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Chemokines Regulate IL-6 and IL-8 Production by Fibroblast-Like Synoviocytes from Patients with Rheumatoid Arthritis[1]

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Rheumatoid arthritis (RA) is characterized by proliferation of synoviocytes that produce inflammatory cytokines and chemokines. The expressed chemokines are thought to be involved in the migration of inflammatory cells into the synovium. In this study we show that CCL2/monocyte chemotactic protein-1, CCL5/RANTES, and CXCL12/stromal cell-derived factor-1 enhanced IL-6 and IL-8 production by fibroblast-like synoviocytes (FLS) from patients with RA, and their corresponding receptors, CCR2, CCR5, and CXCR4, respectively, were expressed by RA FLS. The chemokines stimulated RA FLS more effectively than skin fibroblasts. Culture with CCL2 enhanced phosphorylation of extracellular signal-related kinase 1 (ERK1) and ERK2, but not phosphorylation of p38 or Src. Moreover, activation of ERK1/2 was inhibited by pertussis toxin, a G_i-coupled protein inhibitor, and RS-504393, CCR2 antagonist, suggesting that ERK1/2 was activated by CCL2 via CCR2 and G_i-coupled protein. On the other hand, CCL2, CCL5, and CXCL12 were expressed on RA FLS, and their production was regulated by TNF-α, IL-1β, and TGF-β1. Our results indicate that the chemokines not only play a role in inflammatory cell migration, but are also involved in the activation of FLS in RA synovium, possibly in an autocrine or paracrine manner. ***The Journal of Immunology*, 2001, 167: 5381–5385.**

Approximately 40 chemokines have been identified to date (1). The roles of various chemokines in the migration of specific cell subsets have been intensively investigated (2). Chemokines can induce T cell adhesion by up-regulation of ICAM-1 (3) and can also costimulate T cells and monocytes to induce cytokines or activation markers (4–7). Thus, chemokine and chemokine receptor interaction plays an important role in the pathogenesis of inflammatory diseases.

Rheumatoid arthritis (RA),[3] a chronic inflammatory condition affecting several joints, is characterized by proliferation of synoviocytes in inflamed synovia and expression of inflammatory cytokines and chemokines on synoviocytes (8, 9). Thus, activated synoviocytes seem to contribute to the pathogenesis of RA. Several studies have examined the pathogenic role of chemokine and chemokine receptor interaction in RA (10–15). For example, CCL2 (formerly named as monocyte chemotactic protein-1) and CCL5 (RANTES) are expressed in RA synovium (10–13). Furthermore, we and other groups have recently demonstrated overexpression of CXCR4 by $CD4^+$ memory T cells in RA synovium, and that the unique ligand CXCL12 (stromal cell-derived factor-1) is expressed in RA synovium (14, 15). We also found that CXCL12 costimulates $CD4^+$ T cells to induce cytokine production, activation markers, and proliferation (4). These results suggest that chemokines such as CCL2, CCL5, and CXCL12 play important roles in inflammatory cell migration into RA synovium and stimulation of T cells. However, to our knowledge, the function of the chemokines on fibroblast-like synoviocytes (FLS) has not been analyzed.

The present study was designed to explore novel functions of chemokines in the RA synovium. Specifically, we examined the ability of CCL2, CCL5, and CXCL12 to stimulate FLS of RA patients.

Materials and Methods

Specimens

Synovial tissues were obtained at surgery from RA patients. Signed consent forms were obtained before the operation. The experimental protocol was approved by the ethics committee of the Tokyo Medical and Dental University. RA was diagnosed according to the American College of Rheumatology criteria (16). The synovial tissue was minced and incubated with 0.3 mg/ml collagenase (Sigma, St. Louis, MO) for 1 h at 37°C in DMEM (Sigma). Partially digested pieces of the tissue were pressed through a metal screen to obtain single-cell suspensions. Primary culture skin fibroblasts were established from healthy skin samples.

FACS analysis

FITC-conjugated anti-CD14 (322A-1, Beckman Coulter, Fullerton, CA) mAb, FITC-conjugated anti-HLA class II (9-49, Beckman Coulter) mAb, anti-CCR2 mAb (48607, 121, R&D Systems, Minneapolis, MN), anti-CCR5 mAb (45531.111; R&D Systems), and anti-CXCR4 mAb (44708.111; R&D Systems) were used. FLS from RA patients were adjusted to 1×10^5 cells, incubated with mAb for 30 min, and rinsed with PBS-3% FCS. To analyze chemokine receptor expression, PE-conjugated goat anti-mouse IgG (Southern Biotechnology Associates, Birmingham, AL) was used as a second Ab and analyzed with a FACSCalibur (BD Biosciences, San Jose, CA).

Cell culture

Cells were maintained in high glucose DMEM with 10% FCS (Sigma). RA FLS were used for experiments after five passages. CD14 or HLA class II

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[3] Abbreviations used in this paper: RA, rheumatoid arthritis; FLS, fibroblast-like synoviocytes; PTX, pertussis toxin; ERK, extracellular signal-related kinase; MAP, mitogen-activated protein; JNK, c-Jun amino-terminal kinase.

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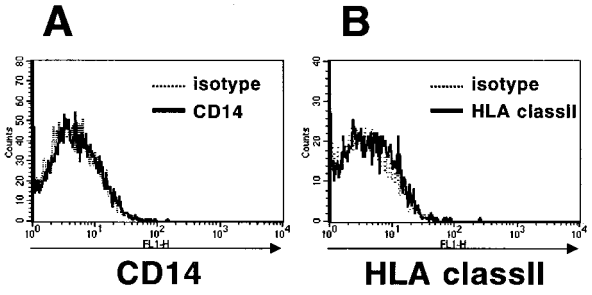

FIGURE 1. Characterization of FLS from patients with RA. RA FLS were stained with anti-CD14 mAb (*A*) or anti-HLA class II mAb (*B*), and the expression was analyzed by FACS. Representative data from one of four RA patients are shown.

were not expressed by RA FLS (Fig. 1), suggesting that macrophages and dendritic cells were not contained in the FLS.

RA FLS and primary culture skin fibroblasts (2×10^4 cells/well) were cultured separately in 96-well culture plate in DMEM with 10% FCS for 24 h. Then the medium was replaced with FCS-free DMEM, followed by further incubation of the cells for 24 h. For blocking CCL2 stimulation, 0.5 μg/ml pertussis toxin (PTX; Calbiochem, La Jolla, CA) or 20 μM RS-504393 (17) was added, and the cells were incubated at 37°C for 30 and 5 min, respectively. In the next step the cells were incubated in a medium supplemented where indicated with CCL2, CCL5 (Wako Pure Chemical Industries, Osaka, Japan), CXCL12, TNF-α, IL-1β, or TGF-β1 (R&D Systems). After incubation at 37°C for 24 h the production of cytokines and chemokines was analyzed as described below.

For Western blot analysis, RA FLS (8×10^5 cells/well) were cultured in 60-mm dishes. The stimulation procedure with CCL2 was same as that described above.

All reagents used in this experiment were certified as endotoxin free by the manufacturers. To confirm it, endotoxin level was measured by a *Limulus* test kit (Pyrogen; BioWhittaker, Walkersville, MD).

RT-PCR

Total RNA was prepared from RA FLS and primary culture skin fibroblasts using the RNeasy Total RNA System (Qiagen, Valencia, CA). The RNA was treated with DNase I (Qiagen). First-strand cDNA was synthesized using oligo(dT) primers and Superscript II reverse transcriptase (Life Technologies, Gaithersburg, MD). The amount of cDNA for amplification was adjusted by the amount of RNA measured by OD meter and also β-actin PCR products. The cDNA was amplified with primers for CCR2 (5′-TGG GAG TTT TGG TGG AGT CCG AT; 3′-GGG GGA TGT GGC CTA AGA AGC AT), CCR5 (5′-CTC AGG GAA TGA AGG TGT CAG A; 3′-TGC TAC TGT TGC ACT CTC CAC AAC T), CXCR4 (5′-GGA CCT GTG GCC AAG TTC TTA GTT; 3′-ACT GTA GGT GCT GAA ATC AAC CCA), and β-actin (5′-GTG GGG CGC CCC AGG CAC CA; 3′-CTC CTT AAT GTC ACG CAC GAT TTC). The PCR conditions were described previously (14). The PCR products were then separated by electrophoresis through 1.5% agarose.

Western blot

RA FLS were collected and lysed with extraction buffer (20 mM HEPES, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin). After 30 min at 4°C debris was eliminated by centrifugation at 14,000 rpm for 30 min, and the supernatant was collected. After measurement of protein concentration with a protein assay kit (Bio-Rad, Richmond, CA), cell lysates were mixed with 6× sample loading buffer containing 6% 2-ME and 10% SDS and stored at 4°C until analysis. Twenty micrograms of protein was separated by 10% SDS-PAGE, transferred to polyvinylidene difluoride membranes, and blocked with 0.05% Tween 20 and 5% BSA overnight. The immunoblots were incubated with anti-phospho-specific extracellular signal-related kinase 1/2 (ERK1/2) rabbit polyclonal Ab (0.5 μg/ml; BioSource, Camarillo, CA), anti-pan ERK1/2 rabbit polyclonal Ab (1/500; Santa Cruz Biotechnology, Santa Cruz, CA), anti-phospho-specific p38 rabbit polyclonal Ab (0.5 μg/ml; BioSource), anti-pan p38 rabbit polyclonal Ab (1/500; Santa Cruz Biotechnology), anti-phospho-specific Src rabbit polyclonal Ab (0.5 μg/ml; BioSource), or anti-pan Src rabbit polyclonal Ab (0.5 μg/ml; BioSource) in PBS with 1% BSA for 1 h. Subsequently, the immunoblots were incubated with protein A conjugated with HRP (Amersham Pharmacia Biotech, Piscataway, NJ) for 1 h. All immunoblots were detected by ECL (Amersham Pharmacia Biotech).

ELISA

Cytokine and chemokine concentrations in the culture supernatant were assayed with ELISA kits for IL-6, IL-8, CCL2, and CCL5 (BioSource). The CXCL12 concentration in the culture supernatant was measured by sandwich ELISA using anti-CXCL12 mAb (79018.111, R&D Systems) and biotinylated anti-CXCL12 Ab (R&D Systems).

Statistical analysis

All data were expressed as the mean ± SEM. Differences in cytokine and chemokine expression between groups were examined for statistical significance using Student's *t* test. A value of $p < 0.05$ denoted the presence of a statistically significant difference.

Results

Chemokine receptor expression on FLS of RA patients

CCR2, one of the receptors for CCL2; CCR5, one of the receptors for CCL5; and CXCR4, a unique receptor for CXCL12 mRNA expression on RA FLS and primary culture skin fibroblasts, were analyzed by RT-PCR, respectively. FLS from all three patients with RA and skin fibroblasts from two healthy donors expressed significant amounts of mRNAs of CCR2, CCR5, and CXCR4 (Fig. 2*A*). Surface CCR2, CCR5, and CXCR4 expression were also analyzed by FACS. FLS from all three patients with RA expressed CCR2, CCR5, and CXCR4 on the cell surface (Fig. 2*B*).

Stimulation of RA FLS by chemokines

FLS from RA patients spontaneously produced IL-6 and IL-8. Culture with CCL2 enhanced the production of IL-6 and IL-8 by RA FLS in a dose-dependent manner (Fig. 3). CCL5 and CXCL12 also enhanced IL-6 and IL-8 production by RA FLS, although the degree

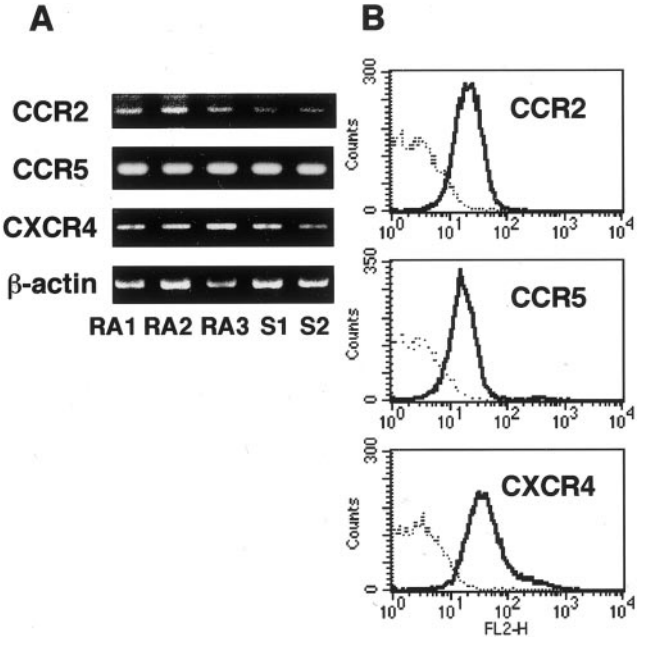

FIGURE 2. Expression of CCR2, CCR5, and CXCR4 on RA FLS and skin fibroblasts. Expression of CCR2, CCR5, CXCR4, and β-actin mRNAs by FLS from three patients with RA and primary culture skin fibroblasts from two healthy donors was analyzed using RT-PCR. PCR products were separated by electrophoresis through 1.5% agarose. RA1–3, RA FLS; S1–2, skin fibroblasts (*A*). Surface expression of CCR2, CCR5, and CXCR4 by RA FLS was analyzed using FACS. Representative data from one of three or four RA patients are shown (*B*). Dotted lines show staining by isotype-matched control mAb.

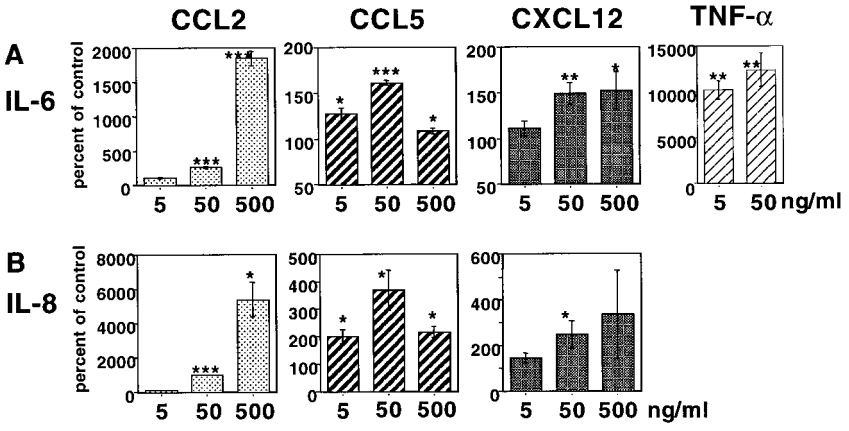

FIGURE 3. CCL2, CCL5, and CXCL12 enhanced IL-6 and IL-8 production by RA FLS. RA FLS (2×10^4 cells/well) were cultured in 96-well culture plate in DMEM with 10% FCS for 24 h. Then, the medium was replaced with FCS-free DMEM for 24 h. Subsequently, the cells were incubated in DMEM supplemented where indicated with CCL2, CCL5, CXCL12, or TNF-α at 37°C for 24 h. IL-6 and IL-8 concentrations in the culture supernatant were assayed by ELISA, and the percentage of control of IL-6 and IL-8 concentrations was calculated. Data are the mean (±SEM) values of three to seven independent experiments analyzed in duplicate (*A*, IL-6; *B*, IL-8). Concentrations of CCL2, CCL5, CXCL12, and TNF-α are indicated. *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0005$.

of the enhancement by CCL5 and CXCL12 was less than that by CCL2, especially when used at 500 ng/ml. Interestingly, CCL5 at 500 ng/ml was less effective than at 50 ng/ml in enhancing IL-6 and IL-8 production. TNF-α also enhanced IL-6 production by RA FLS.

The enhancement of IL-6 production by culture with CCL2 was not inhibited by polymyxin B (Fig. 4), suggesting that a significant amount of endotoxin was not contained in this stimulation procedure. In fact, endotoxin was not detected from any reagents used in this experiment (data not shown). On the other hand, PTX, a G_i-coupled protein inhibitor, partially, but significantly, inhibited CCL2-induced enhancement of IL-6 production by RA FLS (Fig. 5). However, PTX did not inhibit TNF-α-induced enhancement of IL-6 production.

IL-6 was also spontaneously produced by primary culture skin fibroblasts of healthy donors. However, the degree of CCL2- and CCL5-induced enhancement of IL-6 production by skin fibroblasts was less than that by RA FLS (Fig. 6).

Enhancement of ERK1/2 activation by stimulation with CCL2

The mitogen-activated protein (MAP) kinase is known to be important in cell activation. Phosphorylation of MAP kinase, including ERK1/2, p38 MAP kinases, and c-Jun amino-terminal kinase (JNK), induced by culture with CCL2 in RA FLS was analyzed. Phosphorylation of Src protein tyrosine kinase by CCL2 stimulation was also analyzed. ERK1/2, p38 MAP kinase, and Src were activated without any stimulation in RA FLS. Culture with CCL2 enhanced the activation of ERK1/2, but not that of p38 or Src (Fig. 7*A*). JNK was not activated without stimulation, and CCL2 did not induce JNK activation (data not shown). Enhancement of ERK1/2 activation by culture with CCL2 was inhibited by PTX, a G_i-coupled protein inhibitor, and RS-504393, a CCR2 antagonist (Fig. 7*B*).

Production of CCL2, CCL5, and CXCL12 by RA FLS

RA FLS spontaneously produced CCL2 and CXCL12. TNF-α, IL-1β, and TGF-β1 enhanced CCL2 expression by RA FLS, although the degree of the enhancement by TGF-β1 was less than that by TNF-α and IL-1β (Fig. 8). CCL5 expression was not detected on RA FLS under this condition without any stimulation. However, culture with TNF-α and IL-1β, but not that with TGF-β1, induced CCL5 expression. CXCL12 expression was enhanced by TGF-β1, but not by TNF-α or IL-1β (Fig. 8).

Discussion

The major finding of the present study was that CCL2, CCL5, and CXCL12 differentially regulated IL-6 and IL-8 production by FLS of patients with RA. Our results also showed that these chemokines and corresponding receptors were expressed on RA FLS, and that such expression was regulated by TNF-α, IL-1β, and TGF-β1. Thus, our results indicate that the chemokines are not only involved in inflammatory cell migration, but also in the regulation of

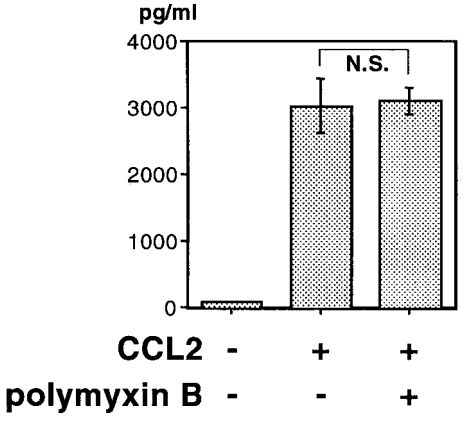

FIGURE 4. Polymyxin B did not inhibit CCL2-induced IL-6 production. RA FLS were incubated in medium supplemented where indicated with 500 ng/ml CCL2 and 50 mg/ml polymyxin B. IL-6 concentrations in the culture supernatant were assayed by ELISA. Representative mean (±SEM) data from one of three independent experiments analyzed in duplicate are shown. The presence of CCL2 and polymyxin B is indicated. N.S., not significant.

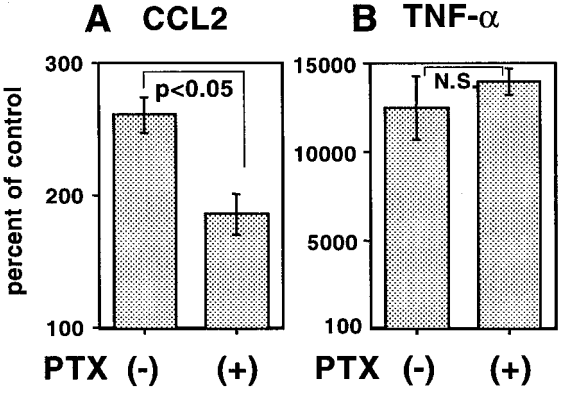

FIGURE 5. PTX inhibited stimulation of RA FLS by CCL2. RA FLS were incubated with 0.5 μg/ml PTX for 30 min at 37°C before stimulation with 50 ng/ml CCL2 (*A*) or TNF-α (*B*). The IL-6 concentration in the culture supernatant was assayed by ELISA, and the percentage of control of the IL-6 concentration was calculated. Data are the mean (±SEM) values of three independent experiments analyzed in duplicate. N. S., not significant.

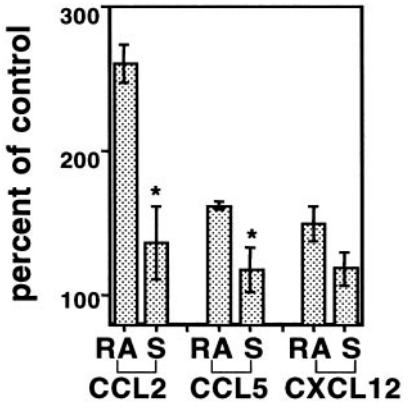

FIGURE 6. Chemokines stimulate RA FLS more effectively than they do skin fibroblasts. RA FLS and primary culture skin fibroblast were stimulated by 50 ng/ml CCL2, CCL5, and CXCL12. IL-6 concentrations in the culture supernatants were assayed by ELISA, and the percentage of the control of the IL-6 concentration was calculated. Data are the mean (±SEM) values of two to seven independent experiments analyzed in duplicate. RA, RA FLS; S, primary culture skin fibroblasts. *, $p < 0.05$.

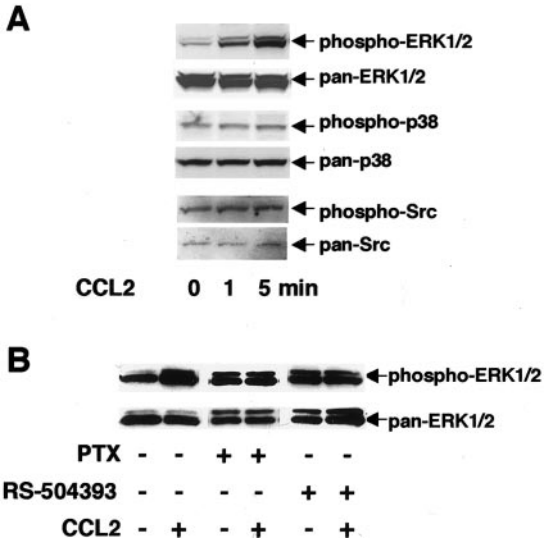

FIGURE 7. CCL2 enhanced activation of ERK1/2. RA FLS (8×10^5 cells/well) were incubated in 60-mm dishes with 50 ng/ml CCL2 for varying time intervals. Cell lysates were analyzed by Western blot with anti-phospho-specific ERK1/2 Ab, anti-pan ERK1/2 Ab, anti-phospho-specific p38 Ab, anti-pan p38 Ab, anti-phospho-specific Src Ab, and anti-pan Src Ab (*A*). RA FLS were incubated with 0.5 μg/ml PTX or 20 μM RS-504393; subsequently 50 ng/ml CCL2 was supplied, and cells were incubated for 5 min. Cell lysates were analyzed by Western blot with anti-phospho-specific ERK1/2 Ab and anti-pan ERK1/2 Ab (*B*). Representative data from one of two to four independent experiments are shown.

RA FLS activation in RA synovium, possibly in an autocrine or paracrine manner.

Previous studies have shown that CCL2 is expressed in RA synovium (10, 11) and is thought to induce monocyte migration into inflamed RA synovium. CCL5 is also detected in RA synovium (12, 13) and is speculated to induce T cell migration into RA synovium. Furthermore, CXCL12 expression is found in RA synovium (14, 15) and induces $CD4^+$ memory T cell accumulation. CCL2, CCL5, and CXCL12 also costimulate T cells (4–6). Hence, these chemokines seem to play important roles in the migration of inflammatory cells into RA synovium and stimulation of T cells. The present study showed that chemokines stimulated IL-6 and IL-8 production by RA FLS, and that these chemokines were expressed on RA FLS in association with their corresponding receptors. Taken together, it is likely that CCL2, CCL5, and CXCL12 stimulate FLS to enhance cytokine and chemokine production in an autocrine or paracrine manner in RA synovium. Moreover, CCL2 and CCL5 are also expressed by macrophage and T cells, respectively, in RA synovium (10, 13), suggesting that the expressed chemokines could be relevant molecules to costimulate RA FLS by macrophages and T cells in RA synovium. TNF-α enhanced IL-6 production by RA FLS more effectively than chemokines. However, since chemokines bind surface proteoglycans (18), they are sequestered and presented to target cells at high concentration within the local microenvironment. Thus, chemokine could contribute to RA FLS activation.

Interestingly, chemokines showed differential effects on RA FLS. For example, CCL2 stimulated RA FLS more effective than CCL5 and CXCL12. Moreover, stimulation by CCL2 and CXCL12, but not CCL5, was dose dependent. In contrast, high concentrations of CCL5 were less effective for enhancement of IL-6 and IL-8 production. Thus, the chemokines might differentially regulate IL-6 and IL-8 production by FLS in RA synovium. In addition, the sensitivity of RA FLS to produce IL-6 in response to CCL2 and CCL5 was increased compared with that of skin-derived fibroblasts. In this respect, our group has reported increased inducibility of $p16^{INK4a}$, a cyclin-dependent kinase inhibitor, in RA FLS, but not in normal skin fibroblasts (19). It might therefore be possible that RA FLS, which exist in a proinflammatory milieu, are intrinsically different from skin fibroblasts.

The current results showed that culture with CCL2 enhanced activation of ERK1/2, but not p38 MAP kinase, JNK, and Src. Moreover, enhancement of ERK1/2 activation was inhibited by PTX and RS-504393. Thus, it was suggested that CCL2 enhanced activation of ERK1/2, via CCR2 and G_i-coupled protein and probably activation of ERK1/2 induced IL-6 and IL-8 production by RA FLS. It was reported that ERK1/2 activation was maximum at 30 s after CXCL12 stimulation on CTS cells (20). However, the current data showed that CCL2-induced enhancement of ERK1/2

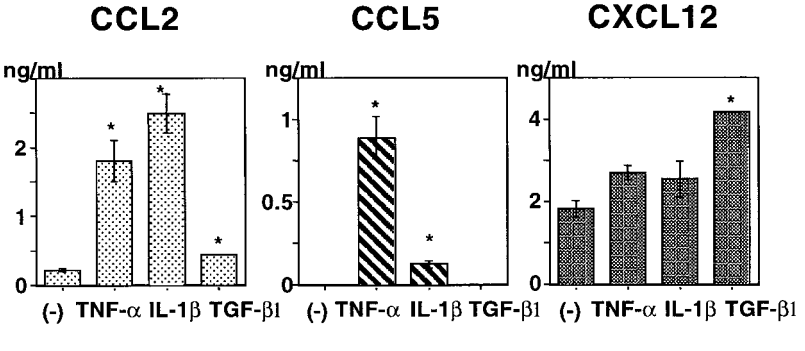

FIGURE 8. Expression of CCL2, CCL5, and CXCL12 on RA FLS. RA FLS were stimulated with 10 ng/ml TNF-α, IL-1β, or TGF-β1. CCL2, CCL5, and CXCL12 concentrations in the culture supernatant were assayed by ELISA. Data are the mean (±SEM) values of three independent experiments analyzed in duplicate. *, $p < 0.05$.

activation on RA FLS was relatively delayed. In this regard it was reported that treatment of CTS cells with CXCL12 resulted in delayed activation of ERK1/2 under stem cell factor stimulation (20). Thus, it is possible that CCL2 may costimulate RA FLS with other factors expressed by RA FLS.

The current data showed that PTX partially inhibited CCL2-induced IL-6 production by RA FLS. It is possible that CCL2 stimulation may be mediated by both PTX-dependent and -independent pathways on RA FLS. In this regard it was reported that CCL5 stimulation involved both PTX-dependent and -independent pathways on T cells (6).

RA FLS are thought to be involved in the pathogenesis of RA (9, 21, 22). These cells produce several types of cytokines and chemokines, such as IL-6, IL-8, GM-CSF, G-CSF, vascular endothelial growth factor, CCL3 (macrophage inflammatory protein-1α), CCL2, CCL5, and CXCL12. IL-6 is an acute phase inflammatory cytokine and augments erosion of the bone. Blockade of IL-6 signal transduction by anti-IL-6R mAb can be potentially useful therapeutically in RA (23). IL-8, which is a CXC chemokine (CXCL8), exhibits proangiogenic activity and induces blood vessel formation and angiogenesis (9). IL-8 also displays a chemoattractant activity for neutrophils and dendritic cells (24, 25). Thus, IL-6 and IL-8, whose production was up-regulated by chemokines, might contribute to the development of RA.

It has been demonstrated that stimulation by CCL2 enhances collagen expression on rat lung fibroblasts (26) and matrix metalloproteinase expression on human primary culture skin fibroblasts and a stable fibroblast cell line (27, 28). The present results showed that not only CCL2, but also CCL5 and CXCL12, stimulated FLS of RA patients to produce IL-6 and IL-8. Such stimulation by the chemokines was more effective in RA FLS compared with skin fibroblasts.

It was reported that the expression of CCL2 on RA FLS is up-regulated by TNF-α, IL-1β, and TGF-β1 (10, 11), and CCL5 expression is up-regulated by TNF-α and IL-1β (12). Our results confirmed the previous studies and also showed that TGF-β1, but not TNF-α and IL-1β, enhanced CXCL12 production by RA FLS. Considered together, these results suggest that each cytokine differentially regulates chemokine expression. Since TNF-α, IL-1β, and TGF-β1 are expressed mainly on macrophages in RA synovium (29), it is speculated the cytokines expressed by macrophages, in turn, stimulate FLS to induce chemokines.

In conclusion, we demonstrated in the present study that CCL2, CCL5, and CXCL12 regulated IL-6 and IL-8 production by RA FLS. Our results suggested that these chemokines not only act as a chemoattractant for inflammatory cells and a costimulator of $CD4^+$ T cells, but also stimulate FLS to induce cytokine and chemokine production by RA synovium. Modulation of the production of these chemokines or their interactions with their receptors could be a new mode of treatment for RA.

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