ARTICLE
Journal of Cellular Biochemistry 112:1219–1227 (2011)

Journal of Cellular Biochemistry

Stromal Cell-Derived Factor-1/CXCR4 Promotes IL-6 Production in Human Synovial Fibroblasts

Hsien-Te Chen,[1,2,3] Hsi-Kai Tsou,[3,4,5] Chin-Jung Hsu,[1,2] Chun-Hao Tsai,[1] Cheng-Hsing Kao,[6,7] Yi-Chin Fong,[1,2] and Chih-Hsin Tang[8,9*]

[1] *Department of Orthopaedics, China Medical University Hospital, Taichung, Taiwan*
[2] *School of Chinese Medicine, China Medical University, Taichung, Taiwan*
[3] *Department of Materials Science and Engineering, Feng Chia University, Taichung, Taiwan*
[4] *Department of Neurosurgery, Taichung Veterans General Hospital, Taichung, Taiwan*
[5] *Department of Rehabilitation, Jen-Teh Junior College of Medicine, Nursing and Management, Miaoli County, Taiwan*
[6] *Department of Spine Surgery, Chi-Mei Medical Center, Tainan, Taiwan*
[7] *Center for General Education, Southern Taiwan University of Technology, Tainan, Taiwan*
[8] *Department of Pharmacology, School of Medicine, China Medical University and Hospital, Taichung, Taiwan*
[9] *Graduate Institute of Basic Medical Science, China Medical University and Hospital, Taichung, Taiwan*

ABSTRACT

The production of chemokine stromal cell-derived factor (SDF)-1 is significantly higher in synovial fluid of patients with osteoarthritis (OA). IL-6 is a multifunctional cytokine that plays a central role in both OA and rheumatoid arthritis. However, the effects of SDF-1α on human synovial fibroblasts are largely unknown. In this study, we investigated the intracellular signaling pathway involved in SDF-1α-induced IL-6 production in human synovial fibroblast cells. SDF-1α caused concentration- and time-dependent increases in IL-6 production. SDF-1α also increased the mRNA and surface expression of CXCR4 receptor in human synovial fibroblasts. CXCR4-neutralizing antibody, CXCR4-specific inhibitor (AMD3100), or small interfering RNA against CXCR4 inhibited the SDF-1α-induced increase of IL-6 expression. The transcriptional regulation of IL-6 by SDF-1α was mediated by phosphorylation of phosphatidylinositol 3-kinase (PI3K)/Akt and activation of the activator protein (AP)-1 component of c-Jun. The binding of c-Jun to the AP-1 element on the IL-6 promoter and the increase in AP-1 luciferase activity was enhanced by SDF-1α. Co-transfection with CXCR4, PI3K, Akt, and c-Jun mutants or siRNA inhibited the potentiating action of SDF-1α on AP-1 promoter activity. Taken together, our results suggest that SDF-1α-increased IL-6 production in human synovial fibroblasts via the CXCR4 receptor, PI3K, Akt, c-Jun, and AP-1 signaling pathways. J. Cell. Biochem. 112: 1219–1227, 2011.

KEY WORDS: IL-6; SDF-1; CXCR4; OA

Osteoarthritis (OA) is a chronic joint disorder characterized by slow progressive degeneration of articular cartilage, subchondral bone alteration, and variable secondary synovial inflammation. The exact etiology of OA is not well understood [Clouet et al., 2009]. In response to macrophage-derived proinflammatory cytokines such as interleukin (IL)-1β and tumor necrosis factor-α (TNF-α), and OA synovial fibroblasts (OASF) produce chemokines that promote inflammation, neovascularization, and cartilage degradation via activation of matrix-degrading enzymes such as matrix metalloproteinases [Mor et al., 2005]. Diagnosis of the disease and the progression of joint damage are mainly based on evaluation of clinical and radiological findings. Molecular markers may serve as promising indicators for OA evaluation because they provide more direct information about local inflammation, alterations in joint tissues, and related bone and cartilage turnover [Wright et al., 2009].

Chemokines are a family of small, soluble peptides that regulate cell movement, morphology, and differentiation. They achieve their

Hsien-Te Chen and Hsi-Kai Tsou contributed equally to this work.

Additional Supporting Information may be found in the online version of this article.

Grant sponsor: National Science Council of Taiwan; Grant number: NSC99-2320-B-039-003-MY3; Grant sponsor: China Medical University Hospital; Grant number: DMR-100-088.

*Correspondence to: Chih-Hsin Tang, Department of Pharmacology, School of Medicine, China Medical University, No. 91, Hsueh-Shih Road, Taichung, Taiwan. E-mail: chtang@mail.cmu.edu.tw

Received 20 December 2010; Accepted 19 January 2011 • DOI 10.1002/jcb.23043 • © 2011 Wiley-Liss, Inc.
Published online 10 February 2011 in Wiley Online Library (wileyonlinelibrary.com).

regulation by signaling through a family of transmembrane G protein-coupled receptors. It has been reported that the concentration of an 8-kDa chemokine, stromal cell-derived factor (SDF)-1, is greatly elevated in the synovial fluid from patients with OA and rheumatoid arthritis (RA) [Kanbe et al., 2002]. Such elevation of SDF-1 concentrations in synovial fluid is due, at least partially, to the stimulated synthesis of SDF-1 by synovial fibroblasts under OA and RA conditions [Kanbe et al., 2002]. The source of SDF-1 in the joint is from synovium, as demonstrated by immunocytochemistry, protein chemistry, and reverse transcription-polymerase chain reaction analysis [Kanbe et al., 2002, 2004]. Interaction of SDF-1 with its specific receptor CXCR4 on the surface of chondrocytes induces the release of MMPs from chondrocytes and contributes to the breakdown of articular cartilage during arthritis [Kanbe et al., 2002; Chiu et al., 2007].

IL-6 is a multifunctional cytokine that plays a central role in both innate and acquired immune responses. IL-6 is the predominant mediator of the acute-phase response, an innate immune mechanism that is triggered by infection and inflammation [Graeve et al., 1993; Grimble, 1998]. IL-6 also plays multiple roles during the subsequent development of acquired immunity against pathogens, including regulation of the expression of cytokines and chemokines, stimulation of Ab production by B cells, regulation of macrophage and dendritic cell differentiation, and the response of regulatory T cells to microbial infection [Graeve et al., 1993; Grimble, 1998]. In addition to these roles in pathogen-specific inflammation and immunity, IL-6 levels are elevated in chronic inflammatory conditions, such as OA and RA [Jones, 2005; Yokota et al., 2006]. Several consensus sequences, including those for NF-κB, CREB, NF-IL-6, and AP-1 in the 5′ promoter region of the IL-6 gene, have been identified as regulatory sequences that induce IL-6 expression in response to various stimuli [Matsusaka et al., 1993; Grassl et al., 1999]. AP-1 is commonly activated in response to inflammatory stimuli and has been implicated in cytokine expression and cellular immune responses [Wagner and Eferl, 2005; Hasselblatt et al., 2007]. Moreover, c-Jun/AP-1 has been implicated in human inflammatory disease [Bantel et al., 2002], suggesting that AP-1 may be an important mediator in intestinal and inflammatory cells.

Previous studies have shown that SDF-1 promotes the inflammatory response [Chiu et al., 2007; Lu et al., 2009]. Although a role for SDF-1 in IL-6 induction has been implicated in some cell types, the signaling pathway for SDF-1 in IL-6 production in synovial fibroblasts has not been extensively studied. In this study, we explored the intracellular signaling pathway involved in SDF-1α-induced IL-6 production in human synovial fibroblasts. The results show that SDF-1α activates CXCR4 receptor and causes the activation of the phosphatidylinositol 3-kinase (PI3K), Akt, and AP-1 pathways, leading to up-regulated of IL-6 expression.

MATERIALS AND METHODS

MATERIALS

Anti-mouse and anti-rabbit IgG-conjugated horseradish peroxidase, rabbit polyclonal antibodies specific for p85, p-p85, Akt, p-Akt, c-Jun, p-c-Jun, lamin B, and the small interfering RNAs (siRNAs) against c-Jun, and a control for experiments using targeted siRNA transfection (each consists of a scrambled sequence that does not lead to specific degradation of any known cellular mRNA) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). PD98059, SB203580, SP600125, Ly294002, and Akt inhibitor (1L-6-hydroxymethyl-chiro-inositol-2-((R)-2-O-methyl-3-O-octadecylcarbonate)) were purchased from Calbiochem (San Diego, CA). Rabbit polyclonal antibody specific for CXCR4 was purchased from R&D Systems (Minneaplis, MN). The recombinant human SDF-1α was purchased from PeproTech (Rocky Hill, NJ). Tanshinone IIA was purchased from BIOMOL (Butler Pike, PA). The IL-6 enzyme immunoassay kit was purchased from Cayman Chemical (Ann Arbor, MI). The siCXCR4 and siCXCR4-mut siRNA were generated in our laboratory (siCXCR4 but not siCXCR4-mut specific inhibited the expression of mRNA and protein level of CXCR4) [Huang et al., 2007]. The p38 dominant negative mutant was provided by Dr. J. Han (South-western Medical Center, Dallas, TX). The JNK dominant negative mutant was provided by Dr. M. Karin (University of California, San Diego, CA). The ERK2 dominant negative mutant was provided by Dr. M. Cobb (South-Western Medical Center, Dallas, TX). The p85α (Δp85; deletion of 35 amino acids from residues 479–513 of p85) and Akt (Akt K179A) dominant-negative mutants were provided by Dr. W. M. Fu (National Taiwan University, Taipei, Taiwan). The human full length CXCR4 was provided by Dr. Jun Komano (National Institute of Infectious Diseases, Japan). The AP-1 luciferase plasmid was purchased from Stratagene (La Jolla, CA). The human IL-6 promoter construct pIL6-luc651(-651/+1), AP-1 site mutation (pIL6-luc651ΔAP1), NF-κB site mutation (pIL6-luc651ΔNF-κB), and C/EBP-β site mutation (pIL6-luc651ΔC/EBP-β) were provided by Dr. Oliver Eickelberg (Department of Medicine II, University of Giessen, Giessen, Germany). The pSV-β-galactosidase vector and luciferase assay kit were purchased from Promega (Madison, WI). All other chemicals were purchased from Sigma–Aldrich (St. Louis, MO).

CELL CULTURES

After approval by the local ethics committee, human synovial fibroblasts were isolated using collagenase treatment of synovial tissues obtained from knee replacement surgeries of 12 patients with OA. Fresh synovial tissues were minced and digested in a solution of collagenase and DNase. Isolated fibroblasts were filtered through 70-μm nylon filters. The cells were grown on plastic cell culture dishes in 95% air/5% CO_2 in RPMI 1640 (Life Technologies) that was supplemented with 20 mM HEPES and 10% heat-inactivated FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (pH adjusted to 7.6). More than 95% of the cells were fibroblasts, as characterized by immunofluorescence staining using an antibody specific for the fibroblast protein marker vimentin (Supplementary Fig. S1). Fibroblasts from passages four to nine were used for the experiments [Tang et al., 2007; Chiu et al., 2009].

MEASUREMENT OF IL-6 PRODUCTION

Human synovial fibroblasts were cultured in 24-well culture plates. After reaching confluency, cells were treated with SDF-1α and then incubated in a humidified incubator at 37°C for 24 h. To examine the

downstream signaling pathways involved in SDF-1α treatment, cells were pretreated with various inhibitors for 30 min before addition of SDF-1α (100 ng/ml) administration. After incubation, the medium was removed and stored at −80°C until the assay was performed. IL-6 in the medium was assayed using IL-6 enzyme immunoassay kits, according to the procedure described by the manufacturer [Tang et al., 2007; Chiu et al., 2009].

QUANTITATIVE REAL-TIME PCR

Total RNA was extracted from synovial fibroblasts with a TRIzol kit (MDBio, Inc., Taipei, Taiwan). The reverse transcription reaction was performed using 2 μg of total RNA (in 2 μl RNase-free water) that was reverse transcribed into cDNA with an MMLV RT kit (Promega) and following the manufacturer's recommended procedures. Quantitative real time PCR (qPCR) analysis was carried out with TaqMan® one-step PCR Master Mix (Applied Biosystems, Foster City, CA). cDNA template (2 μl) was added to each 25-μl reaction with sequence-specific primers and TaqMan® probes. All target gene primers and probes were purchased commercially (β-actin was used an internal control) (Applied Biosystems). qPCR assays were carried out in triplicate on a StepOnePlus sequence detection system. The cycling conditions were: 10-min polymerase activation at 95°C followed by 40 cycles at 95°C for 15 s and 60°C for 60 s. The threshold was set above the non-template control background and within the linear phase of target gene amplification to calculate the cycle number at which the transcript was detected (denoted C_T).

WESTERN BLOT ANALYSIS

Cellular lysates were prepared as described [Tang et al., 2007; Chiu et al., 2009]. Proteins were resolved using SDS–PAGE and transferred to immobilon polyvinyldifluoride membranes. The membranes were blocked with 4% BSA for 1 h at room temperature and then probed with rabbit antibodies against human Akt, p85, p-Akt, or p-p85 (1:1,000) for 1 h at room temperature. After three washes, the blots were incubated with a donkey anti-rabbit peroxidase-conjugated secondary antibody (1:1,000) for 1 h at room temperature. The blots were visualized with enhanced chemiluminescence on Kodak X-OMAT LS film (Eastman Kodak, Rochester, NY).

TRANSFECTION AND REPORTER GENE ASSAY

Human synovial fibroblasts were co-transfected with 0.8 μg luciferase plasmid and 0.4 μg β-galactosidase expression vector. OASF cells were grown to 80% confluency in 12-well plates and then transfected on the following day with Lipofectamine 2000 (LF2000; Invitrogen). DNA and LF2000 were premixed for 20 min and then added to the cells. After 24 h of transfection, the cells were incubated with the indicated reagents. After a further 24 h of incubation, the medium was removed, and cells were washed once with cold PBS. To prepare lysates, 100 μl reporter lysis buffer (Promega) was added to each well, and cells were scraped from dishes. The supernatant was collected after centrifugation at 13,000 rpm for 2 min. Aliquots of cell lysates (20 μl) containing equal amounts of protein (20–30 μg) were placed into wells of an opaque black 96-well microplate. An equal volume of luciferase substrate was added to all samples, and luminescence was measured in a microplate luminometer. The value of luciferase activity was normalized to the transfection efficiency, which was monitored by activity of the co-transfected β-galactosidase expression vector.

CHROMATIN IMMUNOPRECIPITATION ASSAY

Chromatin immunoprecipitation analysis was performed as described previously [Chiu et al., 2009]. DNA immunoprecipitated with an anti-c-Jun Ab was purified and extracted with phenol–chloroform. The purified DNA pellet was subjected to PCR. PCR products were then resolved by 1.5% agarose gel electrophoresis and visualized with UV light [Chiu et al., 2009].

STATISTICS

Data were expressed as means ± SEM. For statistical evaluation, we used the Mann–Whitney *U*-test for non-Gaussian parameters. The difference was considered significant if the *P*-value was <0.05.

RESULTS

SDF-1α INDUCES IL-6 PRODUCTION IN HUMAN SYNOVIAL FIBROBLASTS

Chemokines are involved in the pathology of OA [Borzi et al., 2004]. It has been reported that SDF-1 is greatly elevated in the synovial fluid from patients with OA. Therefore, we used human synovial fibroblasts to investigate the signaling pathways of SDF-1α in the production of IL-6, encoded by an inflammatory response gene. Treatment of OASF with SDF-1α (3–100 ng/ml) for 24 h induced IL-6 production in a concentration-dependent manner (Fig. 1A), and this induction occurred in a time-dependent manner (Fig. 1B). After SDF-1α (100 ng/ml) treatment for 24 h, the amount of IL-6 released had increased in OASF cells (Fig. 1B). In addition, stimulation of cells with SDF-1α also led to increased expression of IL-6 mRNA in a concentration-dependent manner (Fig. 1C). To confirm that this stimulation-specific effect was mediated by SDF-1α in the absence of LPS contamination, we used polymyxin B, an LPS inhibitor. We found that polymyxin B (1 μM) completely inhibited LPS (1 μM)-induced IL-6 release. However, it had no effect on SDF-1α (100 ng/ml)-induced IL-6 release from OASF cells (Fig. 1D). To further determine if SDF-1α induced IL-6 expression required transcription and/or translation, OASF cells were stimulated with SDF-1α in the absence or presence of the transcription inhibitor, actinomycin D, or the translation inhibitor, cycloheximide, and IL-6 expression was determined by ELISA. As shown in Figure 2, SDF-1α-mediated induction of IL-6 expression was abolished by both actinomycin D and cycloheximide, respectively. Taken together, these findings demonstrate that the induction of IL-6 by SDF-1α depends on de novo protein synthesis in human synovial fibroblasts.

SDF-1α/CXCR4 INTERACTION INCREASES IL-6 PRODUCTION IN HUMAN SYNOVIAL FIBROBLASTS

Interaction of SDF-1 with its specific receptor CXCR4 on the surface of oral cancer cells has been reported to induce the release of IL-6 [Tang et al., 2008]. We examined whether SDF-1/CXCR4 interaction was involved in the signal transduction pathway leading to IL-6 expression caused by SDF-1α. Human OASF were treated with SDF-1α for different time intervals, the results from qPCR and flow

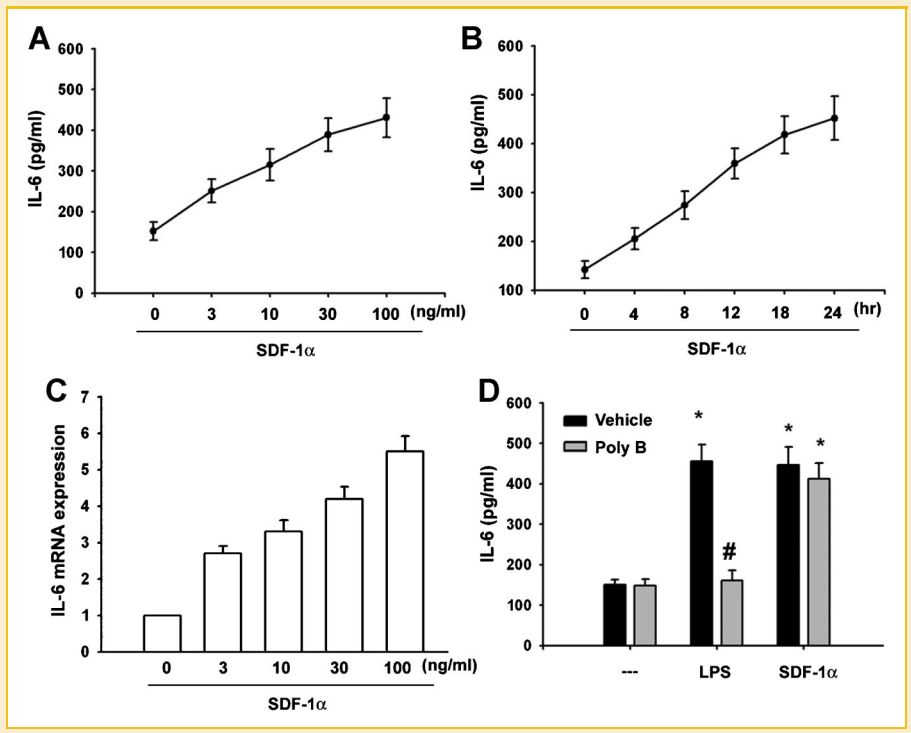

Fig. 1. Concentration- and time-dependent increases in IL-6 production by SDF-1α. OASF were incubated with various concentrations of SDF-1α for 24 h (A) or with SDF-1α (3–100 ng/ml) for 4, 8, 12, 18, or 24 h (B). Media were collected to measure IL-6 ($n = 4$). C: OASF cells were incubated with SDF-1α for 24 h, and IL-6 mRNA was examined by qPCR. D: OASF cells were pretreated with polymyxin B (Poly B, 1 μM) for 30 min followed by stimulation with LPS (1 μM) or SDF-1α (100 ng/ml) for 24 h. $^{*}P < 0.05$ as compared with LPS-treated and control groups. $^{\#}P < 0.05$ as compared with LPS or SDF-1α-treated group.

cytometry indicated that SDF-1α significantly increased mRNA and cell surface expression of CXCR4 (Fig. 3A,B). Transfection of cells with human full length CXCR4 increased the SDF-1α-induced IL-6 expression (Fig. 3C). Pretreatment of cells for 30 min with CXCR4-specific chemical inhibitor AMD3100 (200 and 500 ng/ml), CXCR-4-neutralizing antibody (12G5) (10 μg/ml) but not mouse monoclonal immunoglobulin isotype control (isotype Ab) (10 μg/ml) antagonized the SDF-1α-induced IL-6 expression (Fig. 3D). In addition, transfection of cells with small interfering RNA against CXCR4 (siCXCR4), but not a mutant form of siCXCR4 (siCXCR4-mut) specifically inhibited the expression of IL-6 directed by SDF-1α (Fig. 3E). Therefore, an interaction between SDF-1α and CXCR4 is very important for IL-6 production in human synovial fibroblasts.

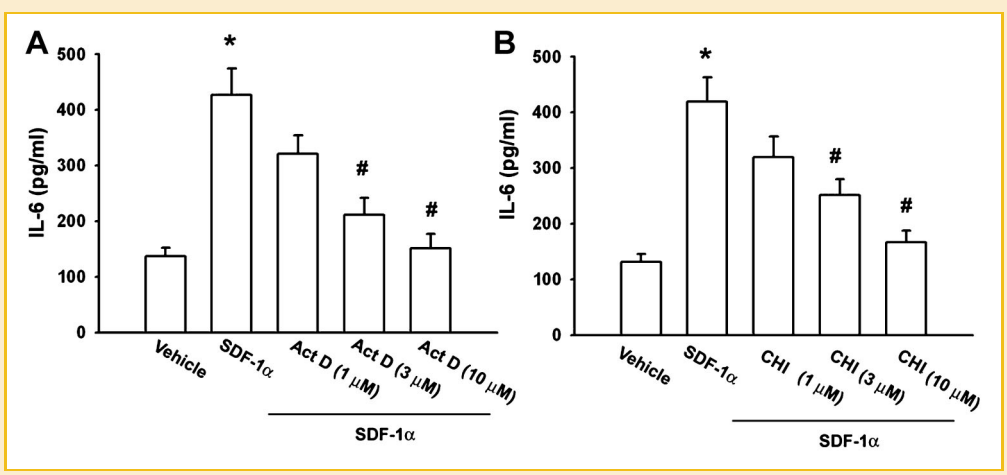

Fig. 2. Actinomycin D or cycloheximide reduced SDF-1α-mediated IL-6 production in synovial fibroblasts. OASF cells were pretreated with or without actinomycin D (Act. D, 1–10 μM; A) or cycloheximide (CHI, 1–10 μM; B) for 30 min, and then incubated in the absence or presence of SDF-1α for 24 h. Media were collected to measure IL-6. Results are expressed of four independent experiments performed in triplicate. $^{*}P < 0.05$ as compared with basal level. $^{\#}P < 0.05$ as compared with SDF-1α-treated group.

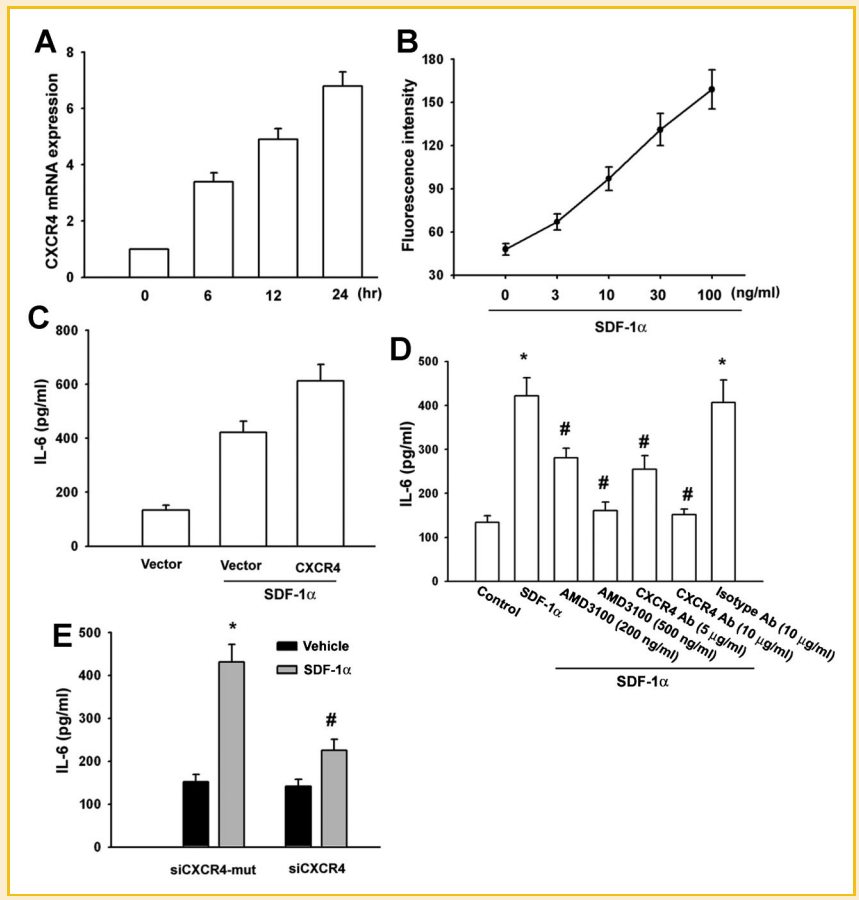

Fig. 3. Involvement of CXCR4 receptor in SDF-1α-mediated IL-6 production in synovial fibroblasts. A,B: OASF cells were incubated with SDF-1α for 24 h, and the mRNA and cell surface expression of CXCR4 was examined by qPCR and flow cytometry. C: OASF cells were transfected with control vector or human full length CXCR4 for 24 h and then stimulated with SDF-1α for 24 h. Media were collected to measure IL-6. D: Cells were pretreated with CXCR-4-neutralizing 12G5 antibody, AMD3100, and isotype antibody for 30 min followed by stimulation with SDF-1α. Media were collected to measure IL-6. E: OASF cells were transfected with CXCR4 or CXCR4-mut siRNA for 24 h and then stimulated with SDF-1α for 24 h. Media were collected to measure IL-6. $^{*}P < 0.05$ as compared with basal level. $^{\#}P < 0.05$ as compared with SDF-1α-treated group.

PI3K/AKT SIGNALING PATHWAY IS INVOLVED IN SDF-1α-MEDIATED IL-6 UPREGULATION

As SDF-1α/CXCR4 interaction has been shown to activate several signaling pathways, including PI3K/Akt and mitogen-activated protein kinase (MAPK), in various cell lines [Bachelder et al., 2002; Lu et al., 2009]. To determine whether ERK, p38, JNK, and PI3K/Akt pathways are involved in SDF-1α-triggered IL-6 production, OASF cells were pretreated with ERK inhibitor PD98059, p38 inhibitor SB203850, JNK inhibitor SP600125, PI3K inhibitor Ly294002, and Akt inhibitor for 30 min and then incubated with SDF-1α for 24 h. As shown in Figure 4A, pretreatment with PI3K or Akt inhibitor but not ERK, p38, or JNK inhibitor reduced SDF-1α-induced IL-6 production, suggesting that PI3K/Akt but not MAPKs may play a role in SDF-1α-induced IL-6 expression in OASF. In addition, transfection of cells with p85 or Akt but not p38, JNK, or ERK mutants also antagonized the potentiating effects of SDF-1α (Fig. 4B). Next, we used Western blot analysis to elucidate the PI3K/Akt signal-transduction mechanism involved in the SDF-1α-induced upregulation of IL-6. Stimulation of cells with SDF-1α increased p85 and Akt phosphorylation (Fig. 4C). Taken together, these data suggest that the activation of the PI3K/Akt pathway is required for the SDF-1α-induced increase of IL-6 in human OASF.

INVOLVEMENT OF AP-1 IN SDF-1α-INDUCED IL-6 PRODUCTION

The promoter region of human IL-6 contains three known *cis*-regulatory elements, namely the AP-1, C/EBP-β, and NF-κB binding sites [Matsusaka et al., 1993; Grassl et al., 1999]. Three different IL-6 promoter constructs containing mutations in the NF-κB, AP-1, or C/EBP-β sites were generated by site-directed mutagenesis. We found that SDF-1α-stimulated luciferase activity was abolished by the AP-1 binding site mutation but not by mutations in the NF-κB or C/EBP-β sites (Fig. 5A). The role of AP-1 was further established with the AP-1 inhibitors, curcumin and tanshinone IIA, which blocked the enhancement of IL-6 production induced by SDF-1α (Fig. 5B). Therefore, the AP-1 binding site is more important than the NF-κB and C/EBP-β sites in SDF-1α-induced IL-6 production. It has been reported that the AP-1 binding site between −283 and −276 is important for the activation of the IL-6 gene [Matsusaka et al., 1993]. AP-1 activation was further evaluated by analyzing the accumulation of phosphorylated c-Jun in the nucleus as well as by a chromatin immunoprecipitation assay. Treatment of cells with

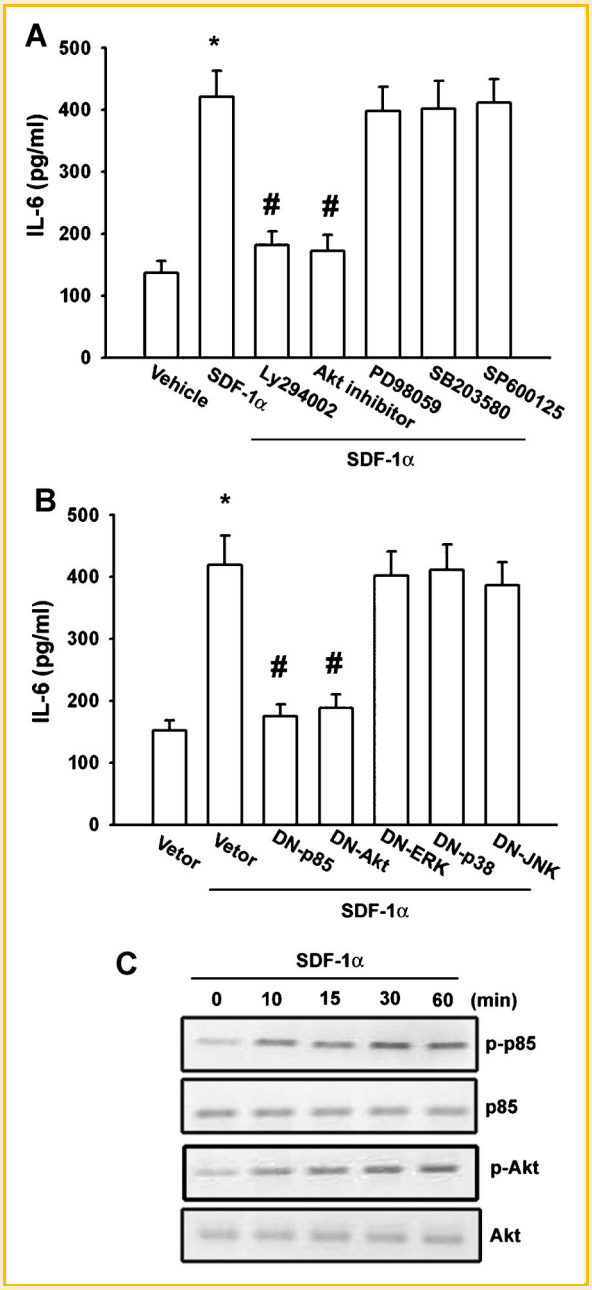

Fig. 4. PI3K/Akt is involved in SDF-1α-induced IL-6 production. A,B: OASF cells were pretreated for 30 min with PD98059 (30 μM), SB203580 (10 μM), SP600125 (10 μM), Ly294002 (10 μM), and Akt inhibitor (10 μM) or transfected with dominant negative (DN) mutant of ERK, p38, JNK, p85, and Akt for 24 h followed by stimulation with SDF-1α for 24 h. Media were collected to measure IL-6. C: Cells were incubated with SDF-1α for indicated time intervals, and p85 and Akt phosphorylation were examined by Western blotting. $^{*}P < 0.05$ as compared with basal level. $^{\#}P < 0.05$ as compared with SDF-1α-treated group.

SDF-1α resulted in a marked accumulation of phosphorylated c-Jun in the nucleus (Fig. 5C). Transfection of cells with c-Jun siRNA suppressed the expression of IL-6 after SDF-1αstimulation (Fig. 5D).

We next investigated whether c-Jun binds to the AP-1 element on the IL-6 promoter after SDF-1α stimulation. The in vivo recruitment of c-Jun to the IL-6 promoter (−312 to −39) was assessed via chromatin immunoprecipitation assay [Chiu et al., 2009]. In vivo binding of c-Jun to the AP-1 element of the IL-6 promoter occurred after SDF-1α stimulation (Fig. 6A). The binding of c-Jun to the AP-1 element by SDF-1α was attenuated by AMD3100, Ly294002, and Akt inhibitor (Fig. 6A). To further confirm that the AP-1 element is involved in SDF-1α-induced IL-6 expression, we performed transient transfection with AP-1 promoter-luciferase constructs. Synovial fibroblasts incubated with SDF-1α showed a 3.2-fold increase in AP-1 promoter activity. The increase in AP-1 activity by SDF-1α was antagonized by CXCR4 Ab, AMD3100, Ly294002, and Akt inhibitor or CXCR4 and c-Jun siRNA or p85 and Akt mutant (Fig. 6B,C). Taken together, these data suggest that the activation of the CXCR4, PI3K, Akt, c-Jun, and AP-1 pathway is required for the SDF-1α-induced increase in IL-6 in human OASF cells.

DISCUSSION

OA is a heterogeneous group of conditions associated with defective integrity of articular cartilage as well as related changes in the underlying bone. The chronic inflammatory process is mediated through a complex cytokine network. It is not yet completely understood which factors are responsible for initiating the degradation and loss of articular tissues. SDF-1 is significantly higher in synovial fluid of patients with OA and rheumatoid arthritis. We identified IL-6 as a target protein for the SDF-1α signaling pathway that regulates the cellular inflammatory response. We showed that potentiation of IL-6 by SDF-1α requires activation of the CXCR4 receptor, PI3K, Akt, and AP-1 signaling pathways. These findings suggest that SDF-1α acts as an inducer of inflammatory cytokines such as IL-6 and enhances the inflammatory response in OA.

The synovium of OA and RA patients produces many types of cytokines and chemokines, such as IL-1, TNF-α, and macrophage inflammatory protein-1 [Yoshihara et al., 2000]. SDF-1 has the additional function to accumulate CD^{4+} memory T cells in the synovium. This indicates that SDF-1 is related to the immune system and the inflammation that attracts lymphocytes to develop arthritis [Blades et al., 2002]. However, the expression of IL-6 by SDF-1α in synovial fibroblasts is mostly unknown. Here, we found that SDF-1α increased IL-6 expression by using qPCR and ELISA analysis, which plays an important role during arthritis. Previous studies have shown that SDF-1α/CXCR4 interactions modulate cell functions [Bartolome et al., 2006; Huang et al., 2009]. In the present study, we used CXCR4-specific chemical inhibitor AMD3100 and CXCR4-neutralizing antibody to determine the role of CXCR4, and we found that they inhibited SDF-1α-induced IL-6 expression, indicating the possible involvement of CXCR4 in SDF-1α-induced IL-6 expression in OASF. This was further confirmed by the result that the small interfering RNA against CXCR4 inhibited the enhancement of IL-6 production by SDF-1α, indicating the involvement of SDF-1/CXCR4 interaction in SDF-1α-mediated induction of IL-6.

A variety of growth factors stimulate the expression of IL-6 genes via signal-transduction pathways that converge to activate AP-1 complex of transcription factors. MAPK pathways (ERK1/2, JNK,

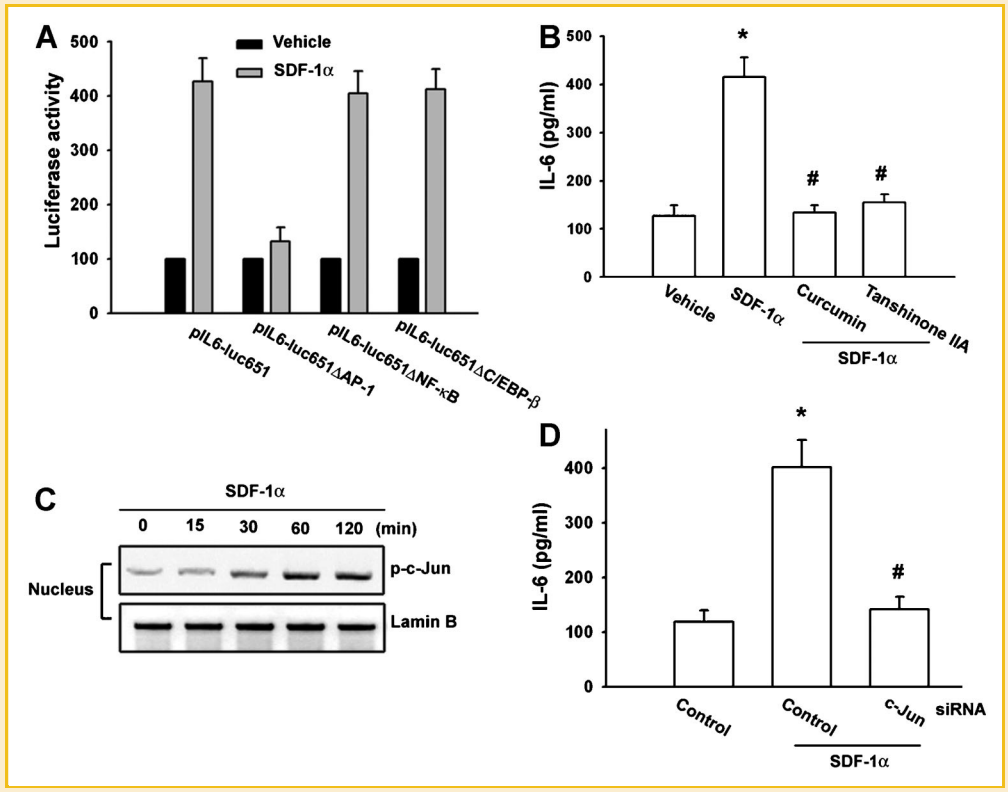

Fig. 5. AP-1 is involved in the potentiation of IL-6 production by SDF-1α. A: OASF cells were transfected with IL-6 luciferase plasmids before incubation with SDF-1α for 24 h. Luciferase activity was then assayed. B: OASF cells were pretreated for 30 min with curcumin and tanshinone IIA followed by stimulation with SDF-1α for 24 h. Media were collected to measure IL-6. C: OASF cells were incubated with SDF-1α for indicated time intervals, and c-Jun phosphorylation in nucleus were determined by Western blotting. D: OASF cells were transfected with c-Jun or control siRNA for 24 h, and then stimulated with SDF-1α for 24 h. Media were collected to measure IL-6. $^{*}P < 0.05$ as compared with basal level. $^{\#}P < 0.05$ as compared with SDF-1α-treated group.

and p38) and PI3K/Akt induce the expression of AP-1 transcription factors. We found that SDF-1α enhanced PI3K and Akt phosphorylation in human OASF. Previous studies have revealed that SDF-1α treatment activates PI3K/Akt in microglia and endothelial cells [Zheng et al., 2008; Lu et al., 2009]. The SDF-1α-directed IL-6 production was effectively inhibited by PI3K or Akt inhibitor, but not by SB203580, SP600125, or PD98059. This was further confirmed by the results that the dominant negative mutant of PI3K and Akt, but not p38, JNK, and ERK, inhibited the enhancement of IL-6 production by SDF-1α. Our data indicate that PI3K/Akt might play an important role in the expression of IL-6 of human OASF cells.

There are several binding sites for a number of transcription factors including NF-κB, CREB, NF-IL-6, and the AP-1 box in the 5′ region of the IL-6 gene [Matsusaka et al., 1993; Grassl et al., 1999]. Recent studies of the IL-6 promoter have demonstrated that IL-6 induction by several transcription factors occurs in a highly stimulus-specific or cell-specific manner [Persson et al., 2005]. The results of our current study show that AP-1 activation contributes to SDF-1α-induced IL-6 production in synovial fibroblasts. Deletion of the AP-1 site reduced SDF-1α-mediated IL-6 promoter activity. Pretreatment of cells with AP-1 inhibitors also reduced SDF-1α-increased IL-6 production. Therefore, the AP-1 binding site is likely to be the most important site for SDF-1α-induced IL-6 production. The AP-1 sequence binds to members of the Jun and Fos families of transcription factors. These nuclear proteins interact with the AP-1 site as Jun homodimers or Jun-Fos heterodimers formed by protein dimerization through their leucine zipper motifs. The results of our study show that SDF-1α induced c-Jun nuclear accumulation. In addition, c-Jun siRNA abolished SDF-1α-induced IL-6 production in OASF cells. Therefore, c-Jun activation mediates by SDF-1α-increased IL-6 expression. Furthermore, SDF-1α increased the binding of c-Jun to the AP-1 element within the IL-6 promoter, as shown by a chromatin immunoprecipitation assay. Binding of c-Jun to the AP-1 element was attenuated by AMD3100, Ly294002, and Akt inhibitor. Using transient transfection with AP-1-luciferase as an indicator of AP-1 activity, we also found that SDF-1α induced an increase in AP-1 activity. In addition, CXCR4 Ab, AMD3100, Ly294002, and Akt inhibitor or CXCR4 and c-Jun siRNA or p85 and Akt mutant reduced SDF-1α-increased AP-1 promoter activity. These results indicate that the interaction between SDF-1α and CXCR4 may act through the PI3K, Akt, c-Jun, and AP-1 pathway to induce IL-6 activation in human OASF cells.

In conclusion, we explored the signaling pathway involved in SDF-1α-induced IL-6 production in human synovial fibroblasts. We found that SDF-1α increased IL-6 production by binding to the CXCR4 receptor and activating PI3K and Akt, which enhanced binding of c-Jun to the AP-1 site and resulted in the transactivation

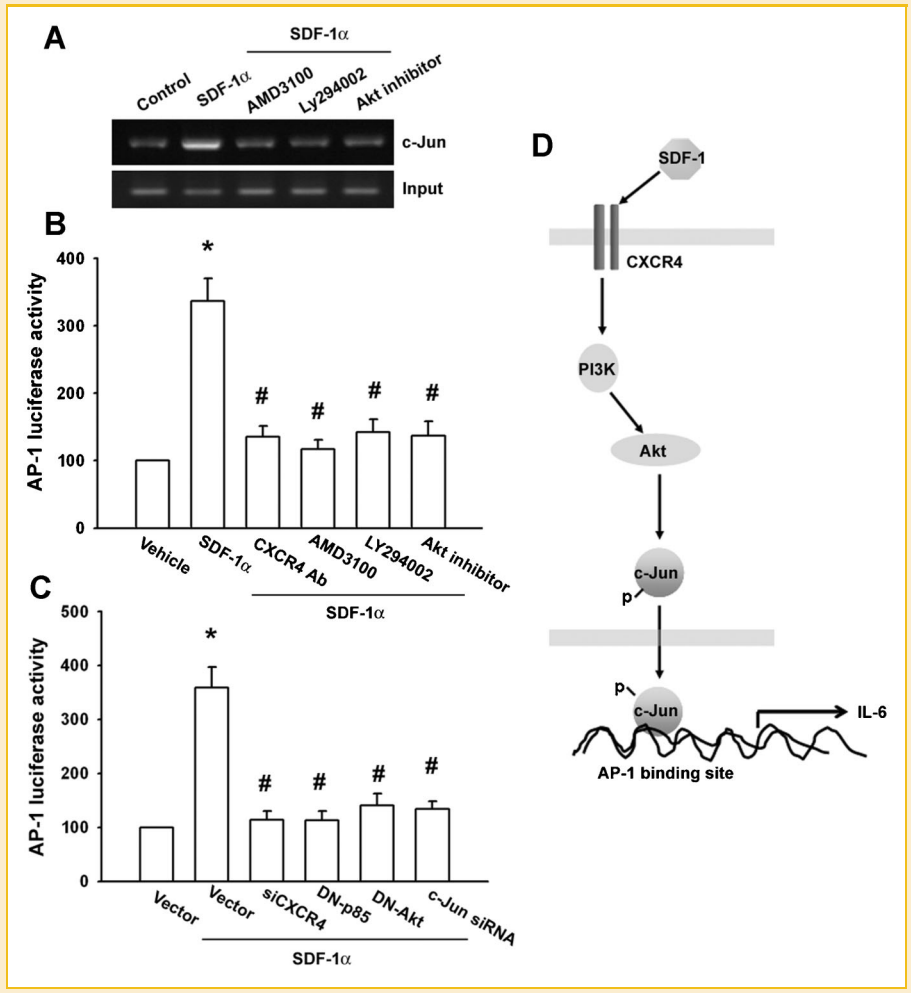

Fig. 6. The CXCR4/PI3K/Akt pathway is involved in SDF-1α-induced AP-1 activation. A: OASF cells were pretreated with AMD3100, Ly294002, or Akt inhibitor, and then stimulated with SDF-1α for 120 min. A chromatin immunoprecipitation assay was then performed. The chromatin was immunoprecipitated with anti-c-Jun. One percent of the precipitated chromatin was assayed to verify equal loading (input). OASF cells were transfected with the AP-1-luciferase expression vector and then pretreated with CXCR4 Ab, AMD3100, Ly294002, and Akt inhibitor or co-transfected with CXCR4 or c-Jun siRNA or the p85 and Akt mutant before incubation with SDF-1α for 24 h (B,C). Luciferase activity was then assayed. $^{*}P < 0.05$ as compared with basal level. $^{\#}P < 0.05$ as compared with SDF-1α-treated group. D: Schematic diagram of the signaling pathways involved in SDF-1α-induced IL-6 production in synovial fibroblasts. SDF-1α increases IL-6 expression by binding to the CXCR4 receptor and activating PI3K and Akt, which enhances binding of c-Jun to the AP-1 site. This results in the transactivation of IL-6 expression.

of IL-6 production (Fig. 6D). These findings may provide a better understanding of the mechanisms of OA pathogenesis.

ACKNOWLEDGMENTS

We thank Dr. Oliver Eickelberg for providing the IL-6 promoter plasmids; Dr. J. Han for providing p38 mutant; Dr. M. Karin for providing JNK mutant; Dr. M. Cobb for providing ERK2 mutant; Dr. Jun Komano for providing human full length CXCR4; Dr. W.M. Fu for providing p85 and Akt mutants. This work was supported by grants from the National Science Council of Taiwan (NSC99-2320-B-039-003-MY3) and China Medical University Hospital (DMR-100-088).

REFERENCES

Bachelder RE, Wendt MA, Mercurio AM. 2002. Vascular endothelial growth factor promotes breast carcinoma invasion in an autocrine manner by regulating the chemokine receptor CXCR4. Cancer Res 62:7203–7206.

Bantel H, Schmitz ML, Raible A, Gregor M, Schulze-Osthoff K. 2002. Critical role of NF-kappaB and stress-activated protein kinases in steroid unresponsiveness. FASEB J 16:1832–1834.

Bartolome RA, Molina-Ortiz I, Samaniego R, Sanchez-Mateos P, Bustelo XR, Teixido J. 2006. Activation of Vav/Rho GTPase signaling by CXCL12 controls membrane-type matrix metalloproteinase-dependent melanoma cell invasion. Cancer Res 66:248–258.

Blades MC, Ingegnoli F, Wheller SK, Manzo A, Wahid S, Panayi GS, Perretti M, Pitzalis C. 2002. Stromal cell-derived factor 1 (CXCL12) induces monocyte migration into human synovium transplanted onto SCID mice. Arthritis Rheum 46:824–836.

Borzi RM, Mazzetti I, Marcu KB, Facchini A. 2004. Chemokines in cartilage degradation. Clin Orthop Relat Res 427 Suppl:S53–S61.

Chiu YC, Yang RS, Hsieh KH, Fong YC, Way TD, Lee TS, Wu HC, Fu WM, Tang CH. 2007. Stromal cell-derived factor-1 induces matrix metalloprotease-13 expression in human chondrocytes. Mol Pharmacol 72:695–703.

Chiu YC, Lin CY, Chen CP, Huang KC, Tong KM, Tzeng CY, Lee TS, Hsu HC, Tang CH. 2009. Peptidoglycan enhances IL-6 production in human synovial fibroblasts via TLR2 receptor, focal adhesion kinase, Akt, and AP-1- dependent pathway. J Immunol 183:2785–2792.

Clouet J, Vinatier C, Merceron C, Pot-vaucel M, Maugars Y, Weiss P, Grimandi G, Guicheux J. 2009. From osteoarthritis treatments to future regenerative therapies for cartilage. Drug Discov Today 14:913–925.

Graeve L, Baumann M, Heinrich PC. 1993. Interleukin-6 in autoimmune disease. Role of IL-6 in physiology and pathology of the immune defense. Clin Investig 71:664–671.

Grassl C, Luckow B, Schlondorff D, Dendorfer U. 1999. Transcriptional regulation of the interleukin-6 gene in mesangial cells. J Am Soc Nephrol 10:1466–1477.

Grimble RF. 1998. Nutritional modulation of cytokine biology. Nutrition 14: 634–640.

Hasselblatt P, Rath M, Komnenovic V, Zatloukal K, Wagner EF. 2007. Hepatocyte survival in acute hepatitis is due to c-Jun/AP-1-dependent expression of inducible nitric oxide synthase. Proc Natl Acad Sci USA 104:17105–17110.

Huang YC, Hsiao YC, Chen YJ, Wei YY, Lai TH, Tang CH. 2007. Stromal cell-derived factor-1 enhances motility and integrin up-regulation through CXCR4, ERK and NF-kappaB-dependent pathway in human lung cancer cells. Biochem Pharmacol 74:1702–1712.

Huang CY, Lee CY, Chen MY, Yang WH, Chen YH, Chang CH, Hsu HC, Fong YC, Tang CH. 2009. Stromal cell-derived factor-1/CXCR4 enhanced motility of human osteosarcoma cells involves MEK1/2, ERK and NF-kappaB-dependent pathways. J Cell Physiol 221:204–212.

Jones SA. 2005. Directing transition from innate to acquired immunity: Defining a role for IL-6. J Immunol 175:3463–3468.

Kanbe K, Takagishi K, Chen Q. 2002. Stimulation of matrix metalloprotease 3 release from human chondrocytes by the interaction of stromal cell-derived factor 1 and CXC chemokine receptor 4. Arthritis Rheum 46: 130–137.

Kanbe K, Takemura T, Takeuchi K, Chen Q, Takagishi K, Inoue K. 2004. Synovectomy reduces stromal-cell-derived factor-1 (SDF-1) which is involved in the destruction of cartilage in osteoarthritis and rheumatoid arthritis. J Bone Joint Surg Br 86:296–300.

Lu DY, Tang CH, Yeh WL, Wong KL, Lin CP, Chen YH, Lai CH, Chen YF, Leung YM, Fu WM. 2009. SDF-1alpha up-regulates interleukin-6 through CXCR4, PI3K/Akt, ERK, and NF-kappaB-dependent pathway in microglia. Eur J Pharmacol 613:146–154.

Matsusaka T, Fujikawa K, Nishio Y, Mukaida N, Matsushima K, Kishimoto T, Akira S. 1993. Transcription factors NF-IL6 and NF-kappa B synergistically activate transcription of the inflammatory cytokines, interleukin 6 and interleukin 8. Proc Natl Acad Sci USA 90:10193–10197.

Mor A, Abramson SB, Pillinger MH. 2005. The fibroblast-like synovial cell in rheumatoid arthritis: A key player in inflammation and joint destruction. Clin Immunol 115:118–128.

Persson E, Voznesensky OS, Huang YF, Lerner UH. 2005. Increased expression of interleukin-6 by vasoactive intestinal peptide is associated with regulation of CREB, AP-1 and C/EBP, but not NF-kappaB, in mouse calvarial osteoblasts. Bone 37:513–529.

Tang CH, Chiu YC, Tan TW, Yang RS, Fu WM. 2007. Adiponectin enhances IL-6 production in human synovial fibroblast via an AdipoR1 receptor, AMPK, p38, and NF-kappa B pathway. J Immunol 179:5483–5492.

Tang CH, Chuang JY, Fong YC, Maa MC, Way TD, Hung CH. 2008. Bone-derived SDF-1 stimulates IL-6 release via CXCR4, ERK and NF-kappaB pathways and promotes osteoclastogenesis in human oral cancer cells. Carcinogenesis 29:1483–1492.

Wagner EF, Eferl R. 2005. Fos/AP-1 proteins in bone and the immune system. Immunol Rev 208:126–140.

Wright AA, Cook C, Abbott JH. 2009. Variables associated with the progression of hip osteoarthritis: A systematic review. Arthritis Rheum 61:925–936.

Yokota K, Miyazaki T, Hirano M, Akiyama Y, Mimura T. 2006. Simvastatin inhibits production of interleukin 6 (IL-6) and IL-8 and cell proliferation induced by tumor necrosis factor-alpha in fibroblast-like synoviocytes from patients with rheumatoid arthritis. J Rheumatol 33:463–471.

Yoshihara Y, Nakamura H, Obata K, Yamada H, Hayakawa T, Fujikawa K, Okada Y. 2000. Matrix metalloproteinases and tissue inhibitors of metalloproteinases in synovial fluids from patients with rheumatoid arthritis or osteoarthritis. Ann Rheum Dis 59:455–461.

Zheng H, Dai T, Zhou B, Zhu J, Huang H, Wang M, Fu G. 2008. SDF-1alpha/CXCR4 decreases endothelial progenitor cells apoptosis under serum deprivation by PI3K/Akt/eNOS pathway. Atherosclerosis 201:36–42.