Simvastatin Inhibits Production of Interleukin 6 (IL-6) and IL-8 and Cell Proliferation Induced by Tumor Necrosis Factor-α in Fibroblast-like Synoviocytes from Patients with Rheumatoid Arthritis

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ABSTRACT. Objective. Rheumatoid arthritis (RA) is a chronic inflammatory disease in which the synovial environment is characterized by aggressive cellular proliferation and intensive immunological activation of fibroblast cells. In particular, macrophage-like synoviocytes and fibroblast-like synoviocytes (FLS) of the hyperplastic lining layer exhibit activated phenotypes. These cells are major sources of proinflammatory cytokines, such as interleukin 6 (IL-6) and IL-8, that play crucial roles in the pathophysiology of RA. Elevated concentrations of these cytokines characterize the synovial inflammation observed in patients with RA.

IL-6 was originally identified as a differentiation factor in culture supernatants and is produced by T cells, monocytes and fibroblasts. IL-6-deficient mice do not develop arthritis or joint destruction in either collagen induced arthritis (CIA) or antigen induced arthritis (AIA) models. IL-6 blockade using a monoclonal antibody directed against the IL-6 receptor has resulted in significant improvements in clinical scores and laboratory indicators in patients with RA.

Among the chemokines, IL-8 is produced by a variety of cells in the inflamed synovial milieu, including synovial tissue macrophages, fibroblasts, and chondrocytes. IL-8 is a strong inducer of neutrophil recruitment and is recognized as an important factor in neutrophil infiltration into the synovial fluids of RA patients. IL-8 expression in rheumatoid synovial tissue has been associated with disease activity. Recent successes in therapeutic targeting of cytokines in RA suggest that IL-8 may be of critical and pathogenic importance in RA.

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in RA joints proliferate aggressively and eventually destroy the articular bones and cartilages\textsuperscript{1,2}. Synovial fluid in RA patients contains high level TNF-\(\alpha\)\textsuperscript{16}. Laboratory experiments and clinical interventions suggest this cytokine may be related to disease activity in RA.

Recently, 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase inhibitors (statins) have been shown to reduce cardiovascular morbidity and mortality\textsuperscript{17,18}. Although these beneficial effects of statins are mediated in part through their lipid-lowering effects, recent studies indicate that statins may have other functions, such as modulating ongoing inflammation within atherosclerotic lesions\textsuperscript{19}, reducing inducible MHC class II expression in immune cells\textsuperscript{20}, and inhibiting lymphocyte function-associated antigen-1/intercellular adhesion molecule-1 interactions via competitive binding\textsuperscript{21}. Indeed, Itô, \textit{et al} reported that statins attenuate IL-6 and IL-8 expression in human vascular smooth muscle cells\textsuperscript{22,23}.

We recently reported that simvastatin treatment significantly improves the clinical indicators of RA in patients with active disease\textsuperscript{24}. In the present study, employing real-time polymerase chain reaction (PCR) analysis, ELISA, and MTT assays, we show that simvastatin \textit{in vitro} inhibits IL-6 and IL-8 production and cell proliferation induced by TNF-\(\alpha\) in FLS from patients with RA.

**MATERIALS AND METHODS**

\textbf{Reagents.} The simvastatin (kindly provided by Merck & Co. Inc., Rahway, NJ, USA) was prepared as a 4 mg/ml (10 mM) stock solution, as reported\textsuperscript{25}. Briefly, 4 mg simvastatin was dissolved in 100 \(\mu\)l of ethanol and 150 \(\mu\)l of 0.1 N NaOH. This solution was incubated at 50°C for 2 h; the pH was adjusted to 7.0 and volume to 1.0 ml. The same solution without simvastatin was prepared for the control experiments.

Other chemicals and materials were purchased from the following sources: RPMI-1640 medium, fetal calf serum (FCS), penicillin, and streptomycin were from Invitrogen (Carlsbad, CA, USA); trypsin/EDTA, collageenate, hyaluronidase, mevalonic acid (MVA), farnesyl-pyrophosphate (FPP), and geranylgeranyl-pyrophosphate (GGPP) were from Sigma Chemical Co. (St. Louis, MO, USA); and recombinant human TNF-\(\alpha\) was from R&D Systems (Minneapolis, MN, USA). IL-6 and IL-8 were detected in cell culture supernatants using ELISA kits purchased from Amersham Biosciences (Piscataway, NJ, USA). Total RNA samples from cultured FLS were purified using the RNeasy mini-kit from Qiagen GmbH (Hilden, Germany). Taqman reverse transcription reagents, Taqman universal PCR master mix, and Taqman predeveloped assay reagents for IL-6, IL-8, and gryeraldehyde-3-phosphate dehydrogenase (GAPDH) were from Applied Biosystems (Foster City, CA, USA). The kit for 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was from Chemicon (Temecula, CA, USA).

\textbf{Preparation and culture of FLS.} Synovial tissues for the preparation of FLS were obtained from 12 RA patients who underwent joint replacement therapy at Saitama Medical School. The patients fulfilled the 1987 revised American College of Rheumatology (ACR; formerly, the American Rheumatism Association) differentiation criteria; written informed consent, approved by the ethics committee of Saitama Medical School, was obtained from each patient prior to the experiment. FLS were isolated and cultured as described\textsuperscript{26}. Briefly, the RA synovial tissues were minced into small pieces and incubated 2 h at 37°C with continuous shaking in RPMI-1640 that contained 0.15 mg/ml collagenase and 0.04% hyaluronidase. After removal of tissue debris by passage through a 70 \(\mu\)m nylon mesh, the collected cells were cultured in RPMI-1640 that contained 10% FCS, 100 U/ml penicillin, and 100 \(\mu\)g/ml streptomycin in a humidified atmosphere with 5% CO\(_2\) at 37°C. After overnight culture, nonadherent cells were removed, and the adherent cells were cultured continuously under the same conditions. Subconfluent cells were trypsinized and recultured. Cells were placed in 24-well plates or 100 mm culture dishes in the culture medium and allowed to grow to subconfluence. The culture medium was then changed to RPMI-1640 containing 1% FCS for the experiments described below. FLS from passages 3 through 7 were used in these experiments. The appropriate volume of the simvastatin stock solution (10 mM) was mixed with the culture medium just prior to addition to the cells, resulting in the final concentration described in the text.

\textbf{Attenuation of simvastatin-mediated inhibition of cell proliferation.} To clarify the mechanism by which simvastatin blocked IL-6 and IL-8 production, subconfluent FLS were cultured for 12 h with or without 1.0 \(\mu\)M simvastatin and 100 \(\mu\)M MV A, 10 \(\mu\)M GGPP, or 10 \(\mu\)M FPP. To clarify the mechanism by which simvastatin blocked IL-6 and IL-8 production, subconfluent FLS were cultured for 12 h with or without 1.0 \(\mu\)M simvastatin and 100 \(\mu\)M MVA, 10 \(\mu\)M GGPP, or 10 \(\mu\)M FPP. Samples (0.5 ml) of the culture medium were obtained at 12 h. After centrifugation at 3000 rpm for 10 min, the supernatants were used for the cytokine assays. Concentrations of IL-6 and IL-8 were measured using ELISA kits according to the manufacture’s instructions. Briefly, the absorbance at 450 nm was measured, and the cytokine concentrations were determined by interpolation of a standard calibration curve.

\textbf{Attenuation of simvastatin-mediated inhibition of IL-6 and IL-8 production.} For analysis of IL-6 and IL-8 production, subconfluent FLS in 24-well dishes were cultured 12 h with or without 0.05--10 \(\mu\)M simvastatin. Samples of culture medium (0.5 ml) were collected at 6 h and 12 h in the time-course experiments. After centrifugation at 3000 rpm for 10 min, the supernatants were used for the cytokine assays. Concentrations of IL-6 and IL-8 were measured using ELISA kits according to the manufacturer’s instructions. Briefly, the absorbance at 450 nm was measured, and the cytokine concentrations were determined by interpolation of a standard calibration curve.

\textbf{Expression of mRNA for IL-6 and IL-8 in FLS.} Total RNA samples were extracted from subconfluent FLS (1 \(\times\) 10\(^6\) cells) in 100 mm dishes using the RNeasy mini kit according to the manufacturer’s instructions. cDNA was synthesized from total RNA by reverse transcriptase reaction using Taqman reverse transcription reagents. The mRNA expression levels were determined by the Taqman PCR system, using the ABI Prism 7000 sequence detection system (Applied Biosystems). For detection of human IL-6 and IL-8 mRNA, we used the Taqman predeveloped assay reagent kit. For standardization, GAPDH also was amplified simultaneously. The PCR mixture (50 ml) consisted of Taqman universal PCR master mix (2x) 25 \(\mu\)l, target primers and probe (20x) 2.5 \(\mu\)l, control primers and probe (20x) 2.5 \(\mu\)l, cDNA 300 ng, and RNase-free water. The quantitative PCR conditions were as follows: 95°C for 10 min, followed by 40 cycles of 2-step PCR, including denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min. For quantitative analysis of mRNA of IL-6 and IL-8, the changes of reporter fluorescence from each reaction-well were evaluated. The threshold cycle of each gene was determined as PCR cycles at which the reporter fluorescence rose above a baseline signal. The difference in threshold cycles between the target gene and GAPDH gives the standardized expression level (\(\Delta\text{Ct}\)). Subtraction of \(\Delta\text{Ct}\) of nontreated FLS from \(\Delta\text{Ct}\) of statin-treated FLS gives the \(\Delta\text{Ct}\) value, which is used to calculate relative expression levels in statin-treated FLS with the formula \(2^{-\Delta\text{Ct}}\). The expression level of each gene was interpreted as fold-increase in statin-treated FLS compared with nontreated controls\textsuperscript{27}.

\textbf{Cell proliferation.} FLS were seeded at 1 \(\times\) 10\(^4\) cells/well in 24-well plates. After 24 h incubation, culture medium was changed to RPMI-1640 containing 0.5% FCS with or without simvastatin (0.05--1.0 \(\mu\)M) and 10 mg/ml TNF-\(\alpha\). Samples were collected at 24, 72, 120, and 168 h (7 days). Cell proliferation was assessed by MTT assay, as described\textsuperscript{28}. Briefly, 50 \(\mu\)l of MTT solution was added to each well. After 3 h, the medium was removed completely from each well, then 200 \(\mu\)l of 40 mM HCl-isopropanol lysis buffer was added to each well. One hundred milliliters of cell lysis were transferred into 96-well plates. The optical density of each well was determined using a microplate reader at 570 nm. The inhibition of cell proliferation was calculated in percentage as below; the difference of OD values between simvastatin-treated and nontreated control FLS was derived from the OD value of nontreated FLS and the calculated numbers were multiplied by 100%.

\textbf{Attenuation of simvastatin-mediated inhibition of cell proliferation.} To clari-
fy the mechanism by which simvastatin inhibited cell proliferation, subconfluent FLS in 24-well plates with or without 1.0 µM simvastatin and 10 ng/ml TNF-α were cultured for 72 h in the presence of 100 µM MVA, 10 µM GGPP, or 10 µM FPP. Cell proliferation rate was assessed by MTT assay as described above.

**Statistical analysis.** For the statistical analysis, we calculated the means and standard errors of the mean. Differences between groups were tested for statistical significance using the Mann-Whitney U test. P values less than 0.05 were considered significant.

**RESULTS**

**mRNA expression patterns of IL-6 and IL-8.** As shown in Figure 1A, after 6 h incubation with simvastatin, expression levels of IL-6 mRNA were reduced significantly to 63% ± 8.7% (0.05 µM simvastatin), 60% ± 9.0% (0.1 µM), 49% ± 4.0% (1.0 µM), and 39% ± 3.0% (10 µM) of the control. After 12 h of incubation with simvastatin, levels of IL-6 mRNA were reduced significantly to 51% ± 14.0% (0.1 µM simvastatin), 37% ± 9.0% (1.0 µM), and 26% ± 6.0% (10 µM) of the control (Figure 1B).

As shown in Figure 2A, after 6 h incubation with simvastatin, expression levels of IL-8 mRNA were reduced significantly to 59% ± 16.0% (0.05 µM simvastatin), 55% ± 10.6% (0.1 µM), 52% ± 15.0% (1.0 µM), and 43% ± 11.4% (10 µM) of the control. After 12 h of incubation with simvastatin, the expression levels of IL-8 mRNA were reduced significantly to 76% ± 6.5% (1.0 µM simvastatin) and 51% ± 4.7% (10 µM) of the control (Figure 2B).

**IL-6 and IL-8 levels in culture supernatant.** After 6 h incubation with simvastatin, the IL-6 levels were reduced significantly to 73.6 ± 6.4 pg/ml (0.05 µM simvastatin), 73.3 ± 11.3 pg/ml (0.1 µM), 60.0 ± 5.7 pg/ml (1.0 µM), and 57.3 ± 4.7 pg/ml (10 µM), while the control level was 108.0 ± 15.6 pg/ml (Figure 3A). After 12 h of incubation with simvastatin, IL-6 levels were reduced significantly to 95.8 ± 10.7 pg/ml (0.05 µM simvastatin), 71.8 ± 6.9 pg/ml (0.1 µM), 59.9 ± 5.9 pg/ml (1.0 µM), and 51.6 ± 4.4 pg/ml (10 µM), compared with the control level (116.4 ± 8.3 pg/ml; Figure 3B).

As shown in Figure 4A, after 6 h incubation with simvastatin, IL-8 levels were reduced significantly to 42.6 ± 3.7 pg/ml (0.05 µM simvastatin), 41.5 ± 6.2 pg/ml (0.1 µM), 32.4 ± 4.8 pg/ml (1.0 µM), and 24.0 ± 5.0 pg/ml (10 µM), while the control level was 63.7 ± 9.3 pg/ml. After 12 h incubation with simvastatin, IL-8 levels were reduced significantly to 63.9 ± 3.4 pg/ml (0.1 µM simvastatin), 57.0 ± 3.5 pg/ml (1.0 µM), and 46.5 ± 1.6 pg/ml (10 µM), compared with the control level (82.1 ± 7.4 pg/ml; Figure 4B).

**Effects of MVA and isoprenoids on simvastatin inhibition of IL-6 and IL-8 production.** After 12 h incubation of FLS with 1.0 µM simvastatin plus MVA, GGPP, or FPP, the inhibitory effect of simvastatin on IL-6 production was clearly reversed by 100 µM MVA or 10 µM GGPP, but not by FPP (Figure 5A). Similarly, after 12 h of incubating FLS with 1.0 µM simvastatin plus MVA, GGPP, or FPP, the inhibitory effect of simvastatin on IL-8 production was completely reversed by 100 µM MVA or 10 µM GGPP, but not by FPP (Figure 5B).

The effects of incubation of FLS with MVA, GGPP, or FPP were minimal in the absence of simvastatin (Figures 5A and 5B).

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**Figure 1.** IL-6 mRNA expression by FLS. FLS were incubated with 0.05–10 µM concentrations of simvastatin for 6 h (A) or 12 h (B). IL-6 mRNA levels were determined by real-time PCR as described in Materials and Methods. Data are shown as value relative to the control (cells cultured in the absence of simvastatin) so that the control value will be 1.0. Values are expressed as mean ± SEM obtained from triplicate experiments using 7 different samples. **p < 0.01 versus control.
Cell proliferation in RA FLS. After 72 h incubation with simvastatin (0–1.0 µM), the inhibition in cell proliferation without TNF-α was significantly increased to 24.4% ± 4.4% (1.0 µM simvastatin) compared with the control (0% indicates the control). After 120 h incubation, the inhibition of cell proliferation was significantly increased to 30.6% ± 4.0% (1.0 µM simvastatin). After 168 h incubation, similar to the data at 120 h, the inhibition of cell proliferation was significantly increased to 36.7% ± 4.2% (1.0 µM simvastatin).

Figure 2. IL-8 mRNA expression by FLS. FLS were incubated with 0.05–10 µM concentrations of simvastatin for 6 h (A) or 12 h (B). IL-8 mRNA levels were determined using real-time PCR. Data are shown as value relative to control (cells cultured in the absence of simvastatin), and are expressed as mean ± SEM from triplicate experiments using 4 different samples. **p < 0.01, *p < 0.05 versus control.

Figure 3. IL-6 levels in FLS culture supernatants. FLS were incubated with 0.05–10 µM concentrations of simvastatin for 6 h (A) and 12 h (B). IL-6 levels in culture supernatants were determined by ELISA. Data are expressed as mean ± SEM of triplicate experiments using 6 different samples. **p < 0.01, *p < 0.05 versus control.
increased to 28.4% ± 4.5% (1.0 µM simvastatin; Figure 6A). After 72 h incubation with simvastatin under activation with 10 ng/ml TNF-α, the inhibition of cell proliferation was significantly increased to 52.2% ± 6.4% (1.0 µM simvastatin). After 120 h incubation with the same conditions as above, the inhibition of cell proliferation was significantly increased to 32.7% ± 6.9% (0.05 µM simvastatin), 37.2% ± 7.7% (0.1 µM), and 65.3% ± 5.5% (1.0 µM). After 168 h incubation, similar to the data at 120 h, the inhibition of cell proliferation was significantly increased to 26.8% ± 7.9% (0.05 µM sim-
vastatin), 28.1% ± 7.6% (0.1 µM), and 58.2% ± 10.6% (1.0 µM; Figure 6B). Taken together with the data above, the inhibitory effect of simvastatin on cell proliferation was observed in a dose-dependent and time-dependent manner and was more prominent when FLS were activated by TNF-α.

Effects of MVA and isoprenoids on simvastatin-induced inhibition of cell proliferation. After incubation of RA FLS for 72 h with 1.0 µM simvastatin and 10 ng/ml TNF-α plus MVA, GGPP, or FPP, the inhibition of cell proliferation by simvastatin was reversed by 100 µM MVA or 10 µM GGPP, but not by FPP (Figure 7). On the other hand, the effects of incubation with MVA, GGPP, or FPP on FLS were minimal without simvastatin. The proliferation of FLS incubated with TNF-α and simvastatin was roughly equal to that of cells incubated without TNF-α or simvastatin.

DISCUSSION
Statins are widely used to treat patients with hyperlipidemia. However, accumulating evidence suggests that statins possess a variety of biological properties including immunomodulatory and antiinflammatory effects. Recently we reported administration of 10 mg of simvastatin significantly reduced clinical scores of patients with active RA with hypercholesterolemia. Leung et al also reported an antiinflammatory effect of simvastatin in the collagen-induced arthritis animal model, and it was suggested that the antiarthritogenic effect might be due to the inhibition of T cell receptor-mediated T cell proliferation. However, the relative amount of simvastatin administered to each mouse in their experiments was much larger than its clinical dose for humans, which gave 0.05–0.1 µM of plasma concentration of simvastatin at pharmacologic conditions. To investigate its beneficial effects on RA, it is important to show the specific mechanism of action of simvastatin in RA at pharmacologic conditions. Thus, we used simvastatin at concentrations of 0.05–10 µM in this study.

Using FLS from patients with RA, our data suggest that the beneficial effect of simvastatin in RA may be obtained by inhibition of production of cytokines like IL-6 and IL-8. Of interest, this effect is significant even at the lowest concentration of simvastatin. The effect also depends on the concentration of simvastatin and on the period of exposure to it. This is
a first study showing that simvastatin affects the production of proinflammatory cytokines and cell proliferation in FLS from patients with RA.

FLS in RA joints probably account for a major source of cytokines such as IL-6 and IL-8. Concentrations of these cytokines were elevated in the synovial fluid and sera from RA patients compared with those from osteoarthritis patients or age-matched controls. IL-6 levels in the synovial fluid correlate with the presence of local IgM rheumatoid factor and the production of systemic acute-phase proteins. Recently, it has been reported that IL-6 blockade using a monoclonal antibody against its receptor is well tolerated and results in significant improvement in clinical and laboratory measures in RA patients. Indeed, the serum and synovial concentrations of these cytokines are especially high in patients with active RA. However, in contrast to these in vivo results, levels of TNF-α or IL-1β mRNA and protein in the FLS and culture supernatants, respectively, could not be detected under our experimental conditions (data not shown). It is possible that the levels of TNF-α and IL-1β are very low in cultured synovial fibroblasts from RA patients.

HMG-CoA reductase, which is the target of statins, catalyzes the conversion of HMG-CoA to MVA. It is well known that MVA is the precursor not only of cholesterol but also of isoprenoid compounds (FPP and GGPP). Takemoto, et al have reported that isoprenoid compounds play an important role in signal transduction pathways by their adhesion to signaling proteins, such as Ras and Rho. Ito, et al also reported that statins attenuated IL-6 and IL-8 expression in human vascular smooth muscle cells, partially through the inhibition of GGPP. Therefore, it is possible that simvastatin suppresses the activities of Rho and Ras-like proteins via depletion of isoprenoid compounds, and subsequently inhibits IL-6 and IL-8 expression by RA FLS.

Nagashima, et al reported that fluvastatin induced mitochondrial- and caspase 3-dependent apoptosis in RA synoviocytes. To rule out the possibility of simvastatin-induced apoptosis in our experiments, the trypan blue dye exclusion test and MTT assay were performed. Cell viability of FLS was always more than 98% after 24 h cell culture with 0.05–1.0 µM simvastatin (data not shown). Therefore, simvastatin-

![Figure 7. Effects of MVA and isoprenoids on simvastatin-induced inhibition of FLS proliferation.](https://example.com/figure7.png)
induced apoptosis of FLS appears to be negligible under our experimental conditions of low-dose simvastatin.

Gitter, et al reported that TNF-α induces the proliferation of fibroblast cell lines. The FLS in RA joints proliferate aggressively and eventually destroy the articular bones and cartilage. Our results indicate that the FLS proliferation induced by TNF-α can be inhibited more significantly by simvastatin in dose- and time-dependent manner than that without TNF-α, and that this inhibition is reversed by MVA and GGPP. Thus, simvastatin inhibits FLS proliferation through blockage of the mevalonate pathway, probably, the inhibition of protein-geranylgeranylation, which might be activated in the presence of TNF-α. This probably means that simvastatin inhibits synovial cell proliferation in RA more effectively than in other conditions, and that simvastatin might be an ideal treatment for RA.

Together, our results suggest that simvastatin has inhibitory effects on IL-6 and IL-8 production and cell proliferation induced by TNF-α in RA FLS. Further in vivo studies are required to elucidate the mechanism that promotes beneficial effects of statins in RA.

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