositol transfer membrane-associated phosphatidylinositol transfer protein 3 (PITPNM3) (4) in the RA synovium, and the stimulatory effects of CCL18 on FLS.

Synovial tissue samples were obtained from patients with RA (n=4) and osteoarthritis (OA) (n=4) undergoing joint replacement surgery. RA patients were 65 (48-85) years old [median (range)], with a disease duration of 9 (2.5-30) years and C-reactive protein level of 1.23 (0.68-2.85) mg/dl. All RA patients were positive for rheumatoid factor and anti-citrullinated protein antibodies. All subjects provided informed consent. The experimental protocol was approved in advance by the Ethics Committee of Tokyo Medical and Dental University.

CCL18-positive cells were observed in the synovial lining, sublining, and perivascular regions of the RA synovium (Figure 1A). CCL18 expression was minimal in the OA synovium (Figure 1C). Western blotting analysis showed that CCL18 expression was significantly higher in the RA synovium than in the OA synovium (Figures 1E and 1F). Double-immunofluorescence staining revealed that most $CD68^+$ macrophages expressed CCL18 (Figures 1G-1I), and von Willebrand factor (vWF)-positive vascular endothelial cells were also positive for CCL18 (Figures 1J-1L). These results indicated that CCL18 expression was increased in the RA synovium and macrophages were the source, which is consistent

with previous studies (2, 3). We also found that endothelial cells expressed CCL18 in RA synovial tissue.

We then analyzed and showed the expression of PITPNM3, a receptor for CCL18, in the synovial lining, sublining, and follicle-like aggregates of RA synovial tissue (Figure 1M). PITPNM3 expression was minimal in the OA synovium (Figure 1O). Increased PITPNM3 expression in the RA synovium was confirmed by western blotting (Figures 1Q and 1R). Double-staining showed that $CD68^+$ macrophages and FLS (vimentin-positive fibroblast-like appearance cells) expressed PITPNM3 (Figures 1S-1X), while vWF^+ endothelial cells did not (data not shown).

The expression of PITPNM3 in *in vitro* cultured FLS established from RA and OA synovial tissues (5) was also analyzed by western blotting. PITPNM3 expression in RA FLS was significantly higher than that in OA FLS (Figures 2A and 2B). To analyze the pathogenic role of CCL18 in RA, we examined the stimulatory effect of CCL18 on RA FLS. FLS established from RA synovial tissue were incubated with CCL18. The production of IL-6, CCL2, and MMP-3 was significantly enhanced by stimulation with CCL18 in a dose-dependent manner (Figures 2C-2E). We also analyzed the effect of CCL18 on the proliferation of FLS. Cellular proliferation was not induced by incubation with CCL18 (Figure 2F). Incubation with CCL18 did not alter the motility of FLS as analyzed by the scrape motility assay (data not shown).

CCL18 expression was shown to be increased in synovial tissue (2, 3), synovial fluid (2, 6), and serum in RA, and serum CCL18 levels correlated with disease severity (7). However, the pathogenic role of CCL18 on RA has yet not been revealed, and the receptor for CCL18 has not been identified. In 2011, Chen *et al.* identified PITPNM3 as a functional receptor for CCL18 (4). In this study, we confirmed that CCL18 was expressed by RA synovial macrophages and also found that endothelial cells expressed CCL18. PITPNM3, a

receptor for CCL18, was also highly expressed in the RA synovium by macrophages and FLS. CCL18 enhanced IL-6, CCL2, and MMP-3 production in RA FLS *in vitro*. IL-6 has a wide range of functions on lymphocytes, hepatocytes, hematopoietic progenitor cells, and fibroblasts, and also plays important roles in autoimmune diseases. The blockade of IL-6 signaling by anti-IL-6 receptor mAb is effective in RA. CCL2 may cause monocyte migration into the synovium (8). MMP-3 is thought to contribute to pannus invasion and cartilage degradation (9). Collectively, CCL18 released from synovial macrophages and endothelial cells in the RA synovium may activate FLS and is partly involved in the pathogenesis of RA. CCL18 could induce the migration of T cells, B cells, monocytes/macrophages, and dendritic cells (10). Therefore, CCL18 may also influence inflammatory cell accumulation into the RA synovium.

CCL18 has been shown to induce the phosphorylation of proline-rich tyrosine kinase 2, focal adhesion kinase, and Src family kinase via PITPNM3 in cancer cells (4). Further studies are needed to clarify the important signaling pathways by which CCL18 stimulation of FLS produces inflammatory mediators.

In summary, the interaction of CCL18 and PITPNM3 could play a role in the pathogenesis of RA by activating FLS in the synovium.

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Figure Legends

Figure 1. Expression of CCL18 and PITPNM3 in the RA synovium. Synovial tissues were obtained from four RA (A and B) and four OA patients (C and D), and CCL18 expression was examined by immunohistochemistry. Samples were stained with anti-CCL18 Ab (LifeSpan BioSciences, Seattle, WA) (A and C) or isotype-matched control Ab (B and D). Original magnification x100. CCL18 expression in RA and OA synovial tissues was also analyzed by western blotting with anti-CCL18 Ab and anti-β-actin mAb (Cell Signaling Technology, Danvers, MA). (E). The relative protein expression of CCL18 to β-actin in RA and OA is shown (F). Values are mean±standard error of the mean (SEM). ***$p<0.001$ (Student's *t* test). Sections of RA synovial tissues were double-stained with anti-CD68 (KP1; DakoCytomation, Carpinteria, CA) or -VWF (F8/86; DakoCytomation) mAb, and anti-CCL18 Ab, and were analyzed by fluorescence microscopy as follows: CD68 (G), CCL18 (H), merged image (I) of (G) and (H), VWF (J), CCL18 (K), merged image (L) of (J) and (K). Arrows indicate double-positive cells. Original magnification x400 in (G) - (I), x200 in (J) - (L). The expression of PITPNM3 in RA (M and N) and OA (O and P) synovial tissues was examined by immunohistochemistry. Samples were stained with anti-PITPNM3 Ab (GeneTex, Irvine, CA) (M and O) or isotype-matched control Ab (N and P). Original magnification x100. PITPNM3 expression in RA and OA synovial tissues was determined by western blotting (Q). The relative expression of PITPNM3 to β-actin in RA and OA is shown (R). Values are mean±SEM. *$p<0.05$ (Student's *t* test). RA synovial tissues were double-stained with anti-CD68 or -vimentin (V9; DakoCytomation) mAb, and anti-PITPNM3 Ab. CD68 (S), PITPNM3 (T), merged image (U) of (S) and (T), vimentin (V), PITPNM3 (W), merged image (X) of (V) and (W). Arrows indicate double-positive cells. Original magnification x400.

Figure 2. Expression of PITPNM3 in cultured FLS and the stimulatory effect of CCL18. The expression of PITPNM3 in *in vitro* cultured FLS from RA and OA synovial tissues was determined by western blotting (A). The relative expression of PITPNM3 to β-actin in RA FLS and OA FLS is shown (B). Values are mean±SEM. *$p<0.05$ (Student's *t* test). FLS (2 x 10^3 cells/well) from RA synovial tissue were cultured overnight in a 96-well plate in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum. Then, the medium was replaced with serum free DMEM followed by the addition of the indicated concentration of CCL18 (R&D systems, Minneapolis, MN). After 72 hours, IL-6, CCL2, and MMP-3 concentrations in the culture supernatant were measured by enzyme-linked immunosorbent assay kits (R&D systems) (C: IL-6, D: CCL2, E: MMP-3), and cell proliferation was examined using a cell counting kit (Cell Counting Kit-8; Dojindo, Kumamoto, Japan) (F). Data are presented as means±SEM of one of three independent experiments analyzed in triplicate. *$p<0.05$, **$p<0.01$, ***$p<0.005$ (a one-way ANOVA with Dunnett's multiple comparison test).

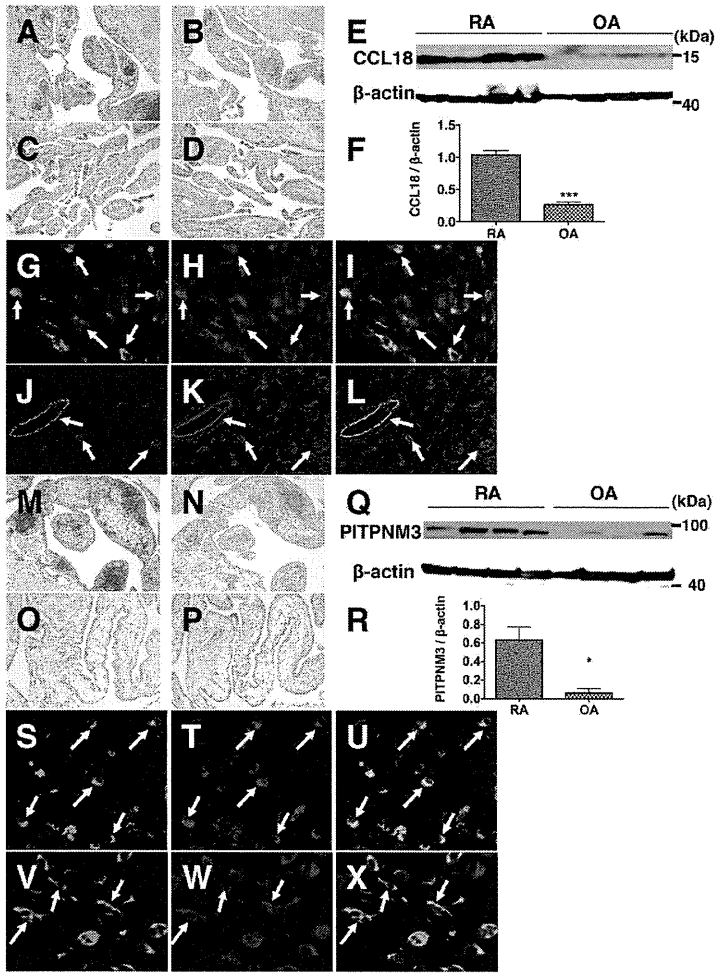

Figure 1

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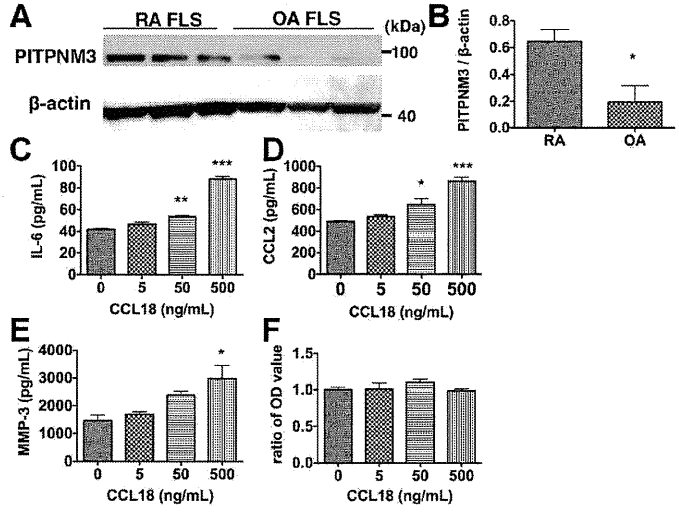

Figure 2

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

Ayako Uto-Konomi [a], Bryan McKibben [b], Julia Wirtz [a], Yayoi Sato [a], Ai Takano [a], Toshihiro Nanki [c], Shinobu Suzuki [a,*]

[a] Department of Molecular & Cellular Biology (MCB), Kobe Pharma Research Institute, Nippon Boehringer Ingelheim Co., Ltd., 6-7-5, Minatojima-Minamimachi Chuo-ku, Kobe, Hyogo 650-0047, Japan
[b] Department of Medicinal Chemistry, Boehringer-Ingelheim Pharmaceutical, Inc., 900 Ridgebury Road, Ridgefield, CT 06877, USA
[c] Department of Medicine and Rheumatology, Graduate School, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan

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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.

* Corresponding author. Fax: +81 78 306 1434.
E-mail address: ars2000hs@gmail.com (S. Suzuki).

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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

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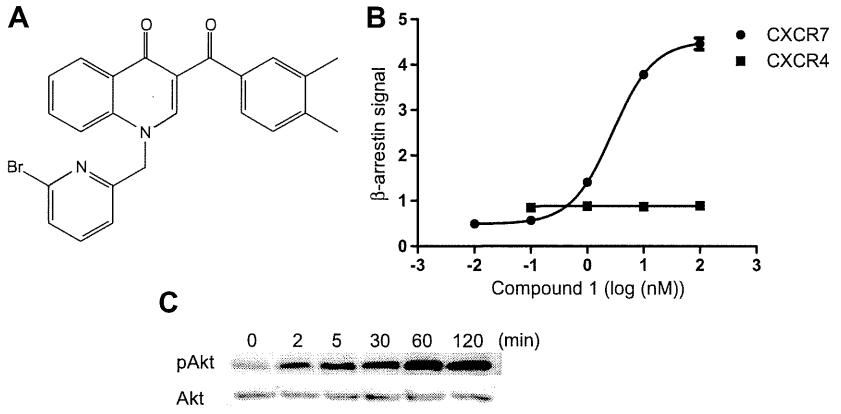

Fig. 1. Generation of Compound 1, an agonist that induces β-arrestin recruitment to CXCR7 and Akt activation. (A) The chemical structure of the CXCR7 agonist, Compound 1. Compound 1 was synthesized internally based on the published patent information from ChemoCentryx. (B) Compound 1 binds to CXCR7 and 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.

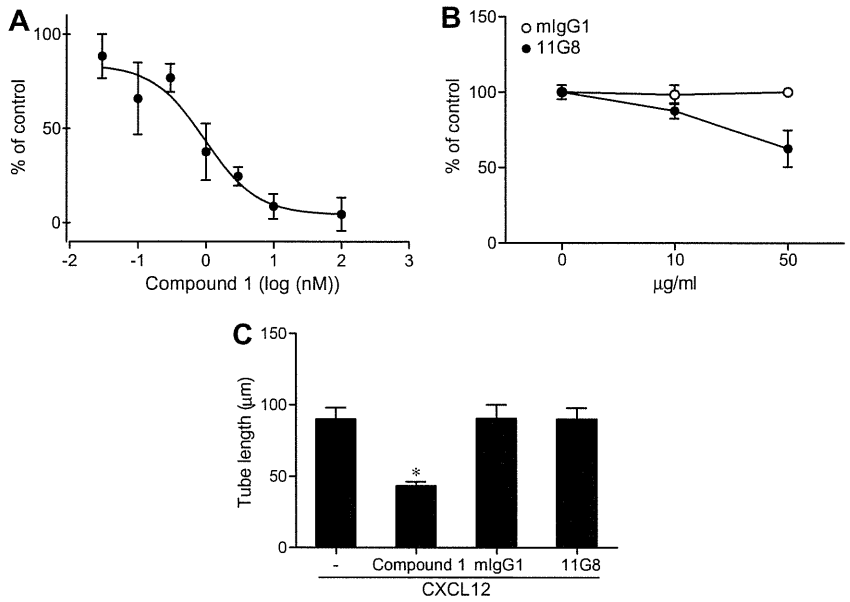

Fig. 2. CXCR7 agonist, but not antagonistic antibody, inhibited CXCL12 induced HUVEC tube formation. (A) Compound 1 inhibited CXCL12-induced angiogenesis. HUVECs were treated with increasing concentrations of Compound 1 for 15 min and seeded on the Matrigel with 100 ng/ml of CXCL12 for 20 h. Data were 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.

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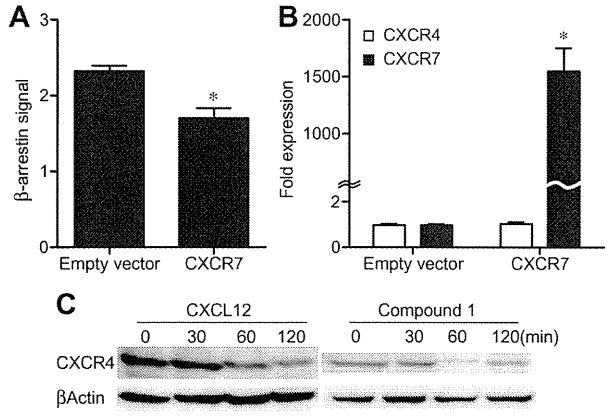

Fig. 3. Stimulation of CXCR7 reduces CXCR4 signaling. (A) The Tango™ CXCR4-bla cell line was transfected with CXCR7 or the empty vector and exposed to 100 ng/ml of CXCL12 for 5 h. $N = 6$, mean ± SEM. $^*P < 0.01$ vs. control (empty vector). (B) The mRNA expression level of CXCR4 and CXCR7 after CXCR7 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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LETTER

Suppression of elevations in serum C reactive protein levels by anti-IL-6 autoantibodies in two patients with severe bacterial infections

Serum C reactive protein (CRP) is generally elevated by infection. We encountered two patients with severe bacterial infections without increases in CRP. Patient 1: A 67-year-old man developed thoracic empyema by *Escherichia coli* and *Streptococcus intermedius*. His body temperature was 36.6°C, his leukocyte count was elevated (9960/μl) and he was negative for CRP (0.01 mg/dl). Despite antibiotic treatment, he died of respiratory failure. Patient 2: A 56-year-old woman developed multiple subcutaneous abscesses by *Staphylococcus aureus*. She had rheumatoid arthritis (RA) for 30 years and was treated with sodium aurothiomalate. Her body temperature was 37.4°C, and leukocyte count (5600/μl) and CRP (0.05 mg/dl) were not elevated. She recovered with antibiotics. Neither patient had a past history of severe bacterial infection.

Since serum CRP is mainly controlled by interleukin (IL)-6,[1] the lack of IL-6 function was suggested. Serum IL-6 was not detected by ELISA in either patient, although it is known to increase with severe infection.[2 3] However, IL-6 production from peripheral blood monocytes with/without lipopolysaccharide stimulation was similar between Patient 1 and healthy controls (data not shown). Soluble IL-6 receptor (IL-6R) and IL-6 signal transducer (IL-6ST) serum levels were also similar between patients and controls (figure 1A,B).

Based on the clinical course and above findings, we hypothesised that patients' sera contain anti-IL-6 antibodies. Figure 1C showed that both patients' sera, but not controls, contained immunoglobulin (Ig)G against IL-6. The presence of anti-IL-6 antibodies was confirmed by western blotting (figure 1D). Isotypes of the autoantibodies of Patients 1 and 2 were IgG4 and IgG1,

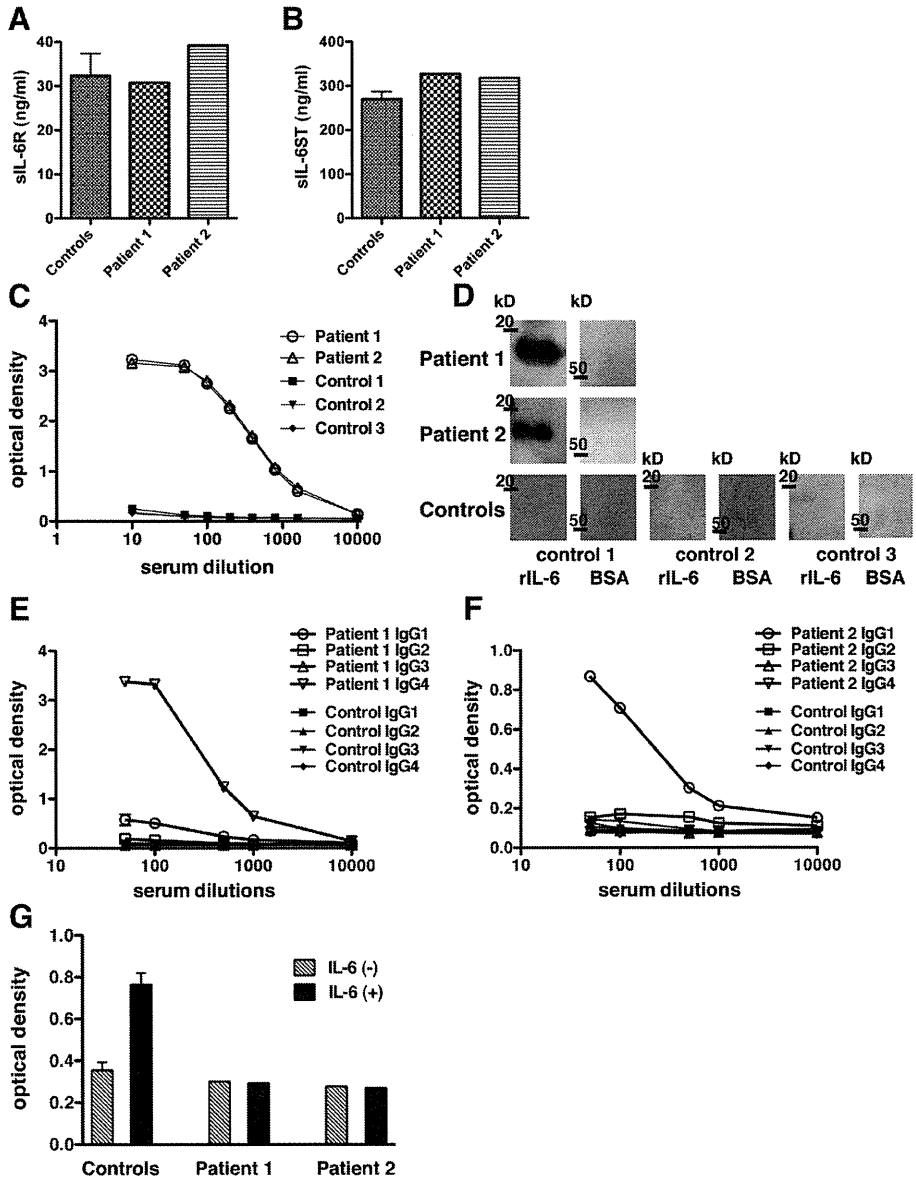

Figure 1 Serum sIL-6R and sIL-6ST levels and the detection of anti-IL-6 autoantibodies in the sera of the two patients. Serum sIL-6R (A) and sIL-6ST (B) levels in three healthy controls and in Patients 1 and 2 were measured using ELISA kits (sIL-6R: Life Technologies, Grand Island, New York, USA; sIL-6ST: R&D Systems, Minneapolis, Minnesota, USA). The ELISA plate was coated with recombinant IL-6 (rIL-6), and then diluted serum samples from three healthy controls and Patients 1 and 2 were incubated on these plates. To measure the titre of anti-IL-6 IgG, horseradish peroxidase (HRP)-conjugated mouse antihuman IgG monoclonal antibody (mAb) was added, 3,3′, 5,5′-tetramethylbenzidine single solution was reacted, and absorbance at 450 nm was measured (C). Control bovine serum albumin (BSA) and rIL-6 were separated on a sodium dodecyl sulphate-polyacrylamide gel. Proteins were then electrotransferred to a polyvinylidene fluoride microporous membrane in a semidry system. Immunoblots were incubated with serum diluted with phosphate-buffered saline (1:200) from three control subjects and Patients 1 and 2. The membrane was incubated with HRP-conjugated mouse antihuman IgG mAb (D). To determine the isotype of anti-IL-6 IgG from Patients 1 and 2, HRP-conjugated mouse antihuman IgG1, IgG2, IgG3 or IgG4 mAb was used for the ELISA (E: Patient 1; F: Patient 2). TF-1 cells were cultured with rIL-6 (5 ng/ml) in a medium containing 10% serum from three healthy controls, Patient 1 or Patient 2 for 3 days. The number of cells was counted with a cell counting kit and reported as optical density units (G). Control data are mean±SD.

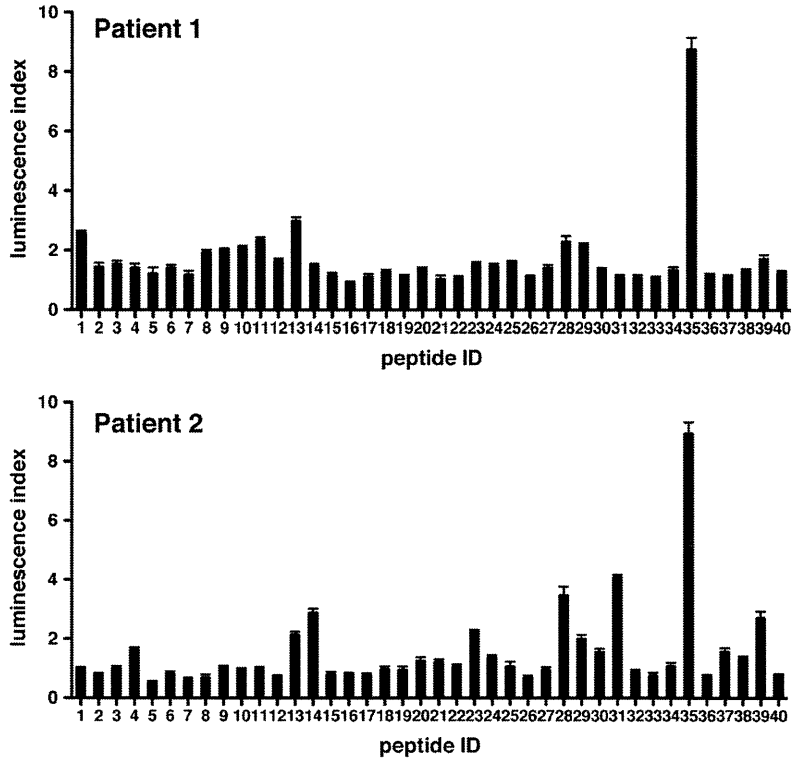

Figure 2 Epitope mapping of anti-IL-6 autoantibodies. N-terminal biotinylated 15-mer peptides overlapping by 10 amino acid residues were generated from the human IL-6 protein sequence, and these peptides were coated on ELISA plates. Serum samples from Patients 1 and 2 were added to these plates, and peptide-bound antibodies were detected. Data are presented as the luminescence index (defined as the luminescence signal with the subtracted peptide-specific background, divided by the sample- and dilution-specific background) measured for each peptide/sample combination. Scores for the 1:200 and 1:400 dilutions are presented as mean ±SD.

respectively (figure 1E,F). The patients' sera suppressed the proliferation of TF-1 cells stimulated with recombinant IL-6 (figure 1G).

These results suggest that anti-IL-6 autoantibodies block IL-6 signalling and inhibit increases in serum CRP. Puel *et al*[4] reported a young boy with repeated cellulitis at 11 and 29 months of age. Serum CRP was negative and anti-IL-6 autoantibodies were detected. Although this case is similar to our patients, to our knowledge, ours are the first reported adult patients negative for CRP despite severe bacterial infections due to anti-IL-6 autoantibodies. Galle *et al*[5] detected anti-IL-6 autoantibodies in three of 4230 healthy subjects. Serum CRP in Patient 2 with RA was occasionally positive until 4 years ago, suggesting that anti-IL-6 autoantibodies developed during that time. The infection may have triggered autoantibody production; however, we could not analyse autoantibodies before infection or after recovery.

IL-6 has important immune functions.[6] Treatment with anti-IL-6R mAb for RA increases the risk of infection such as pneumonia and cellulitis.[7 8] Therefore, patients with anti-IL-6 autoantibodies are immunocompromised and special care for infection should be exercised.

We determined the epitope of anti-IL-6 autoantibodies. Both patients' sera strongly bound to peptide 35, LTKLQAQNQWL QDMT (figure 2), suggesting that this peptide includes the epitope of anti-IL-6 antibodies. This peptide includes a critical amino acid, tryptophan[157], for the binding of IL-6 and IL-6ST to form a dimer of trimers composed of IL-6, IL-6R and IL-6ST.[9]

In conclusion, we detected anti-IL-6 autoantibodies in two patients with severe bacterial infections without elevations in serum CRP.

Toshihiro Nanki,[1,2] Ikumi Onoue,[1,3] Kenji Nagasaka,[4] Aiko Takayasu,[1,3] Masashi Ebisawa,[1] Tadashi Hosoya,[1] Toshizumi Shirai,[5] Takahiko Sugihara,[6] Shinya Hirata,[1] Tetsuo Kubota,[1,3] Masayoshi Harigai,[1,2] Nobuyuki Miyasaka[1]

[1]Department of Medicine and Rheumatology, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, Tokyo, Japan
[2]Department of Pharmacovigilance, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, Tokyo, Japan
[3]Section of Microbiology and Immunology, Graduate School of Health Care Sciences, Tokyo Medical and Dental University, Tokyo, Japan
[4]Department of Rheumatology, Ome Municipal General Hospital, Tokyo, Japan
[5]Department of Cardio-Thoracic Surgery, Ome Municipal General Hospital, Tokyo, Japan
[6]Department of Medicine and Rheumatology, Tokyo Metropolitan Geriatric Hospital, Tokyo, Japan

Correspondence to Dr Toshihiro Nanki, Department of Medicine and Rheumatology, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519 Japan; nanki.rheu@tmd.ac.jp

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Contributors All authors fulfil the authorship criteria. Contributors not fulfilling the authorship criteria are listed in the acknowledgement section.

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ORIGINAL ARTICLE

Clinical characteristics and risk factors for *Pneumocystis jirovecii* pneumonia in patients with rheumatoid arthritis receiving adalimumab: a retrospective review and case–control study of 17 patients

Kaori Watanabe · Ryoko Sakai · Ryuji Koike · Fumikazu Sakai · Haruhito Sugiyama · Michi Tanaka · Yukiko Komano · Yuji Akiyama · Toshihide Mimura · Motohide Kaneko · Hitoshi Tokuda · Takenobu Iso · Mitsuru Motegi · Kei Ikeda · Hiroshi Nakajima · Hirofumi Taki · Tetsuo Kubota · Hirotaka Kodama · Shoji Sugii · Takashi Kuroiwa · Yasushi Nawata · Kazuko Shiozawa · Atsushi Ogata · Shigemasa Sawada · Yoshihiro Matsukawa · Takahiro Okazaki · Masaya Mukai · Mitsuhiro Iwahashi · Kazuyoshi Saito · Yoshiya Tanaka · Toshihiro Nanki · Nobuyuki Miyasaka · Masayoshi Harigai

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Abstract
Objectives To investigate the clinical characteristics and risk factors of *Pneumocystis jirovecii* pneumonia (PCP) in rheumatoid arthritis (RA) patients treated with adalimumab.

Methods We conducted a multicenter, retrospective, case–control study to compare RA patients treated with adalimumab with and without PCP. Data from 17 RA patients who were diagnosed with PCP and from 89 RA

K. Watanabe · R. Sakai · R. Koike · M. Tanaka · T. Nanki · M. Harigai (✉)
Department of Pharmacovigilance, Graduate School of Medical and Dental Sciences, Tokyo Medical Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan
e-mail: mharigai.mpha@tmd.ac.jp

K. Watanabe · R. Sakai · R. Koike · M. Tanaka · Y. Komano · T. Nanki · N. Miyasaka · M. Harigai
Department of Medicine and Rheumatology, Graduate School of Medical and Dental Sciences, Tokyo Medical Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan

R. Koike
Clinical Research Center, Tokyo Medical Dental University Hospital, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan

F. Sakai
Department of Diagnostic Radiology, International Medical Center, Saitama Medical University, 1397-1 Yamane, Hidaka, Saitama 350-1298, Japan

H. Sugiyama
Department of Pulmonary Medicine, National Center for Global Health and Medicine, 1-21-1Toyama, Shinjuku-ku, Tokyo 162-8655, Japan

Y. Akiyama · T. Mimura
Department of Rheumatology and Applied Immunology, Faculty of Medicine, Saitama Medical University, 38 Morohongou, Moroyamamachi, Irumagun, Saitama 350-0495, Japan

M. Kaneko
Kaneko Clinic, 305 Nishiaraijyuku, Kawaguchi, Saitama 333-0083, Japan

H. Tokuda
Department of Respiratory Medicine, Social Insurance Central General Hospital, 3-22-1 Hyakunin-cho, Shinjyuku-ku, Tokyo 169-0073, Japan

T. Iso
Gunma Rheumatism Clinic, 1040 Inomachi, Takasaki, Gunma 370-0004, Japan

M. Motegi
Department of Respiratory Medicine, National Hospital Organization Takasaki General Medical Center, 36 Takamatsu-cho, Takasaki, Gunma 370-0829, Japan

K. Ikeda · H. Nakajima
Department of Allergy and Clinical Immunology, Chiba University Hospital, 1-8-1 Inohana, Chuo-ku, Chiba, Chiba 260-8677, Japan

H. Taki
First Department of Internal Medicine, University of Toyama, 2630 Sugitani, Toyama, Toyama 930-0194, Japan

T. Kubota
Tokyo Medical and Dental University Graduate School of Health Care Sciences, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8510, Japan

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patients who did not develop PCP during adalimumab treatment were collected.
Results For the PCP patients, the median age was 68 years old, with a median RA disease duration of eight years. The median length of time from the first adalimumab injection to the development of PCP was 12 weeks. At the onset of PCP, the median dosages of prednisolone and methotrexate were 5.0 mg/day and 8.0 mg/week, respectively. The patients with PCP were significantly older ($p < 0.05$) and had more structural changes ($p < 0.05$) than the patients without PCP. Computed tomography of the chest revealed ground-glass opacity without interlobular septal boundaries in the majority of the patients with PCP. Three PCP patients died.
Conclusions PCP may occur early in the course of adalimumab therapy in patients with RA. Careful monitoring, early diagnosis, and proper management are mandatory to secure a good prognosis for these patients.

Keywords Adalimumab · *Pneumocystis jirovecii* pneumonia · Rheumatoid arthritis · TNF antagonist

Introduction

Rheumatoid arthritis (RA) is a systemic inflammatory disease characterized by persistent synovitis and structural damage to multiple joints. Tumor necrosis factor (TNF) is abundantly produced in the inflamed synovium and contributes to the imuunopathogenesis of the disease. Adalimumab is the first fully human monoclonal antibody against TNF; treatment with this biologic agent has been well established in patients with RA in multiple clinical trials [1–3]. On the other hand, treatment with adalimumab, as well as infliximab and etanercept, has been associated with increased risk for opportunistic and serious infections in cohort studies using RA patient registries [4–7]. In Japan, strict post-marketing surveillance (PMS) programs have been conducted for patients with RA given TNF antagonists. The numbers of RA patients with *Pneumocystis jirovecii* (*P. jirovecii*) pneumonia (PCP) who were treated with infliximab, etanercept, or adalimumab were 22 (0.4 %) out of 5,000 patients, 25 (0.18 %) out of 13,894 patients, and 25 (0.33 %) out of 7,469 patients, respectively, in these PMS programs [6–8]. Note that these incidence rates of PCP in Japan are apparently higher than the corresponding figure (0.01 %) reported from the United States [9].

H. Kodama
Nagara Orthopaedic Clinic, 3-10-12 Yashiro, Gifu-shi, Gifu 502-0812, Japan

S. Sugii
Department of Rheumatic Diseases, Tokyo Metropolitan Tama Medical Center, 2-8-29 Musashidai, Fucyu-shi, Tokyo 183-8524, Japan

T. Kuroiwa
Department of Medicine and Clinical Science, Gunma University Graduate School of Medicine, 3-39-15 Syowamachi, Maebashi-shi, Gunma 371-8511, Japan

Y. Nawata
Center for Rheumatic Diseases, Chibaken Saiseikai Narashino Hospital, 1-1-1 Izumi-cho, Narashino, Chiba 275-8580, Japan

K. Shiozawa
Rheumatic Diseases Center, Kohnan Kakogawa Hospital, 1545-1 Kanno-cho-saijyo, Kakogawa, Hyogo 675-8545, Japan

A. Ogata
Department of Respiratory Medicine, Allergy and Rheumatic Diseases, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan

S. Sawada
Sekimachi Hospital, 1-6-19 Sekimachikita, Nerima-ku, Tokyo 177-0051, Japan

Y. Matsukawa
Division of Hematology and Rheumatology, Department of Medicine, Nihon University School of Medicine, 30-1 Oyaguchikami-cho, Itabashi-ku, Tokyo 173-8610, Japan

T. Okazaki
Division of Rheumatology and Allergy, Department of Internal Medicine, St. Marianna University School of Medicine, 2-16-1 Sugao, Miyamae-ku, Kawasaki, Kanagawa 216-8511, Japan

M. Mukai
Division of Rheumatology and Clinical Immunology, Department of Medicine Sapporo City General Hospital, Kita 11-jo, Nishi-13 Chome, Chuo-ku, Sapporo 060-8604, Japan

M. Iwahashi
Division of Rheumatology, Higashihiroshima Memorial Hospital, 2214 Saijyocyoyoshiyuki, Higashihiroshima, Hiroshima 739-0002, Japan

K. Saito · Y. Tanaka
The First Department of Internal Medicine, School of Medicine, University of Occupational and Environmental Health, 1-1 Iseigaoka, Yahatanishi-ku, Kitakyushu 807-8555, Japan

N. Miyasaka
Global Center of Excellence (GCOE) Program, International Research Center for Molecular Science in Tooth and Bone Diseases, Tokyo Medical Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan

We have previously described the clinical characteristics and risk factors for PCP in RA patients treated with infliximab [10, 11] and etanercept [12]. These risk factors included older age and presence of coexisting lung diseases for both TNF antagonists, a higher daily dose of prednisolone (PSL) for infliximab, and a higher weekly dose of methotrexate (MTX) for etanercept. Considering the similar incidence of PCP in the PMS programs among the three TNF antagonists, it is clinically important and intriguing to characterize PCP in RA patients given adalimumab and to compare the results with those obtained for RA patients treated with other TNF antagonists.

In this paper, we report detailed clinical, laboratory, and radiographic features of PCP that developed in RA patients during treatment with adalimumab. Furthermore, we compared 17 RA patients receiving adalimumab who developed PCP with 89 RA patients who did not develop PCP during treatment, and identified risk factors for PCP in patients with RA treated with adalimumab.

Materials and methods

Patients

Patients included in the present study fulfilled the 1987 American College of Rheumatology (formerly the American Rheumatism Association) criteria for RA [13] and received adalimumab (40 mg every two weeks) with or without concomitant MTX. Between April 2008 and April 2010, 17 patients with PCP (PCP group) were collected from 16 hospitals through either the PMS program for adalimumab ($n = 16$) or through a voluntary case report by attending physicians at a scientific meeting ($n = 1$). We convened a face-to-face meeting in March 2011 to discuss diagnosis and treatment for the collected cases among the investigators of this study. RA patients who did not develop PCP during adalimumab therapy for at least one year from the first dose of adalimumab (non-PCP group, $n = 89$) were randomly collected from the participating hospitals of this study. Other eligibility criteria for the non-PCP group were registration in the PMS program of adalimumab and the use of adalimumab five times or more. The median (range) observation period for the non-PCP group treated with adalimumab was 365 (63–365) days. To increase the statistical power of this case–control study, the number of patients in the non-PCP group was designed to be about five times as many as that in the PCP group [14].

Diagnostic criteria for PCP

Previously established diagnostic criteria for PCP [15, 16] were used in the present study [10]. A diagnosis of PCP was considered definitive if a patient fulfilled the following four conditions: clinical manifestations (fever, dry cough, or dyspnea), hypoxemia, interstitial infiltrates on chest radiographs, and microscopic detection of *P. jirovecii* in induced sputum or bronchoalveolar lavage fluid. The diagnosis of PCP was considered presumptive if a patient fulfilled all of these conditions except for the microscopic detection of *P. jirovecii* in the absence of other infectious diseases and the presence of either a positive polymerase chain reaction (PCR) test for *P. jirovecii* DNA or increased serum 1,3-β-D-glucan (BDG) levels (Fungitec G test MK; Seikagaku, Tokyo, Japan or Wako β-D-glucan test; Wako Pure Chemical Industries, Tokyo, Japan) [17, 18] along with a response to standard treatments for PCP. Both the PCR test for *P. jirovecii* DNA and that for serum BDG are commercially available, validated, and officially approved as clinical laboratory tests by the Ministry of Health, Labour, and Welfare in Japan.

Collection and analysis of clinical data

Clinical information was collected using a standardized format to evaluate demographic information, Steinbrocker's radiographic stage and functional class [19], comorbidities, concomitant drugs, laboratory data, radiographic data, treatment, and outcome. Chest radiographs and computed tomography (CT) scans were evaluated by a pulmonologist (H.S.) and a diagnostic radiologist (F.S.). CT findings were categorized into three patterns, as we did in previous studies [12, 20]: (a) diffuse ground-glass opacity (GGO) distributed in a panlobular manner; that is, GGO was sharply demarcated from the adjacent normal lung by interlobular septa (type A GGO); (b) diffuse GGO that is homogeneous or somewhat inhomogeneous in distribution but without the sharp demarcation caused by interlobular septa (type B GGO); (c) other patterns, such as mixed consolidation and GGO (type C).

Statistical analyses

Demographic data and baseline data were compared between the PCP and non-PCP groups using the χ^2 test for categorical variables and the Mann–Whitney test for continuous variables. To identify risk factors for PCP, the Cox proportional-hazards regression model was used. All analyses were performed using SPSS software, version 16.0 (SPSS Japan, Tokyo, Japan).

Ethics

The guidelines of the Declaration of Helsinki (revised in 2008) and the ethics guidelines for epidemiologic research in Japan were followed. The study protocol was approved