CCL5/CCR5 Axis Promotes IL-6 Production in Human Synovial Fibroblasts

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Running head: CCL5 induces IL-6 production

Key Words: IL-6; CCL5; CCR5; OA

ABSTRACT

OBJECTIVE: CCL5 (RANTES) was originally identified as a product of activated T cells and plays a crucial role in the inflammatory response. Here, we investigated the intracellular signaling pathways involved in CCL5-induced IL-6 production in human synovial fibroblast cells.

METHODS: CCL5-mediated IL-6 expression was assessed with qPCR and ELISA. The mechanisms of action of CCL5 in different signaling pathways were studied using Western blotting. Knockdown of CCR5 and PKCδ protein was achieved by transfecting of siRNA. Chromatin immunoprecipitation assays were used to study *in vivo* binding of c-jun to the IL-6 promoter. Transient transfection was used to examine IL-6 and AP-1 activity.

RESULTS: Osteoarthritis synovial fibroblasts (OASF) showed significant expression of CCL5 and CCR5, and expression was higher than that in normal SF. Stimulation of OASF with CCL5 induced concentration- and time-dependent increases in IL-6 production. CCL5-mediated IL-6 production was attenuated by CCR5 monoclonal antibody, CCR5 inhibitor (Met-RANTES), and CCR5 siRNA. Pretreatment with a PKCδ inhibitor (rottlerin), c-Src inhibitor (PP2), or AP-1 inhibitor (tanshinone IIA) also blocked the potentiating action of CCL5. Treatment of OASF with CCL5 increased the accumulation of phosphorylated c-Jun in the nucleus, AP-1-luciferase activity, and c-Jun binding to the AP-1 element on the IL-6 promoter. CCL5-mediated AP-1-luciferase activity and c-Jun binding to the AP-1 element were inhibited by Met-RANTES, rottlerin, and PP2.

CONCLUSION: Our results suggest that the interaction between CCL5 and CCR5 increases IL-6 production in human synovial fibroblasts via the PKCδ/c-Src/c-Jun and AP-1 signaling pathway.

INTRODUCTION

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 (1). In response to macrophage-derived proinflammatory cytokines such as interleukin (IL)-1β and tumor necrosis factor-α (TNF-α), OA synovial fibroblasts (OASF) produce chemokines that promote inflammation, neovascularization, and cartilage degradation via activation of matrix-degrading enzymes such as matrix metalloproteinases (2). 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 (3).

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 (4, 5). 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 (4, 5). In addition to these roles in pathogen-specific inflammation and immunity, IL-6 levels are elevated in chronic inflammatory conditions, such as rheumatoid arthritis (RA) (6, 7). 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 (8, 9). AP-1 is commonly activated in response to inflammatory stimuli and has been implicated in cytokine expression and cellular immune responses (10, 11). Moreover, c-Jun/AP-1 has been implicated in human inflammatory disease (12), suggesting that AP-1 may be an important

mediator in intestinal and inflammatory cells.

RANTES (Regulated upon Activation Normal T cell Expressed and Secreted; also known as CCL5) was originally recognized as a product of activated T cells (13). Now widely established as an inflammatory chemokine, CCL5 mediates chemotactic activity in T cells, monocytes, dendritic cells, natural killer cells, eosinophils, and basophils (14-16). CCL5 is associated with chronic inflammatory diseases such as RA, inflammatory bowel disease, and cancer (17, 18). Synovial fluid obtained from patients with RA shows high CCL5 levels, and high serum concentrations of CCL5 have been associated with rapid progression of radiographic changes (19). The percentage of lymphocytes and monocytes expressing CCR5 (receptor for CCL5) in the synovial fluid exceeds by many fold the value observed in peripheral blood (20, 21).

Previous studies have shown that CCL5 promotes the inflammatory response (22, 23). Although a role for CCL5 in IL-6 induction has been implicated in some cell types, the signaling pathway for CCL5 in IL-6 production in synovial fibroblasts has not been extensively studied. In this study, we explored the intracellular signaling pathway involved in CCL5-induced IL-6 production in human synovial fibroblasts. The results show that CCL5 activates CCR5 and causes the activation of the PKCδ, c-Src, 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 β-actin, PKCδ, c-Src, c-Jun, p-c-Jun, lamin B, and the small interfering RNAs (siRNAs) against CCR5, 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). ON-TARGET smart pool PKCδ siRNA and ON-TARGET plus siCONTROL nontargeting pool siRNA were purchased from Dharmacon (Lafayette, CO). Rabbit polyclonal antibodies specific for PKCδ phosphorylated at Thr^{505} and c-Src phosphorylated at Tyr^{416} were purchased from Cell Signaling and Neuroscience (Danvers, MA). GF109203X, rottlerin, and PP2 were purchased from Calbiochem (San Diego, CA). Tanshinone IIA was purchased from BIOMOL (Butler Pike, PA). Mouse monoclonal antibodies specific for CCR5 and Met-RANTES were purchased from R&D Systems (Minneapolis, MN, USA). The IL-6 enzyme immunoassay kit was purchased from Cayman Chemical (Ann Arbor, MI). Recombinant human CCL5 was purchased from PeproTech (Rocky Hill, NJ). The AP-1 luciferase plasmid was purchased from Stratagene (La Jolla, CA). The c-Src dominant negative mutant was a gift from Dr. S. Parsons (University of Virginia Health System, Charlottesville, VA). 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 gifts from 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 obtained 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 15 patients with OA and 8 samples of normal

synovial tissues obtained at arthroscopy from trauma/joint derangement. The synovial fluid concentration of CCL5 was measured with an enzyme-linked immunosorbent assay (ELISA) according to the protocol provided by the manufacturer (Human CCL5 ELISA kit, Alpco Diagnostics; Salem, NH, USA). 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). Fibroblasts from passages four to nine were used for the experiments (24, 25).

Measurement of IL-6 production. Human synovial fibroblasts were cultured in 24-well culture plates. After reaching confluency, cells were treated with CCL5 and then incubated in a humidified incubator at 37°C for 24 hr. To examine the downstream signaling pathways involved in CCL5 treatment, cells were pretreated with various inhibitors for 30 min (the inhibitors were left in the culture medium) before addition of CCL5 (3 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 (24, 25).

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, Madison, WI) and following the manufacturer's recommended procedures. cDNA synthesis was performed in a final volume of 20 μl containing 4 μl of 5 X buffer, 1 μl of dNTPs (mixture of dATP, dCTP, dGTP, and dTTP), 1 μl of oligo (dT), 20 U of RNasin ribonuclease inhibitor, 2 μg of template, 200 U of MMLV, and DEPC-treated water. The reverse transcription

reaction mixture was incubated at 37°C for 60 min and then at 70°C for 5 min to inactivate MMLV. 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). IL-6 mRNA levels were normalized to β-actin mRNA levels and expressed relative to control using the ΔΔCt method.

Western blot analysis. Cellular lysates were prepared as described (24, 25). Proteins were resolved using SDS-PAGE and transferred to Immobilon polyvinyldifluoride membranes. The membranes were blocked with 4% BSA for 1 hr at room temperature and then probed with rabbit antibodies against human PKCδ, p-PKCδ, c-Src, or p-c-Src (1:1000) for 1 hr at room temperature. After three washes, the blots were incubated with a donkey anti-rabbit peroxidase-conjugated secondary antibody (1:1000) for 1 hr at room temperature. The blots were visualized with enhanced chemiluminescence on Kodak X-OMAT LS film (Eastman Kodak, Rochester, NY).

Kinase activity assay. PKCδ and c-Src activity were assessed with a PKC Kinase Activity Assay Kit (Assay Designs, Ann Arbor, MI) or a c-Src Kinase Activity Assay kit (Abnova, Taipei, Taiwan) according to manufacturer's instructions. The kinase activity kits are based on a solid-phase ELISA that uses a specific synthetic peptide as a substrate for PKCδ or c-Src and a polyclonal antibody that recognizes the phosphorylated form of the substrate.

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 hr of transfection, the cells were incubated with the indicated reagents. After a further 24 hr of incubation, the medium was removed, and cells were washed once with cold PBS. To prepare lysates, 100 μl reporter lysis buffer (Promega, Madison, WI) 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 (25). 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 (25).

Statistics. Data were expressed as means ± S.E.M. 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

CCL5 induces IL-6 production in human synovial fibroblasts. Chemokines are involved in the pathology of OA (26). It has been shown that CCL5 enhances IL-6 secretion from RA synovial fibroblasts (27). Therefore, we examined the expression of CCL5 levels in samples from patients with OA. Concentrations of CCL5 in synovial fluid were significantly higher in patients with OA than in controls (Figure 1A). In addition, medium from OASF showed significant expression of CCL5, which was higher than that in medium from normal synovial fibroblasts (Figure 1B). The typical pathology of OA includes chronic inflammation of the synovium that is characterized by infiltration of inflammatory cells and synovial hyperplasia, especially of fibroblast-like synoviocytes. Therefore, we used human synovial fibroblasts to investigate the signaling pathways of CCL5 in the production of IL-6, encoded by an inflammatory response gene. Treatment of OASF with CCL5 (0.3–10 ng/ml) for 24 hr induced IL-6 production in a concentration-dependent manner (Figure 1C), and this induction occurred in a time-dependent manner (Figure 1D). After CCL5 (3 ng/ml) treatment for 24 hr, the amount of IL-6 released had increased in OASF cells (Figure 1D). In addition, stimulation of cells with CCL5 also led to increased expression of IL-6 mRNA in a concentration-dependent manner (Figure 1E). To further confirm that this stimulation-specific effect was mediated by CCL5 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 CCL5 (3 ng/ml)-induced IL-6 release from OASF cells (Figure 1F). To further determine whether CCL5-induced IL-6 expression required transcription or translation, OASF cells were stimulated with CCL5 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 1G&H, CCL5-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 CCL5 occurs at the transcriptional level in

human synovial fibroblasts.

Involvement of CCR5 receptor in the CCL5-mediated increase in IL-6 production. It has been reported that CCL5 exerts its effects through interaction with a specific receptors (28, 29). Stimulation of OASF with CCL5 increased the expression of CCR5 mRNA but not that of CCR1 or CCR3 (Figure 2A), suggesting that the amplification loop enhances the CCL5-CCR5 signaling pathway. Expression of mRNA and cell surface protein expression of CCR5 in OASF were significantly higher than in normal synovial fibroblasts (Figure 2B&C). Pretreatment of OASF cells with CCR5 mAb or a CCR5 inhibitor (Met-RANTES) reduced CCL5-increased IL-6 production (Figure 2D). In addition, transfection of cells with CCR5 siRNA reduced CCR5 protein expression (Figure 2E). Transfection of cells with CCR5 siRNA antagonized CCL5-increased IL-6 expression (Figure 2F). Therefore, an interaction between CCL5 and CCR5 is very important for IL-6 production in human synovial fibroblasts.

The PKCδ and c-Src signaling pathways are involved in the potentiating action of CCL5. Previous studies have shown that PKCδ plays a crucial role in regulating the expression of genes such as those encoding IL-6 and IL-8 (30, 31). To determine whether PKC isoforms are involved in CCL5-triggered IL-6 production, OASF cells were pretreated with either GF109203X, a pan-PKC inhibitor, or rottlerin, a selective PKCδ inhibitor (32), for 30 min and then incubated with CCL5 for 24 hr. As shown in Figure 3A&B, pretreatment with GF109203X or rottlerin reduced CCL5-induced IL-6 production and expression, suggesting that PKCδ may play a role in CCL5-induced IL-6 expression in OASF. Transfection with PKCδ siRNA specifically blocked protein expression of PKCδ (Figure 3C; upper panel). In addition, PKCδ siRNA also reduced CCL5-induced IL-6 expression (Figure 3C). We then directly measured phosphorylation of PKCδ in response to CCL5. Stimulation of OASF cells led to a significant increase in phosphorylation of PKCδ (Figure 3D). In addition, PKCδ

activity was also increased by CCL5 treatment in OASF cells in a time-dependent manner (Figure 3E). Pretreatment of cells with CCR5 Ab, Met-RANTES, or transfection of cells with CCR5 siRNA also reduced CCL5-mediated PKCδ kinase activity (Figure 3F). Based on these results, CCL5 appears to act through a CCR5- and PKCδ-dependent signaling pathway to enhance IL-6 production in human synovial fibroblasts.

PKCδ–dependent c-Src activation has been reported to regulate COX-2 expression (33). We thus investigated the role of Src in mediating CCL5-induced IL-6 expression using the specific Src inhibitor PP2. As shown in Figure 4A&B, CCL5-induced IL-6 expression was markedly attenuated by pretreatment of cells for 30 min with PP2 or transfection of cells for 24 hr with a c-Src mutant. The major phosphorylation site of c-Src at the Tyr^{416} residue results in activation from c-Src autophosphorylation (34). To directly confirm the crucial role of Src in IL-6 expression, we measured the level of Src phosphorylation at Tyr^{416} in response to CCL5. Indeed, treatment of fibroblasts with CCL5 resulted in a time-dependent phosphorylation of c-Src at Tyr^{416} (Figure 4C). Next, we directly examined c-Src kinase activity in response to CCL5. Stimulation of cells with CCL5 also increased the kinase activity of c-Src in a time-dependent manner (Figure 4D). We next evaluated the relationship among CCR5, PKCδ, and c-Src in the CCL5-mediated signaling pathway and found that pretreatment of cells for 30 min with CCR5 Ab, Met-RANTES, or rottlerin markedly inhibited the CCL5-induced c-Src kinase activity (Figure 4E). Based on these results, CCL5 appears to act via CCR5 receptor and the PKCδ- and c-Src-dependent signaling pathway to enhance IL-6 production in human synovial fibroblasts.

Involvement of AP-1 in CCL5-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 (8, 9). 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 CCL5-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 (Figure 5A). The role of AP-1 was further established with the AP-1 inhibitor, tanshinone IIA, which blocked the enhancement of IL-6 production induced by CCL5 (Figure 5B). Therefore, the AP-1 binding site is more important than the NF-κB and C/EBP-β sites in CCL5-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 (9). 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 CCL5 resulted in a marked accumulation of phosphorylated c-Jun in the nucleus (Figure 5C). Transfection of cells with c-Jun siRNA suppressed the expression of c-Jun (Figure 5D). CCL5-induced IL-6 expression was also inhibited by c-Jun siRNA but not by control siRNA (Figure 5E).

We next investigated whether c-Jun binds to the AP-1 element on the IL-6 promoter after CCL5 stimulation. The *in vivo* recruitment of c-Jun to the IL-6 promoter (-312 to -39) was assessed via chromatin immunoprecipitation assay (25). *In vivo* binding of c-Jun to the AP-1 element of the IL-6 promoter occurred after CCL5 stimulation (Figure 6A). The binding of c-Jun to the AP-1 element by CCL5 was attenuated by Met-RANTES, rottlerin, and PP2 (Figure 6A). To further confirm that the AP-1 element is involved in CCL5-induced IL-6 expression, we performed transient transfection with AP-1 promoter-luciferase constructs. Synovial fibroblasts incubated with CCL5 showed a 3.3-fold increase in AP-1 promoter activity. The increase in AP-1 activity by CCL5 was antagonized by Met-RANTES, rottlerin, and PP2 or CCR5, PKCδ and c-Jun siRNA or c-Src mutant (Figure 6B&C). Taken together, these data suggest that the activation of the CCR5, PKCδ, c-Src, c-Jun, and AP-1 pathway is required for the CCL5-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. CCL5 is secreted by several cell types such as endothelial cells, smooth muscle cells, macrophages, and activated T cells (23). Here we found that synovial fluid concentrations of CCL5 were significantly higher in patients with OA than in normal fluid samples. Therefore, CCL5 is a good indicator of OA pathology. We further identified IL-6 as a target protein for the CCL5 signaling pathway that regulates the cellular inflammatory response. We showed that potentiation of IL-6 by CCL5 requires activation of the CCR5 receptor, PKCδ, c-Src, and AP-1 signaling pathways. These findings suggest that CCL5 acts as an inducer of inflammatory cytokines such as IL-6 and enhances the inflammatory response in OA.

CC-chemokine regulated on activation, normal T-cell expression, and presumably secreted CCL5/RANTES mediates its biological activities through activation of the G protein–coupled receptors, CCR1, CCR3, or CCR5, and binds to glycosaminoglycans (35). Stimulation of OASF cells with CCL5 increased the mRNA expression of CCR5 but not CCR1 and CCR3. Therefore, CCR5 is more important than CCR1 and CCR3 in CCL5-mediated IL-6 production. The expression of the CCR5 receptor in human synovial fibroblasts is mostly unknown. Using qPCR analysis, we found that normal and OA synovial fibroblasts express CCR5 receptor. In addition, the expression of mRNA levels and cell surface expression of CCR5 in OASF were significantly higher than in normal synovial fibroblasts. Moreover, CCR5 Ab, Met-RANTES, and CCR5 siRNA reduced CCL5-mediated IL-6 production. Therefore, the interaction between CCL5 and CCR5 is very important for IL-6 production by human synovial fibroblasts.

Several isoforms of PKC have been characterized at the molecular level and have been found to mediate several cellular molecular responses (36). We demonstrated

that the PKC inhibitor GF109203X antagonized the CCL5-mediated potentiation of IL-6 expression, suggesting that PKC activation is an obligatory event in CCL5-induced IL-6 production in these cells. In addition, rottlerin (a specific PKCδ inhibitor) also inhibited CCL5-induced IL-6 production. This was confirmed by the observation that PKCδ siRNA inhibited the enhancement of IL-6 production in synovial fibroblasts. Incubation of OASF cells with CCL5 also increased PKCδ phosphorylation and kinase activity. On the other hand, CCR5 Ab, Met-RANTES, and CCR5 siRNA reduced CCL5-mediated PKC kinase activity. These data suggest that the CCR5 and PKCδ pathways are required for CCL5-induced IL-6 production. Src, a tyrosine kinase, plays a critical role in the induction of chemokine transcription (37). In human synovial fibroblasts, thrombin induces IL-6 expression via c-Src activation (38). Because c-Src has been reported to be a downstream effector of PKCδ (33), we examined the potential role of c-Src in the signaling pathway of CCL5-induced IL-6 expression. Treatment of cells with the c-Src inhibitor PP2 or transfection of cells with a c-Src mutant reduced CCL5-mediated IL-6 production. In addition, we also found that treatment of synovial fibroblasts with CCL5 increased both c-Src phosphorylation at Tyr^{416} and c-Src kinase activity. These effects were inhibited by CCR5 Ab, Met-RANTES and rottlerin, indicating the involvement of CCR5- and PKCδ-dependent c-Src activation in CCL5-mediated IL-6 induction. Taken together, our results provide evidence that CCL5 up-regulates IL-6 in human synovial fibroblasts via the CCR5/PKCδ/c-Src signaling pathway. A similar signaling pathway has also been reported for COX-2 expression, which involves PKCδ-dependent c-Src activation (33). Therefore, this pathway may be important in regulating inflammatory mediators. Whether the other signaling pathway is involved in CCL5-mediated IL-6 production requires further investigation.

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 (8, 9) . 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 (39). The results of our current study show that AP-1 activation contributes to CCL5-induced IL-6 production in synovial fibroblasts. Deletion of the AP-1 site reduced CCL5-mediated IL-6 promoter activity. Pretreatment of cells with an AP-1 inhibitor also reduced CCL5-increased IL-6 production. Therefore, the AP-1 binding site is likely to be the most important site for CCL5-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 CCL5 induced c-Jun nuclear accumulation. In addition, c-Jun siRNA abolished CCL5-induced IL-6 production in OASF cells. Therefore, c-Jun activation mediates by CCL5-increased IL-6 expression. Furthermore, CCL5 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 Met-RANTES, rottlerin, and PP2. Using transient transfection with AP-1-luciferase as an indicator of AP-1 activity, we also found that CCL5 induced an increase in AP-1 activity. In addition, Met-RANTES, rottlerin, and PP2 or CCR5, PKCδ, and c-Jun siRNA or a c-Src mutant reduced CCL5-increased AP-1 promoter activity. These results indicate that the interaction between CCL5 and CCR5 may act through the PKCδ, c-Src, c-Jun, and AP-1 pathway to induce IL-6 activation in human OASF cells.

In conclusion, we explored the signaling pathway involved in CCL5-induced IL-6 production in human synovial fibroblasts. We found that CCL5 increased IL-6 production by binding to the CCR5 receptor and activating PKCδ and c-Src, which enhanced binding of c-Jun to the AP-1 site and resulted in the transactivation of IL-6 production (Figure 6D). These findings may provide a better understanding of the mechanisms of OA pathogenesis.

ACKNOWLEDGMENTS

We thank Dr. S. Parsons for providing the c-Src mutant and Dr. Oliver Eickelberg for providing the IL-6 promoter plasmids. We also thank the Biostatistic Center of China Medical University for statistical supported.

FOOTNOTES

This work was supported by grants from the National Science Council of Taiwan (97-2320-B-039-031-MY3; NSC99-2320-B-039-003-MY3) and China Medical University (CMU98-Tang-Lab).

ABBREVIATIONS

RANTES, Regulated upon Activation Normal T cell Expressed and Secreted; OA, osteoarthritis; OASF, osteoarthritis synovial fibroblasts; RA, rheumatoid arthritis; IL, interleukin; TNF, tumor necrosis factor; siRNA, small interference RNA; qPCR, quantitative real time PCR; ELISA, enzyme-linked immunosorbent assay

FIGURE LEGENDS

Figure 1 Concentration- and time-dependent increases in IL-6 production by CCL5.

(A) Synovial fluid was obtained from normal (n=7) or osteoarthritis patients (n=11) and examined with ELISA for the expression of CCL5. (B) Human synovial fibroblasts were cultured for 48 hr, and media were collected to measure CCL5. OASF were incubated with various concentrations of CCL5 for 24 hr (C) or with CCL5 (3 ng/ml) for 4, 8, 12, 18 or 24 hr (D). Media were collected to measure IL-6. (n =4). *: $p<0.05$ as compared with basal level. (E) OASF cells were incubated with CCL5 for 24 hr, and IL-6 mRNA was examined by qPCR. *: $p<0.05$ as compared with basal level. (F) OASF cells were pretreated with polymyxin B (Poly B, 1 μM) for 30 min followed by stimulation with LPS (1 μM) or CCL5 (3 ng/ml) for 24 hr *: $p<0.05$ as compared with LPS-treated group. OASF cells were pretreated with or without actinomycin D (Act. D, 1-10 μM; G) or cycloheximide (CHI, 1-10 μM; H) for 30 min and then incubated in the absence or presence of CCL5 for 24 hr. Media were collected to measure IL-6. (n =4). Media were collected to measure IL-6. (n =4). *: $p<0.05$ as compared with CCL5-treated group.

Figure 2 Involvement of the CCR5 receptor in CCL5-mediated IL-6 production in synovial fibroblasts.

(A) OASF cells were incubated with CCL5 (3 ng/ml) for 24 hr, and mRNA for CCR1, CCR3, and CCR5 was examined with qPCR. (B) Total RNA was extracted from normal SF and OASF cells and subjected to qPCR analysis for CCR5. (C) Normal SF and OASF cells were cultured for 2 days, and the cell surface expression of CCR5 was analyzed by flow cytometry. Cells were pretreated for 30 min with CCR5 Ab, Met-RANTES (0.5 μg/ml) (D) or transfected with CCR5 siRNA (F) for 24 hr followed by stimulation with CCL5 for 24 hr. Media were collected to measure IL-6. (E) Cells were

transfected with CCR5 siRNA for 24 hr, and the level of CCR5 protein was determined by Western blotting. (n =4). *: $p<0.05$ as compared with CCL5-treated group.

Figure 3 PKCδ is involved in CCL5-induced IL-6 production.

OASF cells were pretreated for 30 min with GF109203X (3 μM) or rottlerin (3 μM) followed by stimulation with CCL5 for 24 hr. Media and total RNA were collected, and the expression of IL-6 was analyzed with ELISA and qPCR (A&B). OASF cells were transfected with PKCδ or control siRNA for 24 hr, the protein levels of PKCδ was determined by Western blotting (C; upper panel). OASF cells were transfected with PKCδ siRNA or control siRNA for 24 hr and then stimulated with CCL5 for 24 hr. Media were collected to measure IL-6 (C; lower panel). Cells were incubated with CCL5 for indicated time intervals, and PKCδ phosphorylation was examined by Western blotting (D). Cells were incubated with CCL5 for indicated time intervals (E) or pretreated 30 min with CCR5 Ab and Met-RANTES or transfected with CCR5 siRNA for 24 hr, followed by stimulation with CCL5 for 60 mine, and PKCδ activity was determined by the PKCδ kinase kit (F). *: $p<0.05$ as compared with CCL5-treated group.

Figure 4 c-Src is involved in CCL5-mediated IL-6 production in synovial fibroblasts.

OASF cells were pretreated for 30 min with PP2 (3 μM) or transfected for 24 hr with the c-Src mutant (DN-c-Src) followed by stimulation with CCL5 for 24 hr. Media and total RNA were collected, and the expression of IL-6 was analyzed with ELISA and qPCR (A&B). Cells were incubated with CCL5 for indicated time intervals, and c-Src phosphorylation was examined by Western blotting (C). Cells were incubated with CCL5 for indicated time intervals (D) or pretreated for 30 min with CCR5 Ab, Met-RANTES, or rottlerin for 30 min, followed by stimulation with CCL5 for 60 min, and

c-Src kinase activity was determined with the c-Src kinase kit (E). *: $p<0.05$ as compared with CCL5-treated group.

Figure 5 AP-1 is involved in the potentiation of IL-6 production by CCL5.

(A) OASF cells were transfected with IL-6 luciferase plasmids before incubation with CCL5 for 24 hr. Luciferase activity was then assayed. (B) OASF cells were pretreated for 30 min with tanshinone IIA followed by stimulation with CCL5 for 24 hr. Media were collected to measure IL-6. (C) OASF cells were incubated with CCL5 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 hr, the protein levels of c-Jun was determined by Western blotting. (E) OASF cells were transfected with c-Jun or control siRNA for 24 hr, and then stimulated with CCL5 for 24 hr. Media were collected to measure IL-6. *: $p<0.05$ as compared with CCL5-treated group.

Figure 6 The CCR5/PKCδ/c-Src pathway is involved in CCL5-induced AP-1 activation.

(A) OASF cells were pretreated with Met-RANTES, rottlerin, or PP2, and then stimulated with CCL5 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 Met-RANTES, rottlerin and PP2 or cotransfected with CCR5, PKCδ or c-Jun siRNA or the c-Src mutant before incubation with CCL5 for 24 hr (B&C). Luciferase activity was then assayed. *: $p<0.05$ as compared with CCL5-treated group. (D) Schematic diagram of the signaling pathways involved in CCL5-induced IL-6 production in synovial fibroblasts. CCL5 increases IL-6 expression by

binding to the CCR5 receptor and activating PKCδ and c-Src, which enhances binding of c-Jun to the AP-1 site. This results in the transactivation of IL-6 expression.

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