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

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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 Gi-coupled protein inhibitor, and RS-504393, CCR2 antagonist, suggesting that ERK1/2 was activated by CCL2 via CCR2 and Gi-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.

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2 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.

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.

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 × 10⁶ 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 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.

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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 were cultured separately in 96-well culture plate in DMEM with 10% FCS for 24 h. For blocking CCL2 stimulation, 0.5 μg/ml loading buffer containing 6% 2-ME and 10% SDS and stored at 4 °C until use. After blocking, the cells were incubated in a medium supplemented 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 AAG AGC AT), CCR5 (5'-CTC AGG GAA TGA AGG TGT CAG A; 3'-ACT TAC TGT TGC ACT CTC CAC AAC T), CXCR4 (5'-GGG CAT GTG GCC AAC AGG TTC TTA GTT; 3'-ACT GTA GGT GCT GAA ATC AAC CCA), and β-actin (5'-GTG GGG GCC CCC AGG CAC CA; 3'-CTC CTG AAT GTA ACGCAC CAT 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 test kit (Pyrogen; BioWhittaker, Walkersville, MD), 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), anti-phospho-p42/44 MAPK rabbit polyclonal Ab (0.5 μg/ml; BioSource), anti-phospho-p38 rabbit polyclonal Ab (1/500; Santa Cruz Biotechnology), anti-phospho-specific Src rabbit polyclonal Ab (0.5 μg/ml; BioSource), or anti-phospho-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. 2A). 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. 2B).

**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 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. 7A). 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. 7B).

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 inflammatory and immune cell responses.

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.
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 stimulate 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. 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 Gα-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
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 could 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 chemotactic 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. Similar 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 chemotactrant 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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