High Concentration Simvastatin Induces Apoptosis in Fibroblast-like Synoviocytes from Patients with Rheumatoid Arthritis

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ABSTRACT. ***Objective.*** We previously reported that 10 mg/day of simvastatin significantly reduced clinical scores of rheumatoid arthritis (RA) in patients with active RA with hypercholesterolemia. We have also reported that a certain pharmacological concentration of simvastatin, i.e., 0.05–0.1 μM, inhibits the production of interleukin 6 (IL-6) and IL-8 and the cell proliferation induced by tumor necrosis factor-α (TNF-α) in fibroblast-like synoviocytes (FLS) derived from patients with RA *in vitro*. We investigated other effects of simvastatin on FLS from the standpoint of cell viability and apoptosis.

Methods. RA FLS were cultured with or without 0.05–50 μM simvastatin for 48 h. Cell viability was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Apoptosis was measured by flow cytometric analysis using propidium iodide and annexin-V. Caspase-3 and -9 activities were analyzed by colorimetric assays.

Results. High concentrations of simvastatin, i.e., 1.0–50 μM, reduced cell viability and induced prominent apoptosis in FLS in a dose-dependent manner. The apoptosis induced by simvastatin was caspase-3- and caspase-9-dependent. These effects were completely reversed in the presence of mevalonic acid or geranylgeranyl-pyrophosphate, but not in the presence of farnesyl-pyrophosphate. Further, a geranylgeranyl transferase inhibitor and a RhoA kinase inhibitor mimicked the effect of simvastatin.

Conclusion. These data, together with our previous report, suggest that low (pharmacological range) and high concentrations of simvastatin affect FLS differently: (1) at a low concentration, it inhibits IL-6 and IL-8 production and the cell proliferation of FLS induced by TNF-α; (2) at high concentrations, it induces apoptosis in FLS. Understanding this dose-dependent biphasic effect of simvastatin may prove important for its clinical applications in the treatment of RA. (First Release Jan 15 2008; J Rheumatol 2008;35:193–200)

Key Indexing Terms:
RHEUMATOID ARTHRITIS FIBROBLAST-LIKE SYNOVIOCYTES CASPASE-3 CASPASE-9 3-HYDROXY-3-METHYLGLUTARYL CO-ENZYME A REDUCTASE INHIBITOR SMALL G PROTEIN SIMVASTATIN

Rheumatoid arthritis (RA) is a chronic inflammatory synovitis of multiple joints dominated by the presence of macrophages, lymphocytes, and synovial fibroblasts in the affected lesions, which eventually leads to the destruction of bone and cartilage[1,2]. In particular, macrophage-like synoviocytes and fibroblast-like synoviocytes (FLS) of the hyperplastic lining layer exhibit active phenotypes. For example, FLS are aggressively proliferating and insufficiently apoptotic, depending on the disease activity of RA[3]. Recent work also suggests that increased proliferation and insufficient apoptosis of FLS might contribute to the pathogenesis of RA[4].

Recently, 3-hydroxyl-3-methylglutaryl-CoA (HMG-CoA) reductase inhibitors (statins) have been shown to reduce cardiovascular morbidity and mortality[5,6]. Although statins work in part via their modification of lipid metabolism, further lines of evidence suggest they have broader properties, such as alterations of inflammatory pathways[7,8].

Activities of statins *in vitro* include suppression of adhesion molecule expression[9], MHC class II expression[10], lymphocyte and macrophage cognate interactions[11], and effects on reactive oxygen species[12] and nitrogen intermediate production[13]. Statins also modify apoptosis in smooth muscle and endothelial cells, leading to altered vascular function and neovascularization[14,15]. These properties suggest the drug's potential to influence chronic inflammatory disease status[16].

HMG-CoA reductase, which is the target of statins, catalyzes the conversion of HMG-CoA to mevalonic acid (MVA)[17]. Inhibition of mevalonate synthesis results in the

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reduction of isoprenoids like geranylgeranyl-pyrophosphate (GGPP) and farnesyl-pyrophosphate (FPP). These isoprenoids are involved in the posttranslational modification of small GTP-binding proteins, including members of the Ras, Rho, and Rac families. Prenylation of small GTP-binding proteins with farnesyl or geranylgeranyl groups are required for their localization to cell membranes and hence for their function[17]. Rho is functionally modified by geranylgeranylation, which is related to cell survival, proliferation, apoptosis, adhesion, differentiation, and invasion[18]. Posttranslational modifications of the Rho superfamily include catalyzation by type I geranylgeranyl transferase (GGTase I)[18]. Protein geranylgeranylation can be selectively inhibited by peptidomimetic inhibitors, such as GGTI-298[19].

Rho-superfamily proteins, e.g., RhoA, are geranylgeranylated and regulate the assembly and organization of the actin cytoskeleton[20]. When cells are stimulated, the activated geranylgeranylated RhoA binds to specific effectors to exert its biological functions. A variety of putative RhoA effectors have been identified[21]. Among them, a serine/threonine kinase, RhoA kinase, regulates cell actions such as the formation of actin stress fibers and focal adhesion[21], DNA synthesis, and the migration of vascular smooth-muscle cell, and angiogenesis[22]. A specific RhoA kinase inhibitor called Y-27632 has been shown to inhibit the RhoA kinase family 100 times more potently than it does other kinases[23].

As statins are believed to have immunosuppressive effects *in vivo*[6], simvastatin was used for the treatment of a murine arthritis model, collagen-induced arthritis (CIA), and ameliorated the progressive course of the disease[24]. It was shown that simvastatin, administered intraperitoneally (IP) at a dosage of 40 mg/kg/day, not only prevented the onset of CIA but also proved effective when administered even after clinically evident onset of the disease. Recently, however, another group showed that simvastatin has no preventive effect on the attenuation of autoimmunity in murine experimental arthritis, an observation that apparently contradicts the previous report[25]. The authors argued that although it was true that simvastatin administered IP at a dosage of 40 mg/kg/day ameliorated CIA, the effect seemed to be a consequence of "side effects" of the treatment. This does not mean, however, that simvastatin at high concentrations is not useful at all. For example, locally administered simvastatin at high concentrations may still be a useful strategy for the treatment of RA.

We have recently reported that a pharmacological concentration of simvastatin inhibits the production of interleukin 6 (IL-6) and IL-8 and the cell proliferation induced by tumor necrosis factor-α (TNF-α) in FLS from patients with RA[26]. We investigated the effect of higher concentrations of simvastatin, namely on apoptosis of the FLS.

MATERIALS AND METHODS

Reagents. Simvastatin, kindly provided by Merck & Co. Inc. (Rahway, NJ, USA), was prepared as a 4 mg/ml (10 mM) stock solution, as described[24,26]. Briefly, 4 mg simvastatin was dissolved in 100 μl of ethanol and 150 μl of 0.1 N NaOH. This solution was incubated at 50°C for 2 h; the pH and volume were adjusted to 7.0 and 1.0 ml, respectively. The same solution without simvastatin was prepared for 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, collagenase, hyaluronidase, mevalonic acid, FPP, GGPP, and caspase-3 and -9 assay kits were from Sigma (St. Louis, MO, USA); GGTI-298 and Y-27632 were from Calbiochem (Schwallbach, Germany). The kit for the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was from Chemicon (Temecula, CA, USA). The apoptosis detection kit was from MBL (Nagoya, Japan).

Preparation and culture of FLS. Synovial tissues for the preparation of FLS were obtained from 12 patients with RA who underwent joint replacement surgery at Saitama Medical University Hospital. All patients fulfilled the 1987 revised American College of Rheumatology (ACR; formerly, the American Rheumatism Association) differentiation criteria[27], and written informed consent, approved by the ethics committee of Saitama Medical University, was obtained from each patient. FLS were isolated and cultured as described[28]. Briefly, synovial tissues from patients with RA 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 μm nylon mesh, the collected cells were cultured in RPMI-1640 that contained 10% FCS, 100 U/ml penicillin, and 100 μg/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 at 3 to 7 passages were used in these experiments. The simvastatin stock solution (10 mM) was mixed with the culture medium just prior to addition to the cells.

Cell viability. FLS were seeded at 1×10^4 cells /well in 24-well plates. After 24 h incubation, culture medium was changed to RPMI-1640 containing 10% FCS with or without simvastatin (0.05–50 μM). Inhibition of GGTase I was accomplished by the addition of GGTI-298. RhoA kinase was inactivated with Y-27632. Samples were collected after 48 h. Cell viability was assessed by MTT assay as described[29]. Briefly, 50 μl of MTT solution was added to each well. After 3 h, the medium was removed completely from each well, and then 200 μl of 40 mM HCl-isopropanol lysis buffer was added to each well. One hundred microliters of cell lysate was transferred into individual wells of 96-well plates. The optical density of each well at 570 nm was determined using a microplate reader. Data are shown as values relative to the control (cells cultured in the absence of simvastatin) so that the control value is 100%.

Detection of apoptosis in RA FLS. FLS were seeded at 1×10^5 cells/well in 100 mm dishes or 1×10^4 cells/well in 24-well plates. After 24 h incubation, culture medium was changed to RPMI-1640 containing 10% FCS with or without simvastatin (0.05–50 μM). Inhibition of GGTase I was accomplished by treating cells with GGTI-298. Inactivation of RhoA kinase was carried out by treating cells with Y-27632. Apoptotic cells were detected by a cell sorter (FACScan, Becton Dickinson, Mountain View, CA, USA) using annexin V staining. Briefly, after 48 h, cells were harvested and washed in ice-cold phosphate buffered saline (PBS). Then 5 μl of $CaCl_2$ was added followed by the addition of annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI). After 10 min of incubation at room temperature in the dark, cells were analyzed by flow cytometry as described[30]. Apoptotic cells were defined as either early apoptotic cells (annexin V-positive and PI-negative) or late apoptotic or necrotic cells (annexin V and PI-positive). Cell debris was excluded by appropriate gating, and data were recorded on a logarithmic scale.

In order to evaluate the dependency of the cell death on caspases, a broad caspase inhibitor, zVAD-fmk (MBL, Nagoya, Japan) was added to FLS 2 h prior to the addition of simvastatin in some experiments.

Analysis of caspase-3 and -9 activities. FLS were seeded at 1×10^5 cells/well in 100 mm dishes. After 24 h incubation, culture medium was changed to RPMI-1640 containing 10% FCS with or without simvastatin (0.05–50 μM). Caspase-3 and -9 activities were analyzed by caspase-3 and -9 colorimetric assays, as described[31]. Briefly, cells were washed in ice-cold PBS and then resuspended in lysis buffer and incubated for 15 min on ice. Cell lysates were obtained after centrifugation at 16,000 rpm for 10 min at 4°C. Cell lysates were added to assay buffer, 2 mM caspase-3 substrate acetyl-Asp-Glu-Val-Asp p-nitroaniline (Ac-DEVD-pNA) or caspase-9 substrate acetyl-Leu-Glu-His-Asp p-nitroaniline (Ac-LEHD-pNA), and incubated at 37°C for 70 min. Absorbance was measured at 405 nm and then caspase-3 and -9 activities were calculated from each calibration curve prepared with defined pNA solution.

Statistical analysis. All the data are expressed as mean ± standard error (SEM). Statistical analysis was performed using Mann-Whitney U-test. P values less than 0.05 were considered significant.

RESULTS

Simvastatin reduced the viability of RA FLS. We initially investigated cell viability in RA FLS. As shown in Figure 1, after 48 h incubation with simvastatin, the rates of cell viability were reduced significantly to 75.0% ± 4.3% (1.0 μM simvastatin), 58.3% ± 3.8% (10 μM), and 33.7% ± 4.1% (50 μM) of the control. Treatment with statins causes mevalonate starvation within cells[17]. MVA metabolism yields a series of isoprenoid compounds, including GGPP and FPP[17]. We postulated that the inhibitory effect of simvastatin might be due to the isoprenoid compounds being deprived by the drug. To test this hypothesis, we incubated FLS with MVA, GGPP, or FPP in the presence of simvastatin. As shown in Figure 2A, after incubation for 48 h, the inhibition of cell viability by simvastatin was completely reversed by 100 μM MVA and 10 μM GGPP but not by FPP, whereas the effects of incubation with MVA, GGPP, or FPP on FLS were minimal in the absence of simvastatin (data not shown). These isoprenoids are involved in the posttranslational modification of proteins, including the

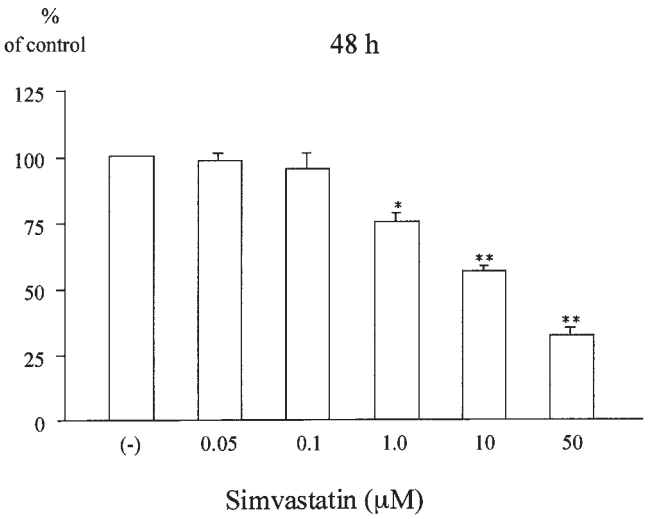

Figure 1. Reduction of cell viability by simvastatin. FLS were incubated with 0.05–10 μM simvastatin or without simvastatin (control) for 48 h. Cell viability was determined by MTT assay. Data were obtained from duplicate experiments using 5 different samples. * $p < 0.05$; ** $p < 0.01$ vs control.

Ras, Rho, and Rac superfamilies[20]. Rho family members are catalyzed by GGTase I, which is inhibited by the selective inhibitor GGTI-298. To determine whether inhibition of GGTase I resulted in a similar inhibitory effect of simvastatin, we incubated FLS with GGTI-298 for 48 h. As expected, the rates of cell viability were reduced significantly to 59.5% ± 4.3% (10 μM GGTI-298) and 45.6% ± 8.4% (20 μM) of the control (Figure 2B). RhoA is an important geranylgeranylated protein and its effector, RhoA kinase, is selectively inhibited by a RhoA kinase inhibitor, Y-27632. To examine whether inhibition of RhoA kinase was similar to the inhibitory effect of simvastatin, we incubated FLS with Y-27632. After 48 h incubation, the rate of cell viability was reduced significantly to 64.6% ± 5.1% (30 μM Y-27632) of the control (Figure 2C), suggesting that RhoA kinase plays an important role in the survival of RA FLS.

Statin-induced apoptotic-cell death in RA FLS. We next investigated whether the cell death induced by simvastatin was due to apoptosis. As shown in Figure 3, simvastatin increased the percentage of apoptotic cells, shown by annexin V-positive staining, in a dose-dependent manner. Of interest, apoptosis was barely observed in the sample treated with a pharmacological concentration (0.05–0.1 μM) of simvastatin. The percentage of annexin V-positive cells was proportional to that of dead cells detected by MTT assay, indicating that the cell death induced by simvastatin is apoptotic cell death.

We then incubated FLS with MVA, GGPP, or FPP in the presence of simvastatin. As shown in Figure 4A, after incubation of RA FLS for 48 h with 10 μM simvastatin and MVA, GGPP, or FPP, the induction of apoptosis by simvastatin was almost completely reversed by 100 μM MVA and 10 μM GGPP but not by FPP. As expected, the effects of incubation with MVA, GGPP, or FPP on FLS were minimal in the absence of simvastatin (data not shown). Because GGPP reversed the effect of simvastatin on FLS, it is that depletion of GGPP that is essential to this activity. To determine whether the inhibition of GGTase I, which reduced GGPP, caused an apoptotic effect similar to that induced by simvastatin, we incubated FLS with GGTI-298. After 48 h incubation, the number of apoptotic cells was increased in a dose-dependent manner (Figure 4B). Because the reduction of GGPP could downregulate the activity of RhoA, it was of interest to determine whether the induction of apoptosis in FLS by simvastatin was related to depleted RhoA activity. To determine whether an inhibition of RhoA kinase that reduced RhoA activity would mimic the effect of simvastatin on FLS, we incubated FLS with Y-27632. As expected, after 48 h incubation, the number of apoptotic cells was also increased in a dose-dependent manner (Figure 4B).

Caspase-3 and -9 activation is induced by simvastatin. As caspase-3 and -9 play a key role in apoptosis[32], we speculated that caspase-3 and -9 were involved in the apoptosis induced by simvastatin. As shown in Figure 5A, after 48 h incubation with simvastatin, caspase-3 activity was increased significant-

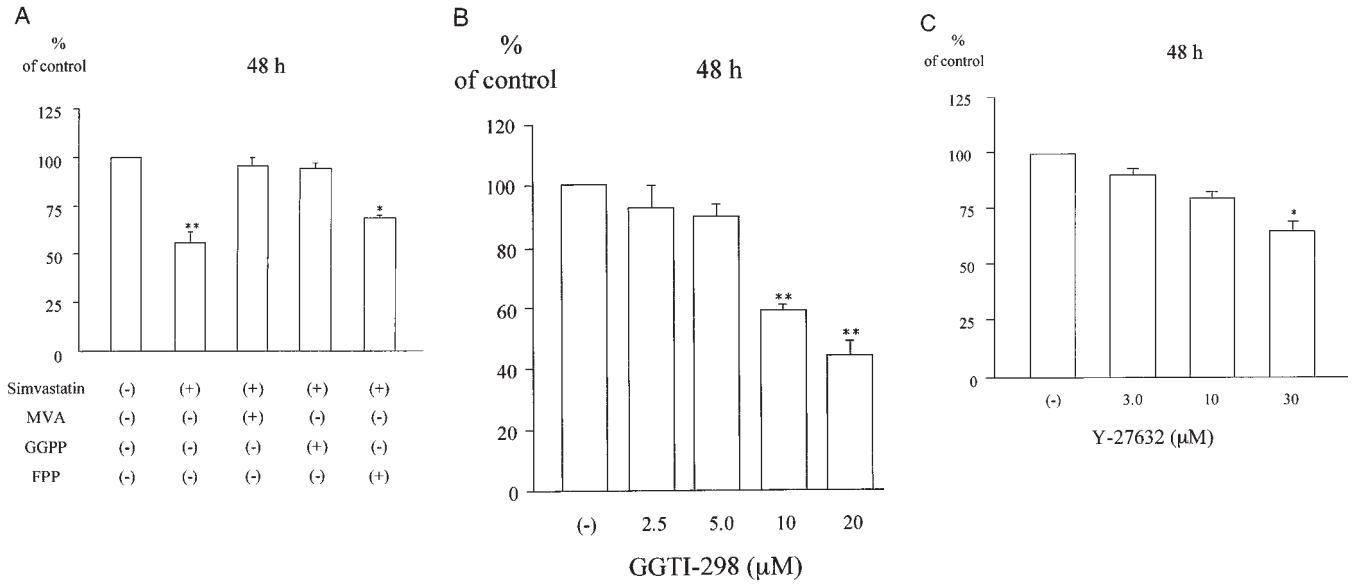

Figure 2. Effects of MVA and isoprenoids on simvastatin-induced reduction of FLS viability. A. FLS were incubated with or without 10 μM simvastatin and 100 μM MVA, 10 μM GGPP, and 10 μM FPP for 48 h. B. FLS were incubated with 2.5–20 μM GGTI-298 for 48 h. C. FLS were incubated with 3.0–30 μM Y-27632. Cell viability was determined by MTT assay. Data were obtained from duplicate experiments using 5 different samples. * $p < 0.05$; **$p < 0.01$ vs control.

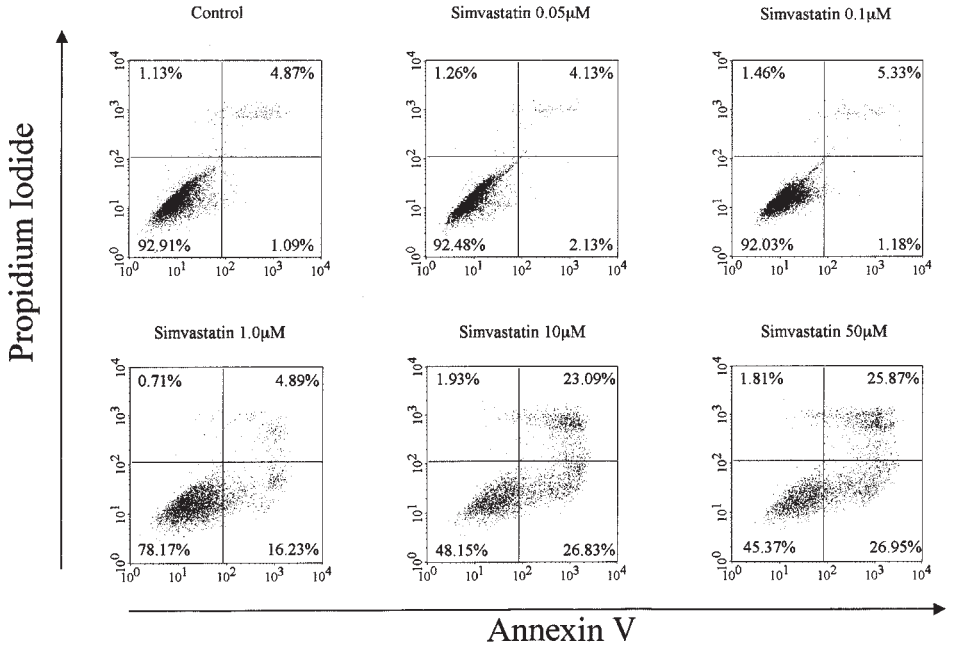

Figure 3. Induction of apoptosis by simvastatin. FLS were incubated with 0.05–10 μM simvastatin or without simvastatin (control) for 48 h and analyzed by flow cytometry. Results are representative of 3 independent experiments performed in triplicate.

ly by 2.14 ± 0.57-fold (1.0 μM simvastatin), 2.87 ± 0.53-fold (10 μM), and 4.30 ± 0.53-fold (50 μM) that of the control. As shown in Figure 5B, under the same conditions, caspase-9 activity was increased significantly by 1.75 ± 0.47-fold (1.0 μM simvastatin), 2.50 ± 0.77-fold (10 μM), and 2.70 ± 0.98-fold (50 μM) that of the control. Taken together, our results strongly suggest that high concentrations of simvastatin induce apoptotic cell death of FLS through the activity of caspases. To our surprise, however, a broad caspase inhibitor, zVAD-fmk, blocked the simvastatin-induced cell death of FLS by only about 40% (data not shown; see below).

DISCUSSION

We previously reported that simvastatin treatment significant-

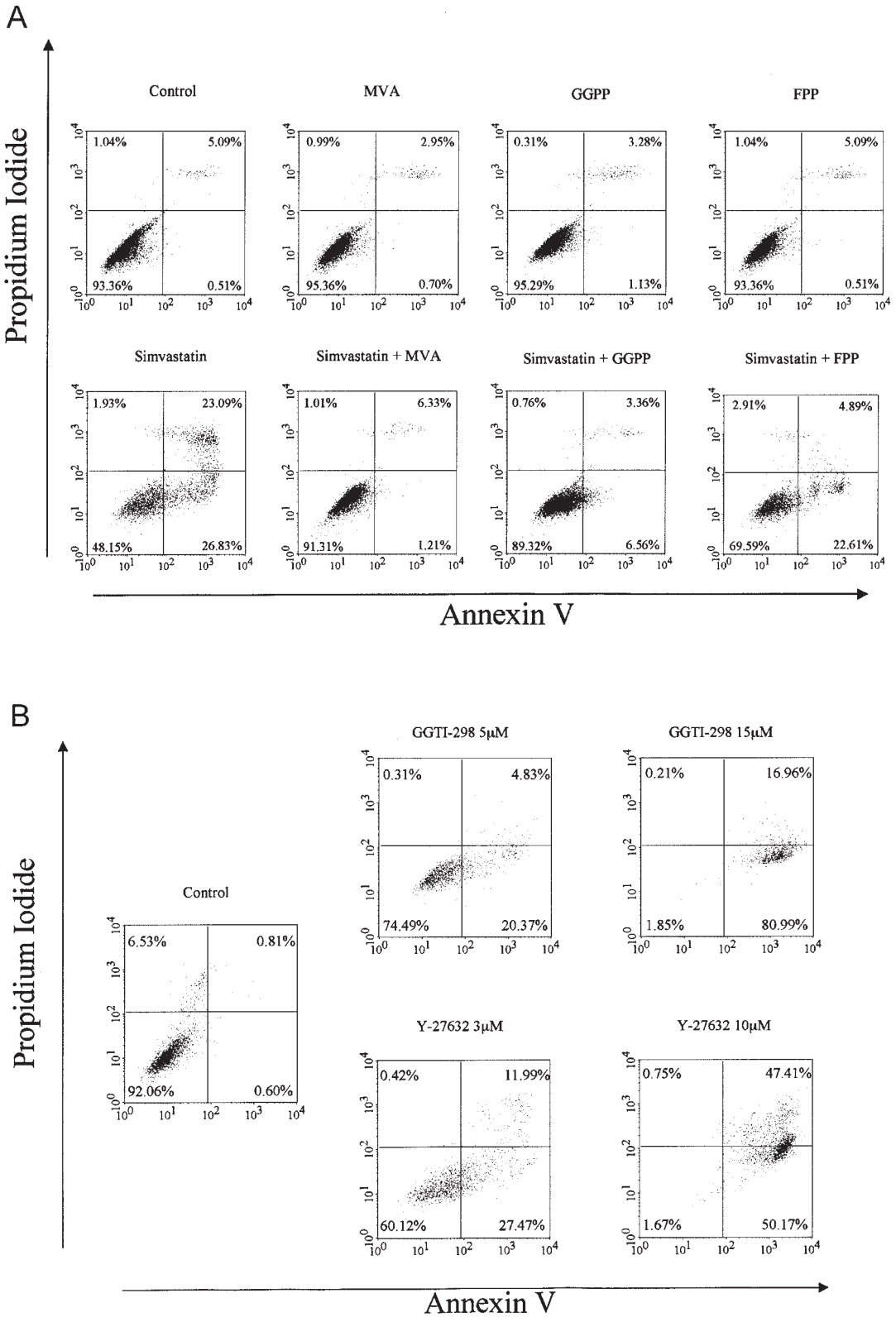

Figure 4. Effects of MVA and isoprenoids on simvastatin-induced apoptosis. A. FLS were incubated with or without 10 μM simvastatin and 100 μM MVA, 10 μM GGPP, or 10 μM FPP for 48 h. B. FLS were incubated with 5.0 or 15 μM concentrations of GGTI-298 and 3.0 or 30 μM concentrations of Y-27632 for 48 h. Apoptotic cells were detected by flow cytometry. Results are representative of 3 independent experiments performed in triplicate.

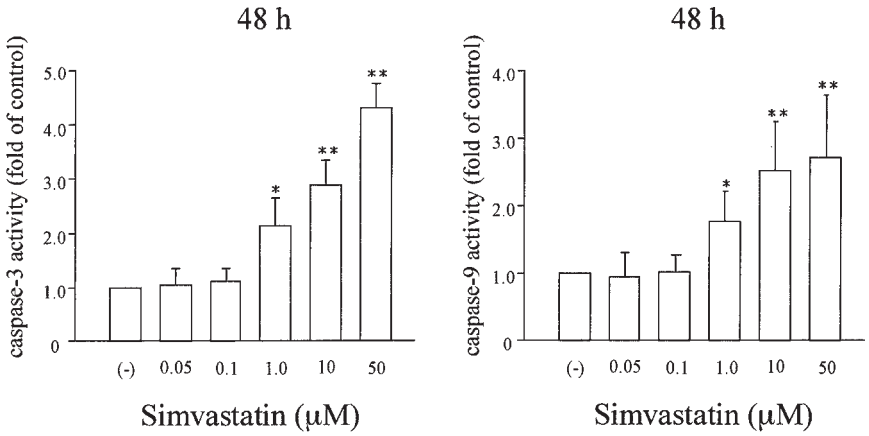

Figure 5. Activation of caspase-3 and -9 by simvastatin. FLS were incubated with 0.05–10 μM simvastatin or without simvastatin (control) for 48 h. Caspase-3 and -9 activities were determined by caspase-3 and -9 assay kits. Data were obtained from duplicate experiments using 3 different samples. **p < 0.01, *p < 0.05 vs control.

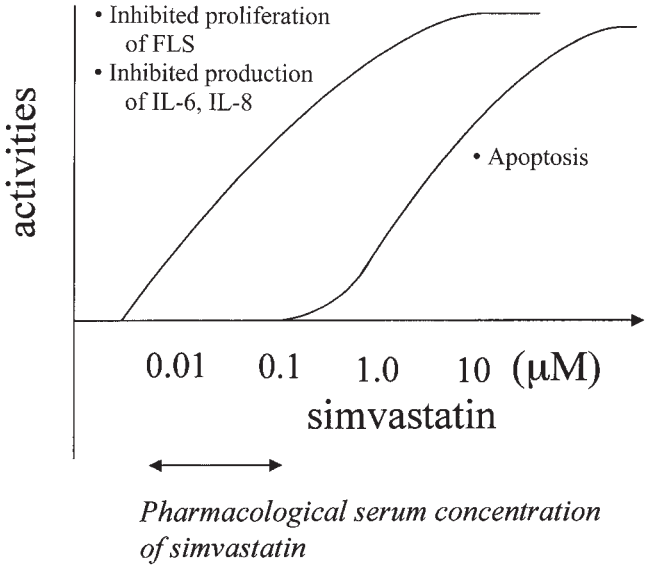

Figure 6. Two different ranges of simvastatin concentration, low (pharmacological range) and high, may affect FLS by 2 different mechanisms. At low concentrations, simvastatin inhibits production of IL-6 and IL-8 in FLS as well as their proliferation induced by TNF-α. At high concentrations, it induces apoptosis in FLS.

ly improves the clinical measures of RA in patients with active disease[32]. We have also reported recently that simvastatin at pharmacological concentrations inhibits production of IL-6 and IL-8 and the cell proliferation induced by TNF-α in FLS from patients with RA[26]. In the present study, we show that simvastatin, at higher concentrations (1.0–50 μM), reduces FLS viability by inducing prominent apoptosis in a dose-dependent manner.

RA is a disease characterized by fibroblastic proliferation, infiltration of the synovial lining by lymphocytes and macrophages, and a paucity of apoptosis[32]. The aggressively increased proliferation and insufficient apoptosis of FLS are implicated in the pathogenesis and progression of RA and disease activity in RA[3]. Application of apoptosis inducers of FLS may be a good strategy to prevent the proliferation of FLS and hence the joint destruction observed in RA.

If statins can specifically cause apoptosis of abnormal FLS, but not normal FLS, in patients with RA, they would be very promising as therapeutic agents. Although statins are known to cause apoptosis in a wide variety of cells[34-36], Nagashima, *et al* showed that fluvastatin, rather than pravastatin, caused more apoptosis of RA-derived FLS than of osteoarthritis (OA)-derived FLS[37]. Connor, *et al*, on the other hand, showed that simvastatin caused more apoptosis of RA FLS than of OA FLS, but only in the presence of TNF-α[38]. Thus, the specificity of statins to induce apoptosis in various cells remains controversial.

As for the mechanisms of apoptosis induced by simvastatin, we first showed that geranylgeranylation of proteins and RhoA kinase is critical for FLS survival. Inhibition of geranylgeranylation, either by depletion of intracellular pools of GGPP through inhibition of HMG-CoA reductase by simvastatin, or by specific inhibition of GGTase I activity by GGTI-298, resulted in induction of apoptosis and reduction of cell viability. In addition, RhoA kinase, which is an effector of geranylgeranylated protein, i.e., RhoA, also induced apoptosis and reduction of cell viability. We furthermore showed that the activities of caspase-3 and -9 were induced by simvastatin in a dose-dependent manner. Thus, simvastatin seems to induce apoptosis through activation of caspases. It is surprising that the broad caspase inhibitor zVAD-fmk only partially prevented the simvastatin-induced cell death of FLS. Our speculation is that simvastatin induced cell death not only by the activation of caspases but also by some caspase-independent pathway(s). There are several reports concerning caspase-independent cell death[39,40] and recently, caspase-independent apoptosis induced by simvastatin was also reported[41]. The authors proposed that apoptosis-inducing factor might be involved in the process.

It is highly likely that the beneficial effect of a therapeutic dose of simvastatin observed in our clinical trial[32] was not from the apoptosis-inducing property of simvastatin, but rather from its effect of reducing cytokine production. However, the fact that a high dose of simvastatin did ameliorate the murine model of arthritis[24] suggests that high concentrations of statin can be a promising agent in the treatment of RA, if only systemic side effects can be avoided. For example, as synovectomy is an established therapy for RA, it may be beneficial to obtain a similar result pharmacologically by administering statins via intraarticular injection. In this way, most of the adverse side effects of systemically administered statins at high concentrations are expected to be avoided. Treatment of animal models of arthritis by such a method or development of new drug delivery systems warrant future studies.

Simvastatin has a concentration-dependent biphasic effect on RA FLS. Low (pharmacological range) and high concentrations of simvastatin affect FLS by 2 different mechanisms, as follows. (1) At low concentrations, simvastatin inhibits the production of IL-6 and IL-8 and the cell proliferation of FLS induced by TNF-α. (2) At high concentrations, simvastatin induces apoptosis in FLS (Figure 6). This biphasic effect is a novel and unique characteristic of simvastatin. If this unique property of this or other statins can be successfully applied in the treatment of RA, a new line of antirheumatic drugs with less adverse side effects compared to existing drugs may be added to the therapeutic repertoire.

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