Proinflammatory Cytokines Synergistically Enhance the Production of Chemokine Ligand 20 (CCL20) from Rheumatoid Fibroblast-like Synovial Cells *in vitro* and Serum CCL20 Is Reduced *in vivo* by Biologic Disease-modifying Antirheumatic Drugs

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ABSTRACT. ***Objective.*** Chemokine ligand 20 (CCL20) is a selective ligand for chemokine receptor 6 (CCR6). We investigated, both *in vitro* and *in vivo*, whether CCL20 is critically involved in the disease process of rheumatoid arthritis (RA).

Methods. *In vitro* study investigated the effect of proinflammatory cytokines and biologic disease-modifying antirheumatic drugs (DMARD) on the production of CCL20 by rheumatoid fibroblast-like synovial cells (FLS). The *in vivo* role of CCL20 was studied by screening for serum CCL20 concentration in patients with RA during the therapeutic course of biologic DMARD, i.e., infliximab, etanercept, and tocilizumab.

Results. Spontaneous CCL20 production from rheumatoid FLS was minimal; however, its production was significantly stimulated by interleukin 1ß (IL-1ß), tumor necrosis factor-α (TNF-α), or IL-17. IL-1ß was the most potent for stimulating the production of CCL20. CCL20 production was synergistically augmented by a combination of IL-1ß, TNF-α, and IL-17. In contrast, interferon-γ suppressed IL-1ß-induced CCL20 production. IL-6, in combination with soluble IL-6 receptor (sIL-6R), did not modulate CCL20 production, whereas IL-1ß-induced, TNF-α-induced, and IL-17-induced production were increased by IL-6. These production levels were clearly suppressed by biologic DMARD *in vitro*. Serum CCL20 was significantly higher in RA than in control subjects, and was clearly decreased by the treatment with infliximab, etanercept, and tocilizumab.

Conclusion. Proinflammatory cytokines modulate the production of CCL20 from FLS. Our data suggest that therapeutic efficacy of biologic DMARD may result from the inhibition of CCL20 production in rheumatoid synovium. (J Rheumatol First Release Oct 1 2009; doi:10.3899/jrheum.090132)

Key Indexing Terms:

BIOLOGIC DISEASE-MODIFYING ANTIRHEUMATIC DRUGS
CHEMOKINE LIGAND 20 FIBROBLAST-LIKE SYNOVIAL CELLS
PROINFLAMMATORY CYTOKINES RHEUMATOID ARTHRITIS

Chemokine ligand 20 (CCL20) is a selective ligand for chemokine receptor 6 (CCR6)[1]. The expression pattern of CCR6 has been revealed in recent investigations; interleukin 17 (IL-17)-producing helper T cells (TH17 cells) highly express CCR6 and also synthesize CCL20[2-4]. TH17 cells are supposed to accumulate in the rheumatoid synovi-

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Accepted for publication June 5, 2009.

um[5-7], and the protein concentrations of CCL20 in both peripheral blood and synovial fluid are high in patients with rheumatoid arthritis (RA)[2,8-11]. These data suggest an interaction of CCL20 with CCR6 in TH17 cells that may perpetuate the chronic inflammatory process of RA. Previous studies have found an inducible effect of proinflammatory cytokines on CCL20 synthesis from fibroblast-like synovial cells (FLS)[8-10]. Since the blockage of the cytokine network by biologic disease-modifying antirheumatic drugs (DMARD) including infliximab, etanercept, and tocilizumab shows remarkable therapeutic efficacy for patients with RA[12-14], the proinflammatory cytokine-biologic DMARD interaction in CCL20 is supposed to be present, although few reports are available.

We showed in our study that FLS-derived CCL20 production is synergistically induced by proinflammatory cytokines, but is inhibited by biologic DMARD and interferon-γ (IFN-γ), which may be reflected in the decrease of serum CCL20 during treatment with infliximab, etanercept, and tocilizumab.

MATERIALS AND METHODS

RA patient samples for the isolation of FLS. Rheumatoid FLS for use in *in vitro* experiments was obtained from 14 patients with RA in the Department of Orthopedic Surgery, Graduate School of Biomedical Sciences, Nagasaki University and Japanese Red Cross Nagasaki Genbaku Hospital at the time of orthopedic surgery. They gave their informed consent to the protocol that was approved by the Institutional Review Board of Nagasaki University and Japanese Red Cross Nagasaki Genbaku Hospital. All patients fulfilled the 1987 criteria of the American College of Rheumatology (ACR)[15] for RA.

Detection of CCL20 in the culture supernatants of FLS in vitro. Rheumatoid FLS were isolated from 14 patients with RA. FLS were grown in 24-well plates (Becton Dickinson, Franklin Lakes, NJ, USA) at 100,000 cells/ml for 48 h at 37°C in a 5% CO_2 atmosphere in RPMI-1640 medium (Wako, Osaka, Japan) supplemented with 10% FBS/1% penicillin/streptomycin (Gibco-BRL, Grand Island, NY, USA). Proinflammatory cytokines of recombinant human IL-1ß (range 1–1000 pg/ml), IL-17 (range 1–1000 ng/ml), IL-6 (100 ng/ml), soluble IL-6 receptor (IL-6R; 100 ng/ml; R&D Systems, Abingdon, UK), and tumor necrosis factor-α (TNF-α; range 1–1000 ng/ml; Upstate Biochemical Co., Lake Placid, NY, USA) were added into the culture either singly or in a variety of combinations for 48 h. The protein concentration of CCL20 was examined using a commercial ELISA detection kit (Quantikine, R&D Systems). In some experiments, FLS were cultured in the presence of proinflammatory cytokines with biologic DMARD [infliximab (Centocor, Malvern, PA, USA), 500 μg/ml; etanercept (Wyeth, Madison, NJ, USA), 500 μg/ml; tocilizumab (Chugai, Tokyo, Japan), 500 μg/ml], and the CCL20 concentration was examined. The effect of IFN-γ (Shionogi, Osaka, Japan), an inhibitory cytokine for TH17, on CCL20 production was also studied. The numbers of individual FLS for each experiment are 14 in Figure 1 and 12 in Figure 2, Figure 3, and Table 1.

Measurement of serum CCL20 concentration in patients with RA during treatment with biologic DMARD. Serum CCL20 concentration in 14 patients with RA (12 women, 2 men) during treatment with biologic DMARD (infliximab, 5 patients; etanercept, 4 patients; and tocilizumab, 5 patients) and 13 healthy controls (11 women, 2 men) were studied using the same ELISA kit. Age, disease duration, and Disease Activity Score 28-erythrocyte sedimentation rate (DAS28-ESR) of the 14 RA patients at entry were 52.6 ± 13.0 years, 7.0 ± 9.4 years, and 6.0 ± 1.2 (high disease activity at the entry), respectively. Age of healthy controls was 36.5 ± 9.5 years; thus, they were statistically younger than patients with RA. We examined correlation of age and serum CCL20 concentration among 13 healthy controls by Spearman's rank correlation, and did not find any association (data not shown). Sex distribution was similar in healthy controls to patients with RA. Therefore, the samples from 13 healthy subjects were employed as control as comparison to patients with RA. CCL20 concentration of 14 patients with RA was serially examined at 3 to 6 months after the treatment with biologic DMARD, which achieved more than the moderate response determined by the EULAR response criteria[16] at the second measurement of CCL20.

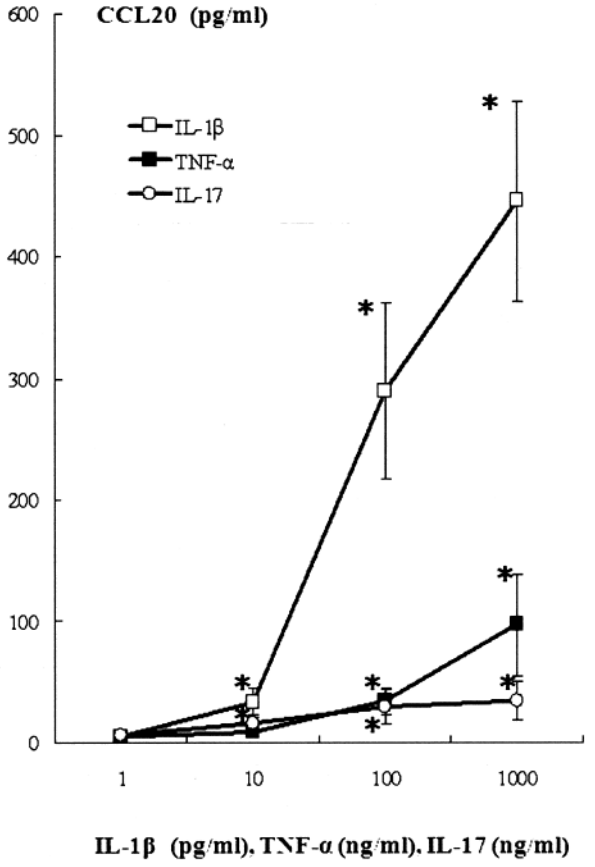

Figure 1. Dose-dependent increase of CCL20 production from cultured fibroblast-like synovial cells (FLS) by interleukin 1ß (IL-ß, tumor necrosis factor-α (TNF-α), and IL-17. Rheumatoid FLS were cultured for 48 h in the presence of 1–1000 pg/ml IL-ß, 1–1000 ng/ml TNF-α, or IL-17, and the CCL20 concentration in the supernatants was examined as described in Materials and Methods. IL-ß, TNF-α, and IL-17, especially IL-1ß, clearly induced CCL20 production from FLS. Results are expressed as means ± standard deviation from 14 independent experiments. *$p < 0.01$, vs 1 ng/ml of each cytokine.

Statistical analyses. Within-group comparisons were made using Mann-Whitney's U-test and Wilcoxon's signed-rank test. The overall significance level for statistical analysis was 5% (2-sided). P values less than 0.05 were considered statistically significant.

RESULTS

Proinflammatory cytokines synergistically stimulate the production of CCL20 from FLS, while TNF inhibitors suppress production. Spontaneous production of CCL20 from

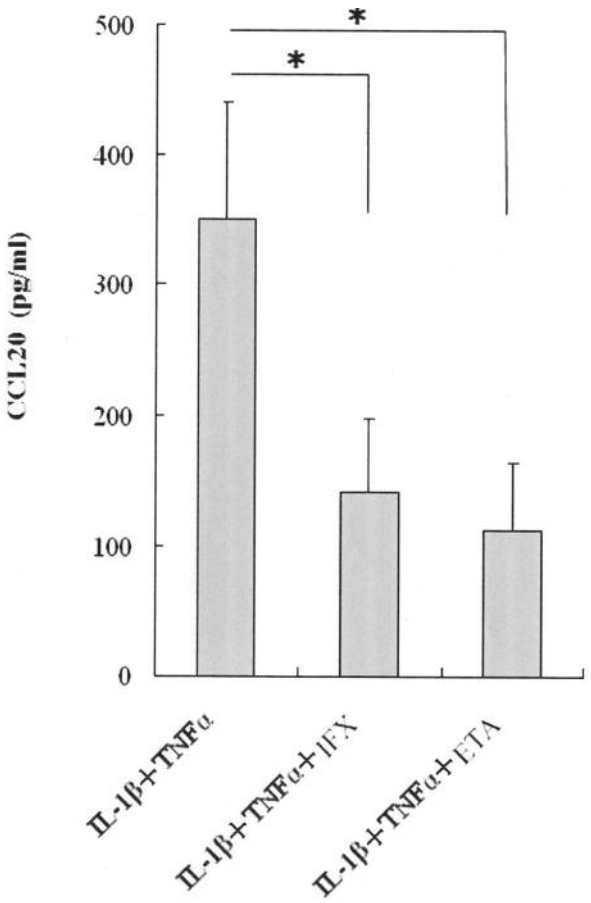

Figure 2. Suppression of CCL20 production from FLS stimulated with IL-1ß and TNF-α by infliximab (IFX) or etanercept (ETA). Rheumatoid FLS were cultured for 48 h in the presence of 100 pg/ml IL-ß and 50 ng/ml TNF-α in the presence or absence of either infliximab or etanercept (500 μg/ml). CCL20 concentration in the supernatants was examined as described in Materials and Methods. Results are expressed as means ± standard deviation from 12 independent experiments. *$p < 0.01$ vs absence of infliximab or etanercept.

FLS was very slight (4.5 ± 5.1 pg/ml, n = 14). We next verified an inducible effect of proinflammatory cytokines in CCL20 production. As reported[8-10], CCL20 production was induced by IL-1ß, TNF-α, or IL-17 in a dose-dependent fashion (Figure 1). The most prominent effect was induced by IL-1ß (Figure 1), and a synergy of these cytokines with each other was demonstrated since the CCL20 value induced by their combination was higher than the sum of each value (Table 1). TH1 cytokine IFN-γ inhibits the development of TH17 cells[17], and accordingly, IFN-γ (100 U/ml) was found to significantly inhibit IL-1ß (100 pg/ml)-induced CCL20 production (58.5% reduction by IFN-γ, Table 1). FLS were used for all experiments. However, all experiments cannot be performed at the same time, and a generation of FLS passage may be different among the experiments. Thus, the baseline CCL20 productions from FLS in the presence of cytokines are different in each figure and table.

We also examined whether proinflammatory cytokine-mediated CCL20 production from FLS is suppressed by TNF inhibitors. These experiments were performed by means of the incubation of FLS stimulated by IL-1ß and TNF-α in the presence of infliximab (500 μg/ml) or etanercept (500 μg/ml). As shown in Figure 2, both infliximab and etanercept *in vitro* clearly suppressed the production of CCL20 by FLS (59.4% reduction by infliximab and 67.9% reduction by etanercept).

IL-6 signal alone does not stimulate CCL20 production, but increases the production of CCL20 stimulated by proinflammatory cytokines, an increase abrogated by tocilizumab. The role of IL-6 in the production of CCL20 was investigated. Based on the previous finding that FLS do not express membrane-bound IL-6R[18,19], these experiments were performed in the presence of soluble IL-6R. As shown in Figure 3, the combination of IL-6 with soluble IL-6R did not increase CCL20 production; however, CCL20 production from FLS stimulated by IL-1ß, IL-1ß+TNF-α, and IL-1ß+IL-17 was clearly increased in the presence of the IL-6 signal. The increase of CCL20 production by IL-6 with soluble IL-6R was abrogated by the anti-IL-6R monoclonal antibody, tocilizumab (500 μg/ml; Figure 3).

Decrease of serum CCL20 concentration in patients with RA by biologic DMARD. Before the treatment, serum CCL20 was significantly higher in patients with RA than in healthy controls (Table 2). Serum CCL20 concentration did not correlate with DAS28-ESR at baseline, probably due to all their disease activity being similarly classified as high disease activity. Biologic DMARD treatment for 3 to 6 months markedly decreased serum CCL20 concentration (Table 2).

DISCUSSION

Recent investigations have revealed that TH17 cells, a T helper subset, specifically produce IL-17, which is critically involved in the disease process of RA[6,7,20]. Prominent TH17 cells are supposed to infiltrate the rheumatoid synovial tissues[5]; thus, a certain RA-specific microenvironment that facilitates the migration of TH17 cells is developed in affected tissues. Since TH17 cells express CCR6[2-4], *in situ* condensation of CCL20, a selective ligand of CCR6, is supposed to perpetuate the rheumatoid synovial inflammation. Therefore, the first half of our study focused on the regulation of CCL20 production from FLS.

The confirmatory results were obtained that IL-1ß, TNF-α, and IL-17 synergistically stimulate the production of CCL20. Cellular subsets observed in the rheumatoid synovial tissues, including TH17 cells, have been shown to produce these proinflammatory cytokines[6,20,21], presumably resulting in the augmentation of CCL20 production from neighboring FLS. Infliximab directly neutralizes TNF-α, and etanercept inhibits the TNF-α-eliciting signal at the TNF receptor. Thus, the result shown in Figure 2 is reasonable, and our data represent the first definitive result to be published.

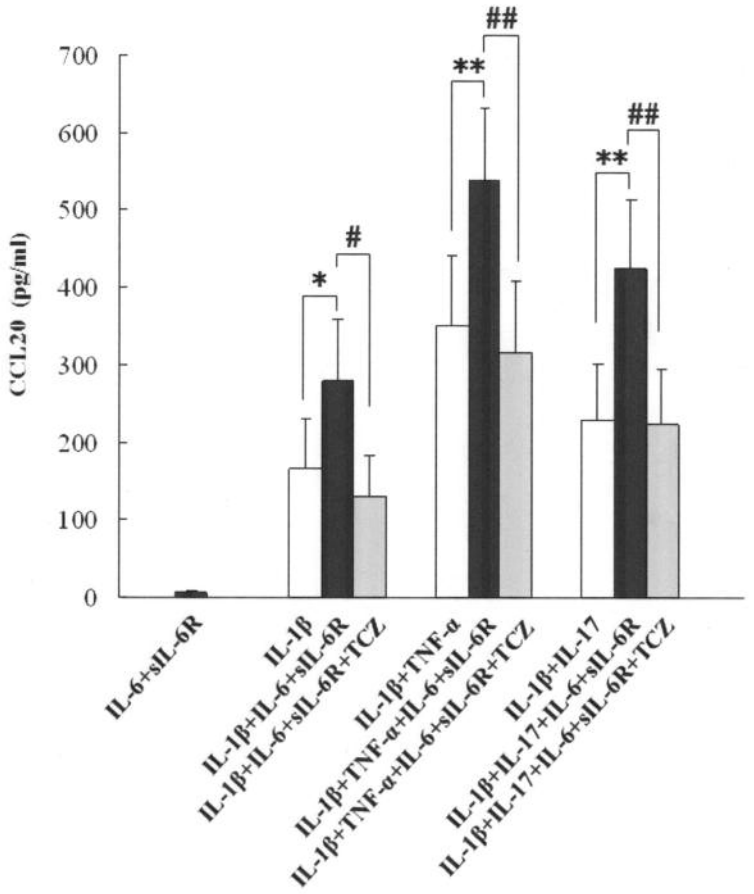

Figure 3. IL-6 combined with soluble IL-6 receptor (sIL-6R) does not induce CCL20 production, whereas it stimulates CCL20 production in the presence of IL-1ß, TNF-α, or IL-17; production is abolished by tocilizumab (TCZ). Rheumatoid FLS were cultured for 48 h in the presence of IL-6 (100 ng/ml) and sIL-6R (100 ng/ml) (1st column); IL-1ß (100 pg/ml), IL-1ß (100 pg/ml) + IL-6 (100 ng/ml), and sIL-6R (100 ng/ml), each in the presence or absence of 500 μg/ml tocilizumab (2nd column); IL-1ß (100 pg/ml) + TNF-α (50 ng/ml), IL-1ß (100 pg/ml) + TNF-α (50 ng/ml) + IL-6 (100 ng/ml), and sIL-6R (100 ng/ml), each in the presence or absence of 500 μg/ml tocilizumab (3rd column); and IL-1ß (100 pg/ml) + IL-17 (50 ng/ml), IL-1ß (100 pg/ml) + IL-17 (50 ng/ml) + IL-6 (100 ng/ml), and sIL-6R (100 ng/ml), each in the presence or absence of 500 μg/ml tocilizumab (4th column). CCL20 concentration in the supernatants was examined as described in Materials and Methods. Results are expressed as means ± standard deviation from 12 independent experiments. IL-6 with sIL-6R did not induce CCL20 production (1st column), whereas it stimulated production in the presence of IL-1ß (2nd column), IL-1ß + TNF-α (3rd column), and IL-1ß + IL-17 (4th column). *$p < 0.05$, **$p < 0.01$ vs absence of IL-6+sIL-6R. These increases were abolished by tocilizumab. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs absence of tocilizumab.

This article has identified a novel role for the IL-6 signal in CCL20 production from FLS. Our data suggest that the IL-6-eliciting signal amplifies the production of CCL20 by IL-1ß, TNF-α, and IL-17, the signals being transmitted through nuclear factor-κB (NF-κB)[22]. Janus kinase and signal transducer and activator of transcription (Jak-STAT), but not NF-κB, is dominantly triggered by IL-6[23], and thus unique phenomena may be induced in the presence of IL-6 with IL-1ß, TNF-α, or IL-17, as represented by our data. Further investigations are necessary to clarify the molecular interactions in FLS stimulated by these cytokines with regard to CCL20 production. It remains unclear whether the amount of biologic DMARD used in *in vitro* experiments can be achieved in the synovial tissues of RA treated by infliximab, etanercept, or tocilizumab. We can determine the serum concentration of infliximab or etanercept in the treated patients to be low compared with the concentrations used in the present *in vitro* study[24,25]. Concentration of biologic DMARD in synovial tissue or synovial fluid of the treated RA patients is not reported; however, the concentrations used in our experiment might be higher. Therefore, CCL20 from cell populations other than FLS are also supposed to

Table 1. Synergistic effects of IL-1ß, TNF-α, IL-17 on CCL20 production by rheumatoid fibroblast-like synovial cells.

Cytokines	Concentrations of CCL20
IL-1ß, 100 pg/ml	166.5 ± 222.8
TNF-α (100 ng/ml)	34.2 ± 40.0
IL-17 (100 ng/ml)	29.4 ± 54.1
IL-1ß + TNF-α (50 ng/ml)	349.8 ± 313.4*
IL-ß+IL-17 (50 ng/ml)	225.8 ± 225.8*
IL-ß+IFN-γ (100 U/ml)	69.1 ± 103.3*

Differences in CCL20 production from FLS stimulated by IL-1ß, IL-1ß+TNF-α, or IL-1ß+IL-17 or IL-1ß+IFN-γ were analyzed using nonparametric paired Wilcoxon test. * $p < 0.01$, significantly different from CCL20 production by IL-1ß stimulation alone.

Table 2. High serum CCL20 concentration is decreased by treatment with biologic DMARD. Data are mean ± standard deviation.

Samples	Concentrations of CCL20
Healthy controls, n = 13	6.6 ± 6.6
RA patients at baseline	49.7 ± 37.5*
RA patients treated with biologics	19.5 ± 13.6**

Differences in concentrations of serum CCL20 between each group were analyzed using nonparametric paired Wilcoxon test. RA: n = 14 (infliximab 5, etanercept 4, tocilizumab 5). * $p < 0.001$, significantly different from controls. ** p , 0.01, significantly different from RA patients at baseline.

involve *in vivo* change of serum CCL20 during the treatment with biologic DMARD in patients with RA. Additionally, our study identified that IFN-γ inhibits CCL20 production from FLS in one effect of the suppression of TH17 function in humans. Indeed, the TH17-dependent arthritis model is inhibited by the introduction of IFN-γ[7].

As far as we know, only a few clinical values of CCL20 during treatment with biologic DMARD in patients with RA can be found in the literature[11]. We have shown here that serum CCL20 declined in patients with RA who responded well to biologic DMARD. The decrease of CCL20 was found in all 3 treatment arms, namely infliximab, etanercept, and tocilizimab, with no statistical difference between the TNF inhibitors (infliximab and etanercept) and tocilizumab (data not shown). Both the TNF inhibitors and tocilizumab inhibited the production of CCL20 from FLS *in vitro*, and probably *in vivo*. TNF-α and IL-6 cooperatively act on the effector cell population to stimulate CCL20 production. Thus, serum CCL20 concentration in patients with RA was clearly downregulated by both of the TNF inhibitors and tocilizumab in a similar fashion.

The CCL20-mediated TH17 cell activation process is supposed to play a central role in the disease process of RA. We do not provide direct evidence of FLS-derived CCL20 interaction with TH17 cells in our study. Development of TH17 cells requires several cytokines, including IL-1, TNF-α, and IL-6, whereas IFN-γ suppresses the process[17,26-28]. CCL20 production from FLS is clearly modulated by IL-1ß, TNF-α, IL-6, and IFN-γ, indicating that proinflammatory cytokine-mediated regulation of CCL20 in FLS may be involved in the TH17 cell-dependent disease process of RA. Further studies are necessary to identify the *in vivo* or *ex vivo* role of FLS-derived CCL20 for the accumulation of TH17 cells in rheumatoid synovial tissues. The CCL20-mediated TH17 cell activation process is supposed to play a central role in the disease process of RA.

Our study gives a possible explanation for why CCL20 is an exacerbating factor in patients with RA. The monitoring of serum CCL20 concentration may reflect the disease activity of RA.

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