

Geranylgeranyl-pyrophosphate regulates secretion of pentraxin 3 and monocyte chemoattractant protein-1 from rheumatoid fibroblast-like synoviocytes in distinct manners

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Abstract

Objectives

We previously reported that 10 mg/day of simvastatin significantly reduced clinical scores of rheumatoid arthritis (RA) in active RA patients with hypercholesterolemia. In this study, we have investigated the mechanism by which simvastatin inhibits the production of the mediators of inflammation, such as pentraxin 3 (PTX3) and monocyte chemoattractant protein-1 (MCP-1), from fibroblast-like synoviocytes (FLS) derived from patients with RA.

Methods

FLS from RA patients were cultured with 0-10 μ M simvastatin for 24 h. ELISA and real-time PCR were used to quantitate the protein level and the mRNA level of PTX3 and MCP-1, respectively.

Results

Simvastatin both reduced the secretion of PTX3 and MCP-1 in FLS cultures and inhibited their mRNA expression in these cells. The effects of simvastatin were all completely reversed in the presence of mevalonic acid or geranylgeranyl-pyrophosphate, but not in the presence of farnesyl-pyrophosphate. The geranylgeranyl transferase inhibitor GGTI-298 and the Rho kinase inhibitor Y-27632 inhibited the production of PTX3 but not of MCP-1.

Conclusions

Although simvastatin inhibited the production of PTX3 and MCP-1 in RA FLS, the mechanisms were quite different. It inhibits PTX3 production in a Rho-dependent manner but MCP-1 production in a Rho-independent manner. These results shed light on novel aspects of the anti-inflammatory mechanisms of simvastatin and may prove its important role in the treatment of rheumatic diseases.

Key words

rheumatoid arthritis, fibroblast-like synoviocytes, 3-hydroxy-3-methylglutaryl co-enzyme A reductase inhibitor, pentraxin 3, monocyte chemoattractant protein-1, small G protein

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Introduction

Pentraxins are a family of evolutionarily conserved, soluble and multi-functional pattern recognition proteins characterised by a cyclic multimeric structure (1). Pentraxins are divided into two groups: short pentraxins and long pentraxins. The short pentraxins consist of C-reactive protein (CRP) and serum amyloid P (SAP) (2). The prototype protein in the long pentraxin group is pentraxin 3 (PTX3). PTX3 has been suggested to play important roles in innate resistance to pathogens, the regulation of inflammatory reactions and the clearance of apoptotic cells (3). While short pentraxins are produced mainly in the liver (4), PTX3 is made by diverse types of cells, including endothelial cells, macrophages and fibroblasts, in response to inflammatory signals (1). Recently, PTX3 levels have been shown to be increased in synovial fluid and synovial tissue from patients with rheumatoid arthritis (RA) (5).

The 3-hydroxyl-3-methylglutaryl-CoA (HMG-CoA) reductase inhibitors, statins, have shown to reduce both morbidity and mortality in many clinical trials (6, 7). Many studies have also demonstrated a wide range of their effects on cells and tissues involved in inflammation and/or autoimmunity. For example, statins attenuated the expression of interferon- γ -induced class II major histocompatibility complex molecules, via the class II transactivator protein, in a variety of cells, such as endothelial cells and monocytes/macrophages, and thus to inhibit T cell activation (8). It has been reported that lovastatin and simvastatin inhibit the interactions between leukocyte function-associated antigen 1 (LFA-1) and intercellular adhesion molecule 1 by binding to a specific recognition site on LFA-1, which is independent of their HMG-CoA reductase activity (9).

HMG-CoA reductase, a target enzyme of statins, catalyses the conversion of HMG-CoA to mevalonic acid (MVA) (10). Inhibiting MVA synthesis results in a reduced pool of isoprenoids, such as geranylgeranyl-pyrophosphate (GGPP) and farnesyl-pyrophosphate (FPP), which are involved in the post-translational modification of small

GTP-binding proteins, including members of the Ras and Rho families, *i.e.*, Rho, Rac and Cdc42. Thus, MVA is considered to antagonise the whole effects of statins. On the other hand, FPP rescues the activation of Ras in the presence of statins. GGPP rescues the activation of Rho family members by way of geranylgeranyl transferase type I (GGTase I), which is inhibited by GGTI-298.

Prenylation of small GTP-binding proteins with farnesyl or geranylgeranyl groups is required for their localisation within cell membranes and hence for their function (11). Ras is farnesylated with FPP, and Rho family members are geranylgeranylated with GGPP and act as pivotal regulators of several signalling networks affecting actin cytoskeleton dynamics, transcriptional regulation, cell cycle progression and membrane trafficking (11). The key enzyme of post-translational modifications of the Rho family is GGTase I (11), which can be selectively inhibited by peptidomimetic inhibitors, such as GGTI-298 (12). When cells are stimulated, geranylgeranylated Rho binds to specific effectors to exert its biological functions, which can be specifically inhibited by Y-27632, a Rho kinase inhibitor (13).

We have previously shown that simvastatin significantly improved clinical indicators in RA patients with active disease (14, 15). We have also reported recently that pharmacological concentrations of simvastatin inhibit production of interleukin 6 (IL-6) and IL-8 and cell proliferation induced by tumour necrosis factor- α (TNF- α) in fibroblast-like synoviocytes (FLS) from patients with RA (16) and that high concentration simvastatin induces apoptosis in FLS from patients with RA (17).

In the present study, we have investigated the effect of simvastatin on the production of PTX3 and compared the mechanism with that on the production of monocyte chemoattractant protein-1 (MCP-1) by FLS derived from patients with RA.

Materials and methods

Reagents

Simvastatin was kindly provided by Merck & Co. Inc. (Rahway, NJ, USA).

Competing interests: none declared.

A 10 mM stock solution was prepared as previously described (16). Briefly, 4 mg simvastatin was dissolved in 100 μ l of ethanol and 150 μ l of 0.1 N NaOH and incubated at 50°C for 2 h; the pH was adjusted to 7.0 and the volume to 1.0 ml. A control solution without simvastatin was prepared in the same way. 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 (MVA), farnesyl-pyrophosphate (FPP) and geranylgeranyl-pyrophosphate (GGPP) were from Sigma (St. Louis, MO, USA); GGTI-298 and Y-27632 were from Calbiochem (Schwalbach, Germany). We previously used GGTI-298 at the concentration of 5 to 15 μ M and Y-27632 at 3 to 10 μ M (17). We therefore originally chose similar concentrations. Y-27632, however, did not affect the production of MCP-1 and PTX-3 at the chosen concentrations. We therefore decided to use Y-27632 at higher concentrations (30–60 μ M). 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, TaqMan Gene Expression Assays for PTX3 and Taqman Pre-Developed Assay Reagents for MCP-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were from Applied Biosystems (Foster City, CA, USA). ELISA kits for PTX3 and MCP-1 were purchased from Perseus Proteomics Inc. (Tokyo, Japan) and GE Healthcare (Buckinghamshire, UK), respectively.

Preparation and culture of FLS

Synovial tissues were obtained from 8 RA and 3 osteoarthritis (OA) patients who underwent joint replacement at Saitama Medical University. The patients with RA fulfilled the revised 1987 American College of Rheumatology differentiation criteria. A written informed consent, approved by the ethics committee at Saitama Medical University, was obtained from each patient, prior to the experiment. FLS

were isolated and cultured as previously described (16). Briefly, synovial tissue was minced into small pieces and shaken for 2 h at 37°C in RPMI 1640 containing 0.15 mg/ml collagenase and 0.04% hyaluronidase. After removing tissue debris using a 70 μ m nylon mesh, the cells were cultured in RPMI 1640 containing 10% FCS, 100 U/ml penicillin and 100 μ g/ml streptomycin in a humidified 5% CO₂ incubator at 37°C. Non-adherent cells were removed the next day and adherent cells were maintained as FLS. Sub-confluent cells were sub-cultured using trypsin/EDTA and plated in 24-well plates or 100-mm culture dishes and again grown to sub-confluence. Experiments were carried out in RPMI 1640 containing 1% FCS using FLS from passages three to seven in a humidified 5% CO₂ incubator at 37°C.

Quantitative PCR

Total RNA was extracted from FLS, using RNeasy Mini Kits according to the manufacturer's instructions. Total RNA samples were reverse transcribed using Taqman Reverse Transcription Reagents. The mRNA expression level was determined using a Taqman PCR system and an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). PTX3 and MCP-1 mRNAs were detected using TaqMan Gene Expression Assays and Taqman Pre-Developed Assay Reagent kits, respectively. GAPDH was amplified simultaneously and used for standardisation. PCR reactions consisted of 25 μ l 2x Taqman Universal PCR Master Mix, 2.5 μ l 20x target primers and probe, 2.5 μ l 20x control primers and probe and 300 ng cDNA, and were made up to 50 μ l with RNase-free water. The conditions for quantitative PCR were: 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 mRNA analysis for PTX3 and MCP-1, changes in the reporter fluorescence from each reaction well were evaluated. For each gene, the threshold cycle (Ct) was defined as the PCR cycle at which fluorescence rose above baseline. The difference between the

threshold cycle of the target gene and that of the control GAPDH gene gave the standardised expression level, Δ Ct. The difference between Δ Ct values for nontreated and simvastatin-treated FLS gave the $\Delta\Delta$ Ct value, which was used to calculate relative expression level in simvastatin-treated FLS as $2^{-\Delta\Delta Ct}$. The expression level of each gene was interpreted as fold-increase in simvastatin-treated FLS compared with that in nontreated controls (16).

ELISA for PTX3 and MCP-1

The detection of PTX3 and MCP-1 proteins in cell supernatants was performed with PTX3 and MCP-1 ELISA kits according to the manufacturer's instructions.

Statistical analysis

All data were expressed as means \pm standard error (SEM). Statistical analysis used the Mann-Whitney U-test with *p*-values <0.05 considered as significant.

Results

Inhibitory effects of simvastatin on PTX3 production in FLS

As shown in Figure 1A, the levels of PTX3 secreted by RA FLS after 24 h-culture in the absence of simvastatin were significantly higher than those with OA. When FLS from RA patients were incubated for 24 h with simvastatin, PTX3 in culture supernatants was reduced significantly, to 70.0 \pm 2.0% of control in the presence of 1.0 μ M simvastatin and 62.0 \pm 6.7% of control in the presence of 10 μ M simvastatin (Fig. 1B). The expression of PTX3 mRNA was also reduced in cultures treated with simvastatin, to 62.3 \pm 11.0%, 38.8 \pm 5.0% and 14.3 \pm 1.2%, compared with control, in the presence of 0.1 μ M, 1.0 μ M and 10 μ M simvastatin, respectively (Fig. 1C).

MVA and GGPP restore the production of PTX3 in FLS in the presence of simvastatin

As shown in Figure 2A, the inhibitory effect of 1.0 μ M simvastatin on PTX3 production in RA FLS was suppressed when the cells were simultaneously treated with 100 μ M MVA or 10 μ M GGPP, but unaffected with FPP. MVA, GGPP and FPP had minimal effects

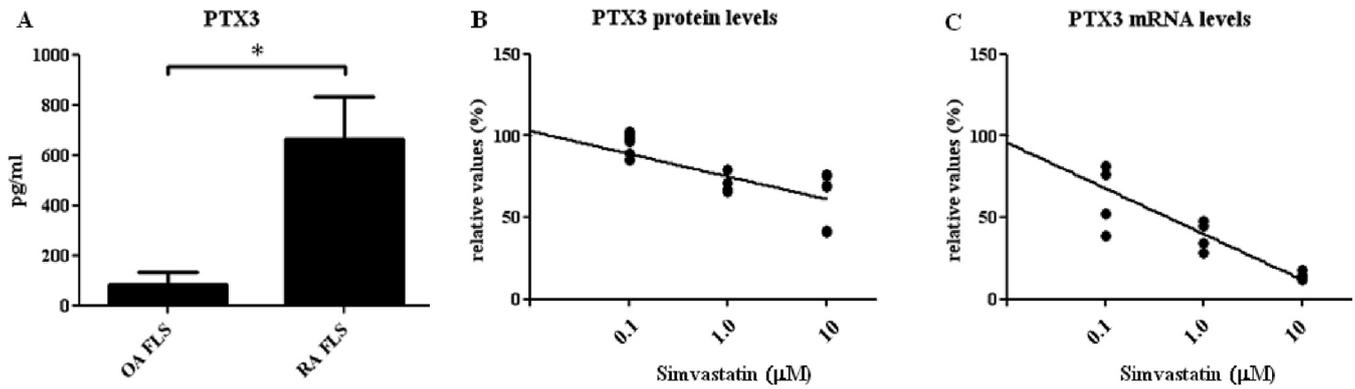


Fig. 1. RA FLS specifically produced PTX3, which was suppressed by simvastatin.

A. FLS from patients with RA or OA were incubated without simvastatin for 24 h. PTX3 levels in culture supernatants were determined by ELISA. Data are expressed as means \pm SEM of duplicate experiments ($n=6$); $*p<0.05$ versus control. B, C. The expression of PTX3 was reduced by simvastatin in a dose-dependent manner both at the protein level (B, $n=6$) and at the mRNA level (C, $n=4$). FLS from RA patients were incubated with 0.1–10 μM simvastatin for 24 h. Data shown are values relative to control cells cultured in the absence of simvastatin. Tendency lines are also shown.

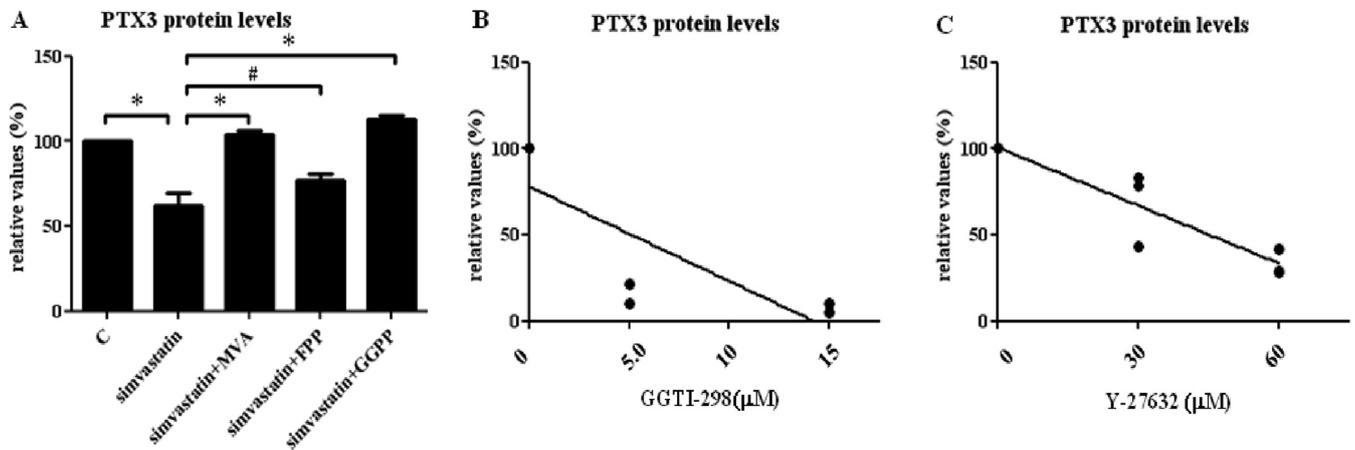


Fig. 2. Effects of MVA and isoprenoids on the simvastatin-mediated suppression of PTX3 production by FLS.

A. MVA and GGPP attenuated suppression of PTX3 production by simvastatin in RA FLS. FLS were incubated with 0.1–10 μM simvastatin for 24 h. PTX3 levels in culture supernatants were determined by ELISA ($n=6$); $*p<0.05$ versus control, # not significant. B, C. An inhibitor of geranylgeranylation, GGTI-298 (B), and a Rho kinase inhibitor, Y-27632 (C), suppressed PTX3 production in RA FLS in a dose-dependent manner. FLS were incubated with 5.0 or 15 μM GGTI-298 or with 30 or 60 μM Y-27632 for 24 h. Tendency lines are shown.

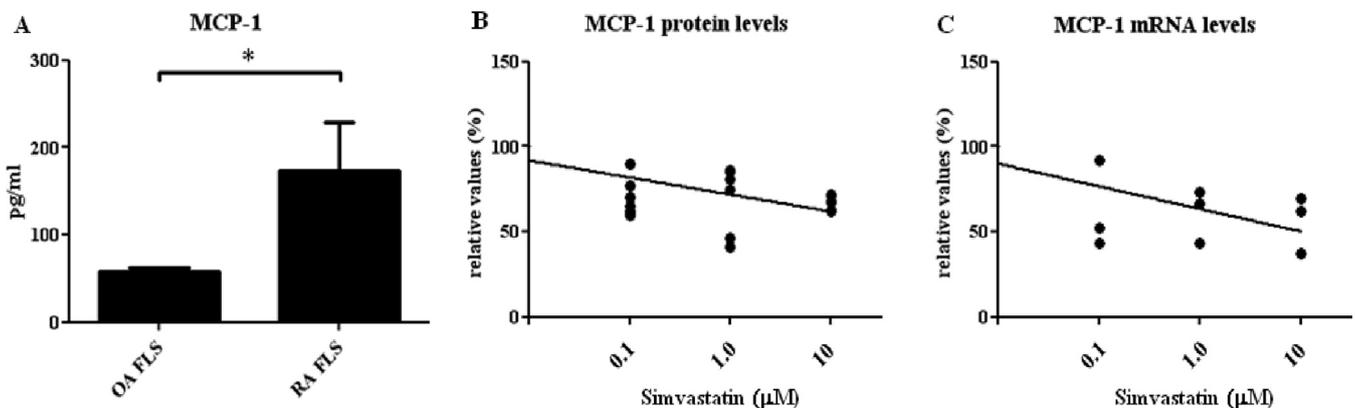


Fig. 3. RA FLS specifically produced MCP-1, which was suppressed by simvastatin.

A. FLS from patients with RA or OA were incubated without simvastatin for 24 h. MCP-1 levels in culture supernatants were determined by ELISA ($n=6$); $*p<0.05$ versus control. B, C. The expression of MCP-1 was suppressed by simvastatin in a dose-dependent manner both at the protein level (B, $n=6$) and at the mRNA level (C, $n=3$). FLS from RA patients were incubated with 0.1–10 μM simvastatin for 24 h. Data shown are values relative to control cells cultured in the absence of simvastatin. Tendency lines are shown.

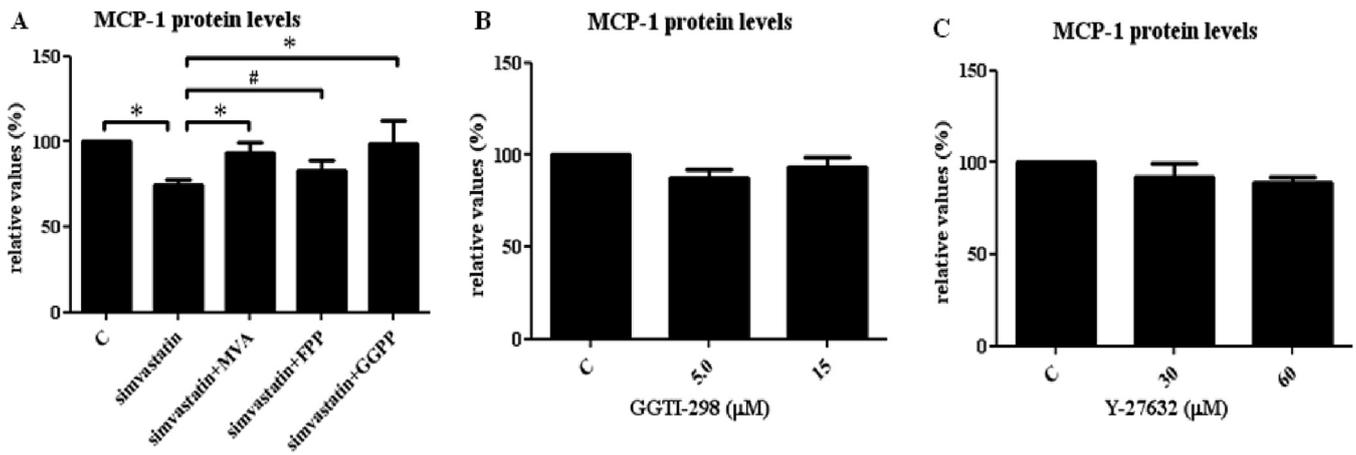


Fig. 4. Effects of MVA and isoprenoids on the simvastatin-mediated suppression of MCP-1 production by FLS. **A.** MVA and GGPP attenuated suppression of MCP-1 production by simvastatin in RA FLS. FLS were incubated with 0.1–10 μM simvastatin for 24 h. MCP-1 levels in culture supernatants were determined by ELISA ($n=6$); * $p<0.05$ versus control, #not significant. **B.** **C.** The production of MCP-1 was not affected by GGTI-298 (**B.**, $n=3$) and Y-27632 (**C.**, $n=3$).

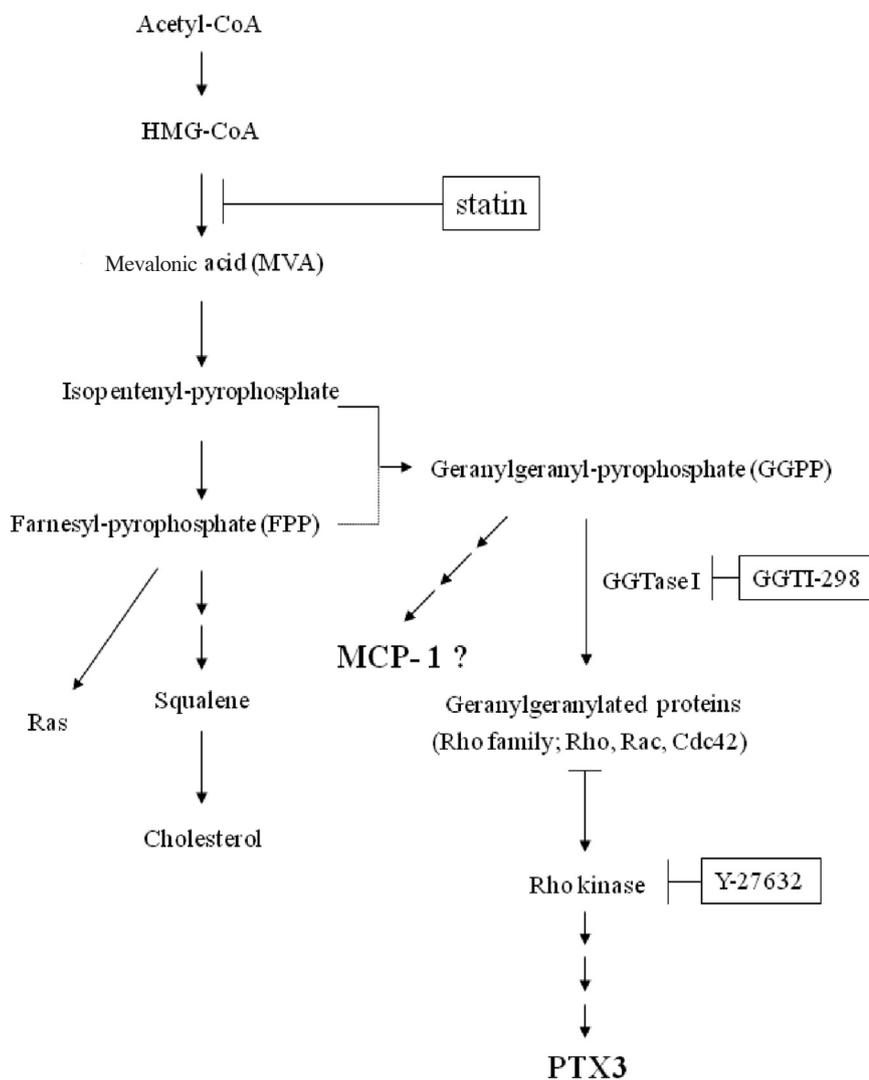


Fig. 5. Simvastatin affects FLS by two different mechanisms. Simvastatin both reduced the secretion of PTX3 and MCP-1 in FLS. Whereas it inhibits PTX3 production in a Rho-dependent manner, it inhibits MCP-1 production in a Rho-independent manner.

on PTX3 production in the absence of simvastatin (data not shown).

Inhibitory effects of GGTI-298 and Y-27632 on PTX3 production in FLS

When FLS were incubated for 24 h with the GGTI-298, the PTX3 protein secreted into culture supernatants was reduced to $17.7\pm 3.7\%$ and $6.8\pm 1.6\%$ of control, with 5.0 μM and 15 μM GGTI-298, respectively (Fig. 2B). When FLS were incubated with Y-27632 for 24 h, PTX3 levels were reduced to $68.0\pm 12.6\%$ and $32.8\pm 4.4\%$ of control, with 30 μM and 60 μM Y-27632, respectively (Fig. 2C).

Inhibitory effects of simvastatin on MCP-1 production in RA FLS

So far, most of the molecules that we discovered to be reduced by simvastatin were regulated in a Rho-kinase dependent manner (16, 17). In our screening process, however, we also discovered a molecule, MCP-1, that was reduced by simvastatin in a Rho-kinase independent manner. The chemokine MCP-1 has been suggested to be a potential therapeutic target in RA. Its level increases in the peripheral blood, synovial fluid and synovial tissue in patients with RA, and it is known to be a potent chemoattractant for monocytes/macrophages and T cells (18).

As shown in Figure 3A, the levels of MCP-1 secreted by RA FLS after 24 h-culture in the absence of simvasta-

tin were significantly higher than those with OA.

When FLS from RA patients were incubated for 24 h with simvastatin, the MCP-1 levels in culture supernatants were reduced significantly, to $69.3 \pm 6.2\%$, $69.0 \pm 10.0\%$ and $67.1 \pm 1.7\%$, compared with control, in the presence of 0.1 μM , 1.0 μM and 10 μM simvastatin, respectively (Fig. 3B). The expression of MCP-1 mRNA was also reduced to $62.6 \pm 15.1\%$, $61.0 \pm 9.3\%$ and $56.2 \pm 9.7\%$, compared to controls, in the presence of 0.1 μM , 1.0 μM and 10 μM simvastatin, respectively (Fig. 3C).

MVA and GGPP restore the production of MCP-1 in FLS in the presence of simvastatin

As shown in Figure 4A, the inhibitory effect of 1.0 μM simvastatin on MCP-1 production in RA FLS attenuated if the cells were simultaneously treated by 100 μM MVA or 10 μM GGPP, but unaffected by FPP. MVA, GGPP and FPP had minimal effects on MCP-1 production in the absence of simvastatin (data not shown).

No inhibitory effects of GGTI-298 and Y-27632 on MCP-1 production in FLS

When FLS were incubated with GGTI-298 or Y-27632 for 24 h, the levels of MCP-1 were not affected by these reagents in contrast to those of PTX3 (Fig. 4B).

Discussion

In our previous reports, the production of IL-6 and IL-8 was also found to be regulated by the GGPP-dependent pathway (16). Since the expression of IL-6, IL-8 and MCP-1 in RA synovium appears to be associated with disease activity (19, 20, 21), simvastatin is thought to act via these pathways to show beneficial effects on RA patients. Furthermore, it has been demonstrated that MCP-1 is stored and released from vesicles in FLS and that high density lipoproteins (HDL) inhibit the release of MCP-1 (22). Since statins are known to increase the level of plasma HDL (23), simvastatin may inhibit both the production and release of MCP-1 from FLS. In the present study, we have shown

that simvastatin inhibited the production of PTX3 and MCP-1 on FLS from patients with RA. In addition, we have demonstrated that GGPP prevents the simvastatin-induced inhibition of PTX3 and MCP-1 production, suggesting that GGPP is critical for PTX3 and MCP-1 production in these cells. In accordance with our previous reports (16, 17), we now provide an additional evidence that simvastatin has beneficial effects on activated FLS from patients with RA.

It has been shown that the main source of PTX3 in the synovium of RA patients is pannus, in which monocytes/macrophages, FLS and endothelial cells are rich (5). It is tempting to speculate that PTX3 participates in synovial membrane injury by amplifying complement-mediated tissue damage (3). Based on these reports, our result may imply that simvastatin could improve the synovial membrane injury in RA patients.

On the other hand, among chemokines, MCP-1 is known to be a potent mediator for recruiting monocytes/macrophages and T cells (24). These cells have been shown to be directly involved in the induction and perpetuation of synovitis and subsequent joint destruction in RA (21). It has been shown that arthritis in MRL/lpr mice is suppressed by treating with anti-MCP-1 monoclonal antibodies before the disease-onset (25). Several lines of evidence have suggested MCP-1 plays important roles in monocyte recruitment and developing atherosclerosis (26, 27). Taken together with a poor-prognostic link between atherosclerosis and RA (28), MCP-1 may be one of the target molecules in the treatment of RA from both an anti-inflammatory and an anti-atherosclerotic aspects. Thus, simvastatin could reduce not only cardiovascular morbidity and mortality but also improve clinical measures in RA patients.

Surprisingly, treating FLS with GGTI-298 or Y-27632 significantly inhibited the production of PTX3, but had no effect on MCP-1 production. We speculated that Rho/Rho kinase may be involved in regulating the production of PTX3 but not MCP-1 in FLS (Fig. 5). In fact, it has been reported that in human endothelial cells and macrophages, statins inhibit CCR2/MCP-1

receptor expression via Rho-independent pathway (29). Therefore, in FLS it is possible that simvastatin inhibits the production of MCP-1 via Rho-independent pathway.

In conclusion, although simvastatin inhibits both PTX3 and MCP-1 production in RA FLS, the mechanisms are quite different. Simvastatin inhibits PTX3 production in a Rho-dependent manner but MCP-1 production in a Rho-independent manner. Understanding the multiple mechanisms by which simvastatin reduces these inflammatory mediators, we may be able to finely regulate the pathological conditions of patients with rheumatic diseases, such as RA.

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