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RESEARCH PAPER

Statins suppress interleukin-6-induced monocyte chemo-attractant protein-1 by inhibiting Janus kinase/signal transducers and activators of transcription pathways in human vascular endothelial cells

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Background and purpose: The mechanisms of anti-inflammatory actions of statins, 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase inhibitors, remain unclear. We investigated the effects of statins on interleukin (IL)-6-induced monocyte chemo-attractant protein (MCP)-1 expression and monocyte chemotaxis.
Experimental approach: Cultures of human aortic endothelial cells (HAECs) were stimulated with IL-6 in the absence and presence of statins. Gene expression and protein secretion of MCP-1, phosphorylation of Janus kinase (JAK) and the signal transducers and activators of transcription (STAT) pathway, and human monocyte migration were examined.
Key results: IL-6 plus its soluble receptor sIL-6R (IL-6/sIL-6R) promoted THP-1 monocyte migration, and increased gene expression and protein secretion of MCP-1, more than IL-6 alone or sIL-6R alone. Various statins inhibited IL-6/sIL-6R-promoted monocyte migration and MCP-1 expression in HAECs. Co-incubation of mevalonate and geranylgeranyl pyrophosphate, but not farnesyl pyrophosphate, reversed the inhibitory effects of statins on MCP-1 expression. Geranylgeranyl transferase inhibitor, but not farnesyl transferase inhibitor, suppressed IL-6/sIL-6R-stimulated MCP-1 expression. IL-6/sIL-6R rapidly phosphorylated JAK1, JAK2, TYK2, STAT1 and STAT3, which were inhibited by statins. Transfection of STAT3 small interfering RNA (siRNA), but not STAT1 siRNA, attenuated the ability of IL-6/sIL-6R to enhance THP-1 monocyte migration. In addition, statins blocked IL-6/sIL-6R-induced translocation of STAT3 to the nucleus.
Conclusions and implications: Statins suppressed IL-6/sIL-6R-induced monocyte chemotaxis and MCP-1 expression in HAECs by inhibiting JAK/STAT signalling cascades, explaining why statins have anti-inflammatory properties beyond cholesterol reduction.
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Keywords: statin; interleukin-6; MCP-1, JAK; STAT; atherosclerosis; cells; endothelium; inflammation

Abbreviations: CCR2, C-C chemokine receptor 2; FCS, fetal calf serum; FPP, farnesyl pyrophosphate; FTI, farnesyl transferase inhibitor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GGPP, geranylgeranyl pyrophosphate; GGTI, geranylgeranyl transferase inhibitor; gp130, glycoprotein130; HAECs, human aortic endothelial cells; HMG-CoA, 3-hydroxy-3-methylglutaryl CoA; IL-6, interleukin-6; IL-6R, interleukin-6 receptor; JAK, Janus kinase; MCP-1, monocyte chemo-attractant protein-1; PMSF, phenyl methyl sulphonyl fluoride; sIL-6R, soluble interleukin-6 receptor; siRNA, small interfering RNA; STAT, signal transducers and activators of transcription

Introduction

Cytokines transmit their signals through a family of cytoplasmic tyrosine kinases known as Janus kinases (JAKs) (Rane and Reddy, 2002). Interleukin (IL)-6 is a multifunctional cytokine that activates not only JAK1, JAK2 and TYK2, but also signal transducers and activators of transcription (STAT) 1 and STAT3 (Heinrich *et al.*, 1998). Phosphorylations of STAT1 and STAT3, and their subsequent translocation to the nucleus activate transcription of various gene expressions. IL-6 exerts biological actions through its receptor complex, glycoprotein (gp) 130 and IL-6 receptor (IL-6R) (Heinrich *et al.*, 1998; receptor nomenclature follows Alexander *et al.*, 2008). Gp130 only transmits signals when IL-6 is bound to IL-6R (Murakami

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et al., 1993). The cells lacking surface IL-6R subunit are not responsive to IL-6, but will respond to IL-6 in the presence of the soluble form of IL-6R subunit (sIL-6R) (Romano *et al.*, 1997). Therefore, it is possible that the addition of sIL-6R may influence the biological actions of IL-6, depending on the type of cells.

Previous investigations have demonstrated that IL-6 induces monocyte chemo-attractant protein (MCP)-1 (Biswas *et al.*, 1998; Coletta *et al.*, 2000; Rott *et al.*, 2003). MCP-1 is a member of the C-C chemokines, and plays an important role in the monocyte/macrophage chemotaxis involved in acute and chronic inflammation (Schall and Bacon, 1994; Rollins, 1997). Recently, atherosclerosis is considered a chronic inflammatory disease leading to acute clinical events (Ross, 1999; Libby, 2002). MCP-1 mRNA and protein secretion are augmented in animal and human atherosclerosis (Ylä-Herttuala *et al.*, 1991). The atheroma-forming cells also express C-C chemokine receptor 2 (CCR2), a receptor for MCP-1, and the activation of MCP-1/CCR2 pathway induces cell adhesion molecules, pro-inflammatory cytokines and chemokines, accelerating atherosclerosis formation in animal models (Aiello *et al.*, 1999; Namiki *et al.*, 2002). In contrast, inhibition of MCP-1/CCR2 pathway prevents initiation and development of atherosclerotic lesions (Boring *et al.*, 1998; Gosling *et al.*, 1999). Additionally, MCP-1 is constitutively secreted from a variety of cells, such as endothelial cells, vascular smooth muscle cells and monocytes (Rollins, 1997).

Statins, 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase inhibitors, have pleiotropic actions, including anti-inflammatory effects that extend beyond cholesterol reduction (Takemoto and Liao, 2001). However, the precise mechanisms by which statins inhibit inflammation remain unclear. Previous investigators have demonstrated that statins suppressed MCP-1 expression in both inflammation and atherosclerosis, and indicated that statins exerted their anti-inflammatory and anti-atherosclerotic effects, at least in part through inhibiting MCP-1 (Martinez-Gonzalez *et al.*, 2001; Kleemann *et al.*, 2003; Veillard *et al.*, 2006). We hypothesized that statins could suppress IL-6-induced monocyte chemotaxis through inhibiting MCP-1 expression. Therefore, the present study was designed to investigate the influences of statins on IL-6-induced monocyte chemotaxis and MCP-1 expression in human aortic endothelial cells (HAECs), especially in regard to JAK/STAT signalling pathways.

Methods

Cell culture of HAECs

HAECs were purchased from Clonetics (San Diego, CA, USA), and cultured as previously described with some modifications (Jougasaki *et al.*, 1998; Ichiki *et al.*, 2008). HAECs were cultured at 37°C in 5% CO_2 and 95% air in a humidified atmosphere. At confluence, HAECs appeared as typical 'cobblestone'-patterned monolayers. HAECs in the third to sixth passage were used in the present experiments. THP-1 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). THP-1 cells were cultured in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum (FCS) and 5×10^{-5} mol·L^{-1} β-mercaptoethanol.

Total RNA extraction and ribonuclease protection assay

Total RNA was extracted from HAECs using Pure Link Micro-to-Midi total RNA extraction kit (Invitrogen, Calsbad, CA, USA). A biotin-labelled antisense RNA probe cocktail was transcribed from a set of custom-designed cDNA templates (BD Biosciences Pharmingen, San Jose, CA, USA) using MAXIscript *in vitro* transcription kit (Ambion, Austin, TX, USA). Full-length probe sizes for MCP-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were 232 and 124 bp, respectively, while protected fragment sizes were 203 and 96 bp respectively. The biotin-labelled antisense probes were hybridized to 5 μg of total RNA, and subjected to RNase digestion with an RPA III kit (Ambion). The RNase-protected fragments were purified, resolved on 6% denaturing Tris–borate–EDTA–urea–polyacrylamide gels (Invitrogen) and transferred to nylon membranes. The protected fragments were visualized by incubation of the membranes with an alkaline phosphate/streptavidin solution with BioDetect chemiluminescence reagent (Ambion). The intensities of the blots of MCP-1 mRNA were quantified using LAS-3000 Lumino Image Analyser (Fujifilm, Tokyo, Japan), and normalized to those of GAPDH mRNA. Yeast RNA served as a negative control.

Western immunoblot analysis

HAECs were washed and immediately lysed in ice-cold cell lysis buffer with 1 mmol·L^{-1} phenyl methyl sulphonyl fluoride (PMSF) and protease inhibitor cocktail, Complete Mini. After measuring the protein concentrations, cell lysates (10 μg) were resuspended in SDS loading buffer, sonicated, boiled for 5 min and separated by NuPAGE 4–12% bis-Tris gels (Invitrogen). The proteins were transferred to PVDF membranes (Invitrogen) by electroblotting, and reacted with phosphorylation state-specific antibodies at concentrations suggested by the manufacturers. The proteins were visualized by alkaline phosphate-conjugated goat anti-rabbit IgG and a chemiluminescence detection system (Cell Signaling Technology, Beverly, MA, USA). The membranes were stripped with ReBlot Plus Recycling Kit (Chemicon International, Temecula, CA, USA), and reprobed with respective total antibodies.

Measurement of MCP-1 protein secreted from HAECs

Protein concentrations of MCP-1 were determined by using commercially available ELISA kit according to the manufacturer's instruction (R&D Systems, Minneapolis, MN, USA). Intra-assay and inter-assay variations are 5 and 6% respectively.

Chemotaxis assay

Chemotactic activity was determined using a microchemotaxis chamber with polyvinylpyrrolidone-free polycarbonate filter (5 μm pore size). The culture medium from treated cells was transferred to the lower chamber of ChemoTx microplates (Neuro Probe Inc., Gaithersburg, MD, USA). An aliquot of THP-1 monocytic cell suspension (2×10^6 cells·mL^{-1}) was added to the upper compartment, and the number of THP-1 cells migrated to the lower chamber was counted with a haemocytometer. The culture medium from

untreated HAECs was used to determine basal migration, and served as a control. The culture medium from untreated HAECs supplemented with recombinant human MCP-1 (1–100 ng·mL^{-1}) served as a positive control. To assess MCP-1-specific chemotaxis, anti-human MCP-1 polyclonal antibody was added at 80 μg·mL^{-1} to neutralize the secreted MCP-1. Normal goat IgG was used as a negative control for MCP-1 neutralization study.

Transfection with small interfering RNA (siRNA)
Transfection with siRNA was performed according to the manufacturer's protocol (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Transfection complexes were prepared using siRNA reagent, transfection medium and STAT1 siRNA or STAT3 siRNA, and delivered to cell monolayers with 100 nmol·L^{-1} final concentration of siRNA duplexes. A scrambled control siRNA was used as a negative control.

Immunocytochemistry for localizations of phospho-STAT3
HAECs plated on a BioCoat slide glass (BD Biosciences, Bedford, MA, USA) were fixed with 4% paraformaldehyde. The slides were blocked with normal horse serum for 1 h, and then incubated with rabbit anti-human phospho-STAT3 polyclonal antibody at a dilution of 1:100. After overnight incubation, the slides were washed and incubated with goat anti-rabbit IgG-Alexa 594 at a dilution of 1:100 for 1 h, and counterstained for nuclei with Hoechst 33342 (50 ng·mL^{-1}, Invitrogen, Carlsbad, CA, USA) for 5 min. Stained slides were mounted and analysed by fluorescence microscopy (Olympus, Tokyo, Japan).

Statistical analysis
Results of quantitative studies are expressed as means ± SEM. Each data point represents the average of three to six experiments. Statistical comparisons were performed by using ANOVA for repeated measures followed by Fisher's least significant difference test when appropriate. Comparisons between groups were performed by using Student's unpaired *t*-test. Statistical significance was accepted for P value less than 0.05.

Materials
Cell culture supplies such as Medium 199, RPMI 1640, heat-inactivated FCS, fungizone and penicillin–streptomycin were purchased from Gibco, Invitrogen. AG490, JAK3 inhibitor II, piceatannol, FTI-276 and GGTI-286 were obtained from Calbiochem (La Jolla, CA, USA). Fludarabine was purchased from Toronto Research Chemicals (North York, Canada). Fluvastatin was a generous gift from Tanabe Seiyaku Co., Ltd. (Osaka, Japan). Simvastatin was purchased from Wako Pure Chemical (Osaka, Japan), and atorvastatin was obtained from LKT Laboratories (St Paul, MN, USA). Mevalonate, farnesyl pyrophosphate (FPP), geranylgeranyl pyrophosphate (GGPP) and β-mercaptoethanol were obtained from Sigma (St Louis, MO, USA). Recombinant human IL-6 and sIL-6R were purchased from PeproTech (London, UK). Recombinant human MCP-1, normal goat IgG and goat anti-human MCP-1 polyclonal antibody were purchased from R&D Systems. Cell lysis buffer and SDS loading buffer were purchased from Cell Signaling Technology. PMSF and protease inhibitor cocktail (Complete Mini) were obtained from Roche Diagnotics GmbH (Mannheim, Germany). The rabbit polyclonal antibodies specific for JAK1, JAK2, TYK2 and phospho-TYK2 (Tyr1054/1055) were purchased from Santa Cruz Biotechnology. The mouse monoclonal antibody against β-actin was obtained from Santa Cruz Biotechnology. The rabbit polyclonal antibodies specific for phospho-JAK1 (Tyr1022/Tyr1023), phospho-JAK2 (Tyr1007/Tyr1008), STAT1, phospho-STAT1 (Tyr701), STAT3 and phospho-STAT3 (Tyr705) were purchased from Cell Signaling Technology. The specific siRNA duplexes targeted against human STAT1 and STAT3 were obtained from Ambion and Santa Cruz Biotechnology, respectively.

Results

IL-6 plus sIL-6R (IL-6/sIL-6R) stimulates MCP-1 *gene expression, and statins inhibit* MCP-1 *gene activation*
HAECs were incubated with IL-6 plus sIL-6R (IL-6/sIL-6R) for various time periods, and total RNA was subjected to ribonuclease protection assay. MCP-1 mRNA increased at 1 h after incubation with IL-6/sIL-6R, and decreased thereafter (Figure 1A). To elucidate the combinatorial effects, HAECs were incubated with IL-6 alone or sIL-6R alone at a concentration of 10^{-9} mol·L^{-1} each for 1 h, and *MCP-1* gene expression was compared with that stimulated with IL-6/sIL-6R. Although IL-6 itself increased MCP-1 mRNA as compared with control untreated cells, the increment of MCP-1 mRNA was much less as compared with IL-6/sIL-6R (Figure 1B). On the other hand, sIL-6R, used alone, did not significantly increase *MCP-1* gene expression. Treatment of HAECs with various statins such as fluvastatin, simvastatin or atorvastatin inhibited IL-6/sIL-6R-induced MCP-1 mRNA in HAECs (Figure 1C).

IL-6/sIL-6R induces phosphorylation of JAK/STAT pathway, and statins inhibit this phosphorylation
HAECs were exposed to IL-6/sIL-6R for different time periods (2–60 min), and protein extracts were analysed by Western immunoblotting. IL-6/sIL-6R induced phosphorylations of JAK1, JAK2, TYK2, STAT1 and STAT3 within 5 min, peaking between 15 and 30 min, and declining at 60 min (Figure 2A). The levels of respective total proteins remained generally unaffected (lower panels of each blot). IL-6/sIL-6R-induced phosphorylations of JAK1, JAK2, TYK2, STAT1 and STAT3 were more intense than those induced by IL-6 alone or sIL-6R alone (data not shown). To examine the influences of statins on JAK/STAT pathway, HAECs were pretreated with statins and then exposed to IL-6/sIL-6R. Total cellular protein extracts prepared after 15 min of IL-6/sIL-6R treatment were analysed for JAK/STAT activation. As shown in Figure 2B, statin reduced IL-6/sIL-6R-induced phosphorylations of JAK1, JAK2, TYK2, STAT1 and STAT3. The total protein levels of JAK/STAT system detected by total antibodies remained

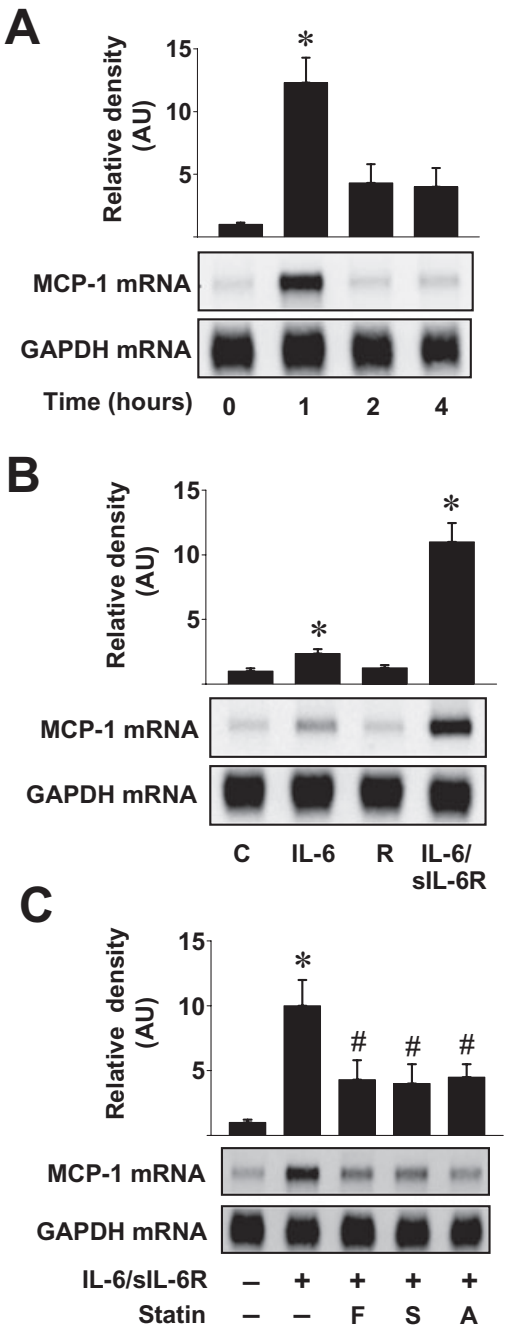

Figure 1 IL-6 plus its soluble receptor sIL-6R (IL-6/sIL-6R) increases *MCP-1* gene expression, and various statins inhibit *MCP-1* gene activation induced by IL-6/sIL-6R. GAPDH mRNA served as a loading control. Bars represent mean ± SEM of three independent experiments. (A) HAECs were treated with 10^{-9} mol·L^{-1} IL-6/sIL-6R for various time periods, and MCP-1 mRNA was determined by ribonuclease protection assay. *P < 0.05 versus time zero. (B) HAECs were incubated with IL-6 alone, sIL-6R alone (R) or IL-6/sIL-6R at a concentration of 10^{-9} mol·L^{-1} each for 1 h, and MCP-1 mRNA was determined by ribonuclease protection assay. *P < 0.05 versus untreated control cells (C). (C) HAECs were pretreated with either fluvastatin (F, 10 μmol·L^{-1}), simvastatin (S, 10 μmol·L^{-1}) or atorvastatin (A, 10 μmol·L^{-1}) for 24 h, and then incubated with IL-6/sIL-6R for 1 h. *P < 0.05 versus untreated control cells. #P < 0.05 versus IL-6/sIL-6R.

constant and were not affected by the treatments of HAECs with statins (lower panels of each blot).

IL-6/sIL-6R stimulates MCP-1 protein secretion from HAECs, and statins inhibit IL-6/sIL-6R-stimulated MCP-1 secretion

HAECs were treated with IL-6 alone, sIL-6R alone or IL-6/sIL-6R at a concentration of 10^{-9} mol·L^{-1} each for 24 h, and the supernatant was assayed by sandwich ELISA. IL-6/sIL-6R stimulated the secretion of MCP-1 protein from HAECs, and the secretion rate of MCP-1 by treatment with IL-6/sIL-6R was more than that with IL-6 alone or with sIL-6R alone (Figure 3A). MCP-1 protein secretion augmented by IL-6/sIL-6R was inhibited by pretreatment with various statins (Figure 3A). Suppression of MCP-1 protein secretion by statin was dose dependent, and was seen at concentrations greater than 0.1 μmol·L^{-1} (Figure 3B).

IL-6/sIL-6R enhances THP-1 monocyte chemotaxis, and various statins block IL-6/sIL-6R-mediated enhancement of THP-1 monocyte chemotaxis

Migration of THP-1 cells towards the culture medium from IL-6/sIL-6R-treated HAECs increased as compared with the culture medium from untreated cells. IL-6/sIL-6R-mediated monocyte chemotaxis was greater than that after IL-6 alone or sIL-6R alone (data not shown). Migration enhanced by IL-6/sIL-6R was significantly inhibited by pre-incubation of the culture medium with goat anti-human MCP-1 polyclonal antibody, whereas goat IgG had no effects on THP-1 monocyte migration (Figure 3C), indicating that IL-6/sIL-6R-induced THP-1 monocyte migration was, at least in part, due to the chemotactic actions of MCP-1. Migration of THP-1 cells in response to recombinant human MCP-1 (1–100 ng·mL^{-1}) also increased in a dose-dependent manner (data not shown). We then examined the effects of various statins on IL-6/sIL-6R-enhanced THP-1 monocyte chemotaxis. HAECs were pretreated with fluvastatin, simvastatin and atorvastatin at a concentration of 10 mol·L^{-1} each, and then exposed to IL-6/sIL-6R. All statins suppressed THP-1 monocyte migration enhanced by IL-6/sIL-6R (Figure 3C).

Mevalonate and GGPP, but not FPP, reverse the inhibitory effects of statin on IL-6/sIL-6R-induced MCP-1 activation

To examine the role of mevalonate metabolites in statin-mediated down-regulation of MCP-1, HAECs were incubated with statin in the presence of either mevalonate, FPP or GGPP followed by treatment with IL-6/sIL-6R. Co-incubation of mevalonate and GGPP, but not FPP, reversed the inhibitory effects of statin on gene expression of *MCP-1* in HAECs (Figure 4A). Similarly, co-incubation of mevalonate and GGPP, but not FPP, reversed the inhibitory effects of statin on MCP-1 protein secretion from HAECs (data not shown).

Geranylgeranyl transferase inhibitor, but not farnesyl transferase inhibitor, attenuates IL-6/sIL-6R-induced MCP-1 activation

HAECs were pretreated with either a specific farnesyl transferase inhibitor FTI-276 or a specific geranylgeranyl

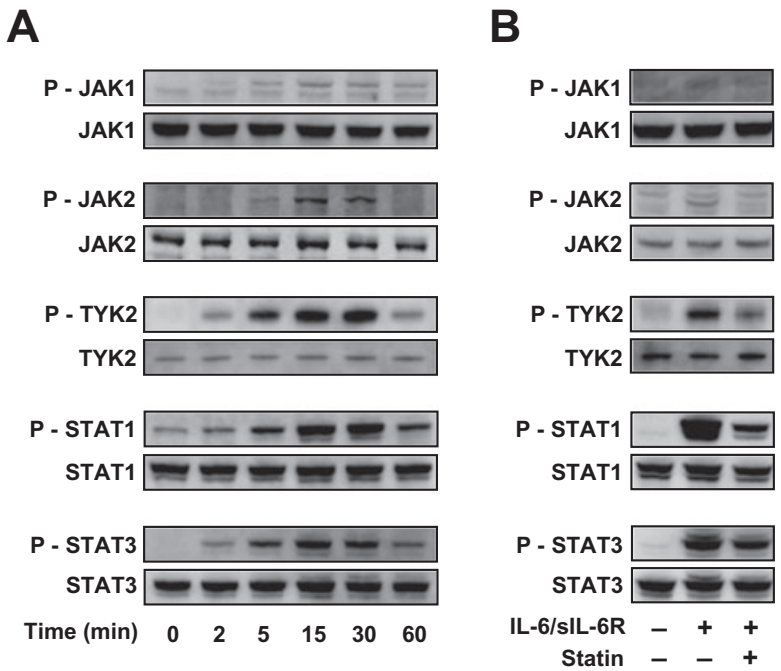

Figure 2 IL-6/sIL-6R induces phosphorylations of JAK1, JAK2, TYK2, STAT1 and STAT3 in HAECs, and statins inhibit these phosphorylations. Cell lysates were analysed by Western immunoblotting with phosphospecific JAK1, JAK2, TYK2, STAT1 and STAT3 antibodies. The blots were stripped and reprobed with the antibodies which detected the total amount of the respective proteins (lower panels of each blot). Representative blots of three independent experiments. (A) HAECs were treated with IL-6/sIL-6R (10^{-9} mol·L^{-1}) for the indicated time periods. (B) HAECs were pretreated with 100 μmol·L^{-1} fluvastatin for 24 h, and then incubated with IL-6/sIL-6R for 15 min.

transferase inhibitor GGTI-286, followed by stimulation with IL-6/sIL-6R. GGTI-286 at a concentration of 10 μmol·L^{-1} inhibited *MCP-1* gene induction by IL-6/sIL-6R without affecting GAPDH mRNA levels (Figure 4B). However, farnesyl transferase inhibitor FTI-276 had no effects on *MCP-1* gene induction. Similarly, IL-6/sIL-6R-enhanced MCP-1 protein secretion from HAECs was inhibited by GGTI-286, but not by FTI-276 (data not shown).

Effects of JAK/STAT inhibitors on IL-6/sIL-6R-induced MCP-1 *gene expression*

To examine whether JAK/STAT pathway is indeed involved in IL-6/sIL-6R-induced *MCP-1* gene expression, HAECs were pretreated with JAK inhibitors such as AG490 or JAK3 inhibitor II, followed by IL-6/sIL-6R treatment. AG490 and JAK3 inhibitor II dose-dependently attenuated gene expression of *MCP-1* stimulated by IL-6/sIL-6R (Figure 5A). AG490 and JAK3 inhibitor II also inhibited phosphorylations of JAK1, JAK2, TYK2, STAT1 and STAT3 stimulated by IL-6/sIL-6R (Figure 5B). To elucidate which STAT protein (STAT1 or STAT3) is more important in the induction of MCP-1 expression, HAECs were pretreated with a specific STAT1/3 inhibitor piceatannol or a specific STAT1 inhibitor fludarabine, followed by IL-6/sIL-6R treatment. Piceatannol dose-dependently inhibited gene expression of *MCP-1* (Figure 6A), and phosphorylation of both STAT1 and STAT3 stimulated by IL-6/sIL-6R (Figure 6B). In contrast, although fludarabine attenuated phosphorylation of STAT1 stimulated by IL-6/sIL-6R (Figure 6B), it had no effects on phosphorylation of STAT3 (Figure 6B) or *MCP-1* gene induction (Figure 6A).

Effects of JAK/STAT inhibitors on IL-6/sIL-6R-induced MCP-1 protein secretion and enhancement of THP-1 monocyte chemotaxis

To evaluate whether inhibition of JAK/STAT pathway affected IL-6/sIL-6R-induced MCP-1 protein secretion and THP-1 monocyte migration, HAECs were pretreated with AG490, JAK3 inhibitor II, piceatannol or fludarabine, followed by stimulation with IL-6/sIL-6R. AG490, JAK3 inhibitor II and piceatannol, but not fludarabine, inhibited MCP-1 protein secretion stimulated by IL-6/sIL-6R (Figure 7A). Similarly, although fludarabine had no effects on IL-6/sIL-6R-induced THP-1 monocyte migration, AG490, JAK3 inhibitor II and piceatannol suppressed THP-1 monocyte chemotaxis enhanced by IL-6/sIL-6R (Figure 7B).

Transfection of STAT3 siRNA, but not STAT1 siRNA, attenuates THP-1 monocyte chemotaxis enhanced by IL-6/sIL-6R

Involvement of STAT protein (STAT1 or STAT3) in the migration of THP-1 cells was further confirmed by the transfection studies. Antibiotic-free HAECs were transfected with either STAT1 siRNA or STAT3 siRNA, and then stimulated with IL-6/sIL-6R. Efficacy of transfection of siRNA was shown in Figure 8A. As shown in Figure 8B, transfection of STAT3 siRNA blunted THP-1 chemotaxis induced by IL-6/sIL-6R. However, although transfection of STAT1 siRNA tended to decrease

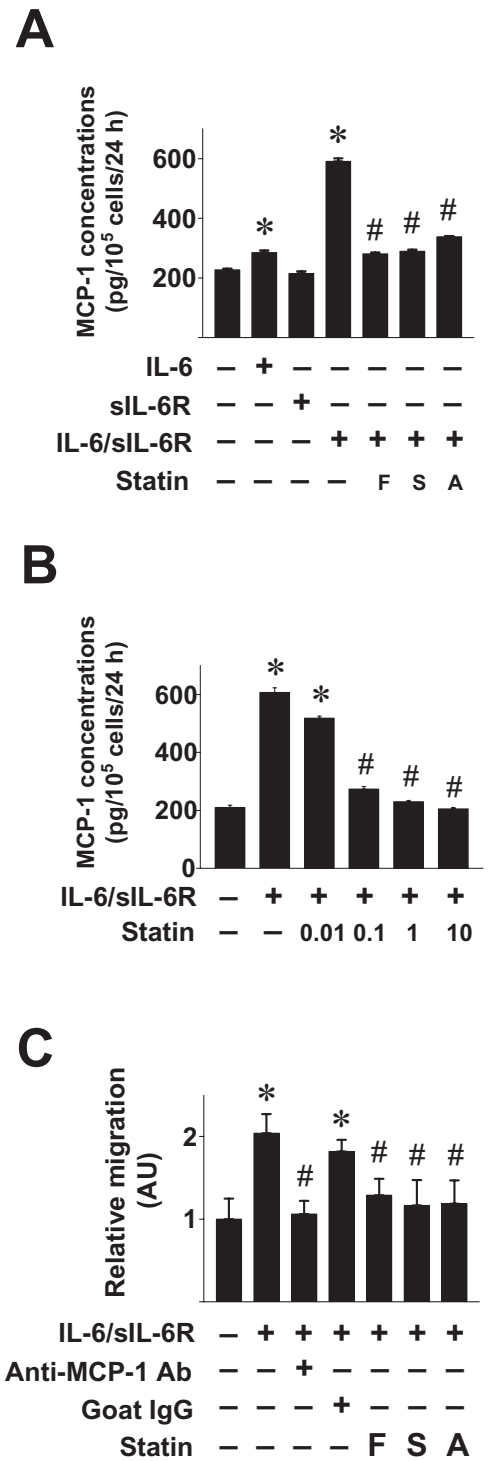

Figure 3 Effects of statins on MCP-1 secretion and chemotactic actions. (A) MCP-1 concentrations in the supernatant from HAECs as measured by sandwich ELISA. IL-6/sIL-6R stimulated secretion of MCP-1 from HAECs as compared with IL-6 alone (10^{-9} mol·L^{-1}) or sIL-6R alone (10^{-9} mol·L^{-1}). Various statins such as fluvastatin (F, 10 μmol·L^{-1}), simvastatin (S, 10 μmol·L^{-1}) and atorvastatin (A, 10 μmol·L^{-1}) inhibited MCP-1 protein secretion from HAECs. Values are means ± SEM ($n = 6$). *$P < 0.05$ versus untreated. #$P < 0.05$ versus IL-6/sIL-6R. (B) Fluvastatin dose-dependently suppressed MCP-1 protein secretion at the dosage greater than 0.1 μmol·L^{-1}. Values are means ± SEM ($n = 6$). *$P < 0.05$ versus untreated. #$P < 0.05$ versus IL-6/sIL-6R. (C) THP-1 monocyte migration as determined by chemotaxis assay. Relative migration indicates the ratio of migrating THP-1 cells towards culture medium from HAECs treated with various reagents relative to those from untreated cells. THP-1 monocyte chemotaxis was promoted in response to culture medium treated with 10^{-9} mol·L^{-1} IL-6/sIL-6R. Pre-incubation of the culture medium from IL-6/sIL-6R-treated HAECs with neutralizing goat anti-MCP-1 polyclonal antibody (80 μg·mL^{-1}), but not with goat IgG, resulted in an inhibition of chemotaxis. Various statins such as fluvastatin (F, 10 μmol·L^{-1}), simvastatin (S, 10 μmol·L^{-1}) and atorvastatin (A, 10 μmol·L^{-1}) inhibited IL-6/sIL-6R-induced THP-1 monocyte chemotaxis. Bars represent mean ± SEM of three independent experiments. *$P < 0.05$ versus untreated. #$P < 0.05$ versus IL-6/sIL-6R.

THP-1 monocyte migration, this effect was not statistically significant (Figure 8B).

Immunocytochemical studies

To determine if statin affected the translocation of STAT3 to the nucleus by inhibiting IL-6/sIL-6R-stimulated STAT3 phosphorylation, HAECs were pre-incubated with statin followed by treatment with IL-6/sIL-6R for 30 min. Immunocytochemistry revealed that IL-6/sIL-6R stimulated the phosphorylation of STAT3 and its translocation to the nucleus, which was inhibited by statin (Figure 9). Addition of mevalonate reversed the phosphorylation of STAT3 and its translocation to the nucleus (Figure 9). IL-6/sIL-6R-stimulated phosphorylation of STAT3 and its translocation to the nucleus was also blocked by AG490, JAK3 inhibitor II and piceatannol (data not shown).

Discussion

Atherosclerosis is now considered a chronic inflammatory disease that leads to acute cardiovascular events (Ross, 1999; Libby, 2002). Statins, HMG-CoA reductase inhibitors, decrease cardiovascular events, not only through lowering cholesterol levels, but also through their pleiotropic properties, including anti-inflammatory actions (Takemoto and Liao, 2001). The present study has demonstrated that statins suppress IL-6-induced *MCP-1* gene expression and protein secretion in HAECs, as well as monocyte migration by inhibiting JAK/STAT signalling pathway, supporting the proposition that statins have anti-inflammatory properties beyond cholesterol-lowering effects.

Monocytes/macrophages and endothelial cells are the major key players implicated in atherogenesis. Monocyte adhesion to the vascular endothelial cells and subsequent migration into the subendothelial space are the critical events in the inflammatory processes, as well as in the initiation and progression of atherosclerosis (Ross, 1999; Libby, 2002). MCP-1 plays a crucial role in the monocyte recruitment into the vessel wall, not only in the inflammatory diseases, but also in the atherosclerotic diseases (Charo and Taubman, 2004). Previous investigators reported that various statins suppress MCP-1 expression in both inflammation and atherosclerosis (Martinez-Gonzalez *et al.*, 2001; Kleemann *et al.*, 2003; Veillard *et al.*, 2006). In the current study, we have shown that statins, such as fluvastatin, simvastatin and atorvastatin, not only inhibit IL-6/sIL-6R-mediated gene induction and protein

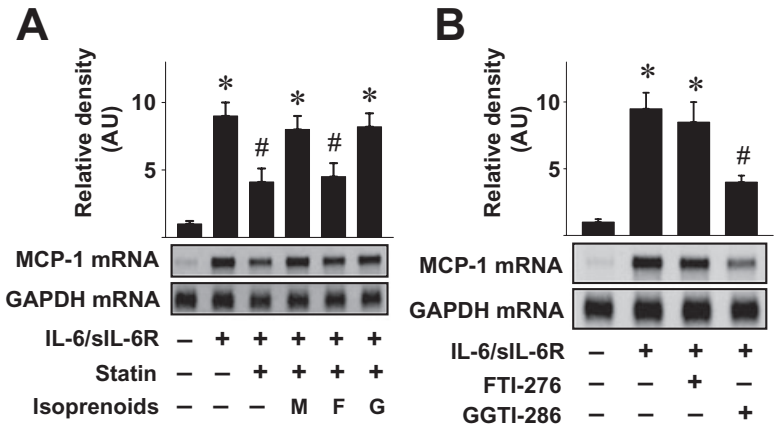

Figure 4 Effects of mevalonate metabolites, farnesyl transferase inhibitor (FTI-276) and geranylgeranyl transferase inhibitor (GGTI-286) on gene expression of *MCP-1* in HAECs. GAPDH mRNA served as a loading control. Bars represent mean ± SEM of three independent experiments. (A) Co-incubation with mevalonate (M, 100 μmol·L^{-1}) and GGPP (G, 10 μmol·L^{-1}), but not FPP (F, 10 μmol·L^{-1}), reversed the inhibitory effects of 10 μmol·L^{-1} fluvastatin on gene expression of *MCP-1* stimulated by 10^{-9} mol·L^{-1} IL-6/sIL-6R. *$P < 0.05$ versus untreated. #$P < 0.05$ versus IL-6/sIL-6R. (B) Pretreatment with GGTI-286 (10 μmol·L^{-1}), but not FTI-276 (10 μmol·L^{-1}), inhibited *MCP-1* gene expression induced by IL-6/sIL-6R. *$P < 0.05$ versus untreated. #$P < 0.05$ versus IL-6/sIL-6R.

secretion of MCP-1 in human vascular endothelial cells, but also inhibit monocyte chemotaxis, providing a new insight into the role of statins in IL-6-mediated inflammation and atherosclerosis. By inhibiting gene expression and protein secretion of MCP-1, statins exert anti-inflammatory and anti-atherosclerotic properties in IL-6-mediated processes.

The therapeutic plasma concentrations of statins in humans are considered to be lower than the statin concentrations used in the present study. In pharmacokinetic studies, Tse *et al*. (1992) reported that the maximal plasma concentrations of fluvastatin in human subjects receiving 40 mg of fluvastatin daily were almost 1 μmol·L^{-1}. In the present study, HAECs were incubated with 10–100 μmol ·L^{-1} statins to demonstrate the inhibition of both *MCP-1* gene expression and phosphorylation of JAK/STAT pathways. As for MCP-1 protein expression, fluvastatin suppressed MCP-1 secretion at concentrations greater than 0.1 μmol·L^{-1}. Therefore, the plasma concentrations of statin seen in the clinical studies could be effective in the suppression of MCP-1, at least in terms of MCP-1 protein secretion. The statin concentrations in the present study have also been used in earlier *in vitro* cell culture experiments (Bellosta *et al*., 1998; Guijarro *et al*., 1998; Kaneider *et al*., 2002). In addition, the effective concentrations of statin might be different among the type of cells. Thunyakitpisal and Chaisuparat (2004) examined the effects of simvastatin on matrix metalloproteinase-9 expression in the osteoblasts, osteosarcoma cells and fibrosarcoma cells, and found that the statin concentrations needed to suppress matrix metalloproteinase-9 expression depended on the type of cells. Lastly, the duration of HAECs exposed to statins should be taken into account in the *in vitro* cell culture experiments. As the time of exposure of cells to statins is very short, usually only for hours, any significant inhibition of *MCP-1* gene and protein expression induced by IL-6 might require higher concentrations of statins in the *in vitro* experiments. The clinical relevance of the statin concentrations in the *in vitro* study needs further investigations.

Metabolism of mevalonate yields isoprenoid compounds, such as FPP and GGPP. These isoprenoids are necessary for the post-translational isoprenylation of various proteins that are involved in cell signalling pathways (Goldstein and Brown, 1990; Casey, 1995). We are interested in the role of mevalonate metabolites in IL-6/sIL-6R-mediated *MCP-1* gene expression and protein secretion in HAECs. With the usage of various mevalonate metabolites, the current study convincingly showed that the inhibitory actions of statins on IL-6/sIL-6R-mediated MCP-1 expression were reversed by the presence of mevalonate and GGPP, but not by FPP. Additionally, geranylgeranyl transferase inhibitor GGTI-286, but not farnesyl transferase inhibitor FTI-276, had inhibitory effects on IL-6/sIL-6R-induced MCP-1 expression. Moreover, statin-mediated inhibitory actions on STAT3 phosphorylation and translocation to the nucleus were reversed completely by mevalonate. These findings suggest that the protein geranylgeranylation plays a crucial role in statin-mediated suppression of IL-6/sIL-6R-induced MCP-1, and that the migration inhibitory effects of statins are probably dependent on the suppression of protein geranylgeranylation. This idea is supported by an earlier report that protein geranylgeranylation is important in regulating chemotactic migration in human THP-1 cells (Wong *et al*., 2001). As statins inhibit Rho and its downstream target Rho kinase, by blocking the synthesis of GGPP (Rolfe *et al*., 2005), further studies investigating the role of Rho proteins in statin-diminished MCP-1 expression are needed.

IL-6 is a multifunctional cytokine that acts on a variety of target cells to regulate their growth and differentiation (Heinrich *et al*., 1998). There are two receptor components for IL-6 signal transduction. IL-6 is bound to IL-6R, and binding of IL-6 to IL-6R then induces gp130 dimerization (Murakami *et al*., 1993). Cells expressing only gp130 subunits, but lacking surface IL-6R subunits, do not respond to IL-6; however, these cells can respond to IL-6 in the presence of sIL-6R, a soluble form of IL-6R (Romano *et al*., 1997). Indeed, addition of sIL-6R is reported to trigger the biological actions of IL-6 (Tamura *et al*., 1993). Other investigators reported that exogenous IL-6 administration failed to activate STAT3 in the malignant cervical carcinoma cells, in

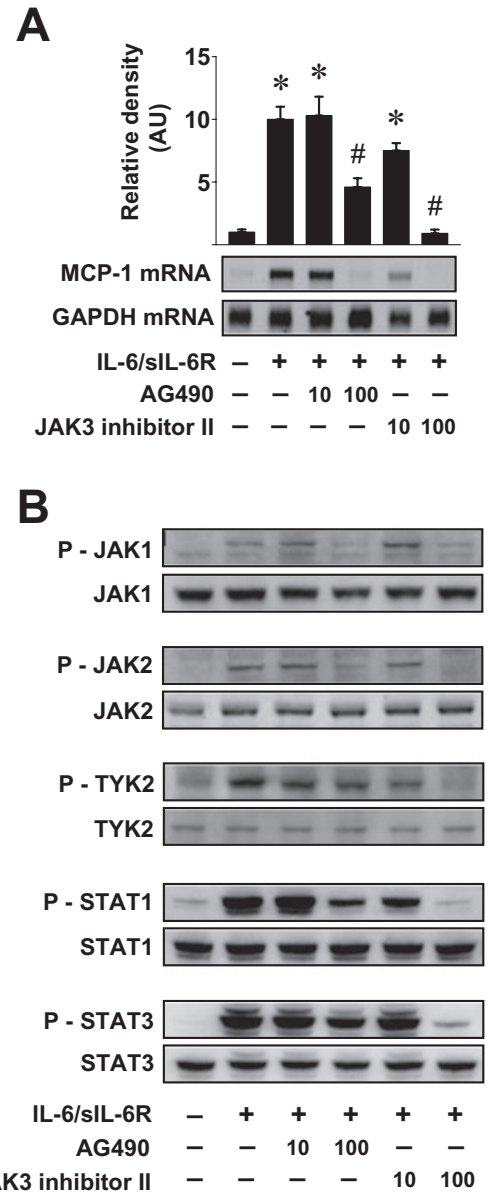

Figure 5 Effects of JAK inhibitors on IL-6/sIL-6R-induced gene expression of *MCP-1* and phosphorylation of JAK/STAT pathway. (A) Pretreatment with either AG490 (100 μmol·L^{-1}) or JAK3 inhibitor II (100 μmol·L^{-1}) inhibited IL-6/sIL-6R-stimulated MCP-1 mRNA. GAPDH mRNA served as a loading control. Bars represent mean ± SEM of three independent experiments. *P < 0.05 versus untreated. #P < 0.05 versus IL-6/sIL-6R. (B) Pretreatment with either AG490 (100 μmol·L^{-1}) or JAK3 inhibitor II (100 μmol·L^{-1}) inhibited phosphorylation of JAK1, JAK2, TYK2, STAT1 and STAT3 induced by IL-6/sIL-6R.

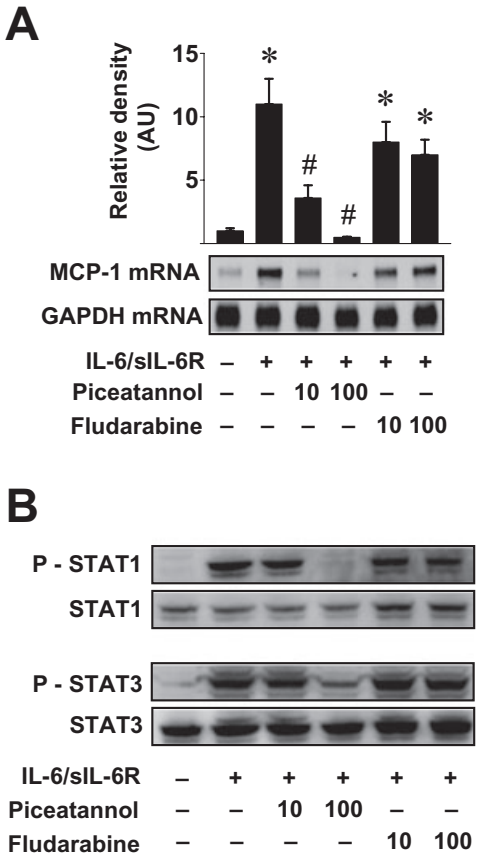

Figure 6 Effects of STAT inhibitors on gene expression of *MCP-1* and phosphorylation of STAT1 and STAT3. (A) Piceatannol (10 and 100 μmol·L^{-1}) dose-dependently inhibited IL-6/sIL-6R-induced *MCP-1* gene expression in HAECs. In contrast, fludarabine (10 and 100 μmol·L^{-1}) did not significantly reduce IL-6/sIL-6R-induced *MCP-1* gene expression in HAECs. GAPDH mRNA served as a loading control. Bars represent mean ± SEM of three independent experiments. *P < 0.05 versus untreated. #P < 0.05 versus IL-6/sIL-6R. (B) Piceatannol (10 and 100 μmol·L^{-1}) dose-dependently inhibited phosphorylation of both STAT1 and STAT3. Fludarabine attenuated phosphorylation of STAT1 stimulated by IL-6/sIL-6R, but it had no effects on phosphorylation of STAT3. Representative blots of three independent experiments.

which surface expression of IL-6R was undetectable, and that addition of sIL-6R restored IL-6 responsiveness and enhanced activation of STAT3, followed by increased production of MCP-1 (Hess *et al.*, 2000). In the current study, the addition of sIL-6R enhanced the biological actions of IL-6 to promote THP-1 monocyte chemotaxis, and to induce gene expression and protein secretion of MCP-1 in HAECs. The human vascular endothelial cells are thus considered to be cells that need additional sIL-6R to maximize the biological actions of IL-6.

In summary, we demonstrated that, compared with IL-6 alone or sIL-6R alone, IL-6 together with sIL-6R markedly activated the JAK/STAT signalling pathway in human vascular endothelial cells, leading to induction of gene expression and protein secretion of MCP-1, and caused enhanced monocyte migration. By inhibiting JAK/STAT phosphorylation and by preventing STAT protein, especially STAT3, from translocating to the nucleus, statins suppressed downstream *MCP-1* gene up-regulation, as well as enhanced monocyte migration by IL-6/sIL-6R in HAECs. Although further studies are required, the current study provides a new insight regarding the mechanism by which statins could suppress the recruitment of monocytes into the subendothelial regions of the vessel wall in IL-6-associated inflammation and atherosclerosis.

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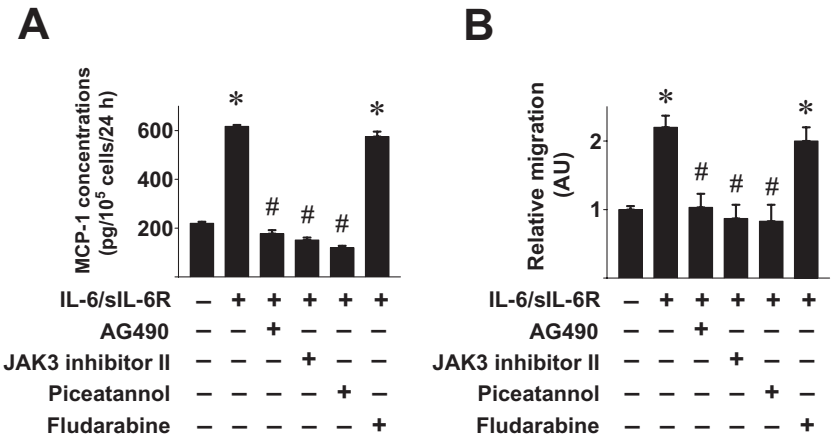

Figure 7 Effects of STAT inhibitors on MCP-1 secretion and chemotactic actions. (A) MCP-1 concentrations in the supernatant as measured by sandwich ELISA. Pretreatment with either AG490 (100 μmol·L^{-1}), JAK3 inhibitor II (100 μmol·L^{-1}) and piceatannol (100 μmol·L^{-1}), but not fludarabine (100 μmol·L^{-1}), inhibited MCP-1 secretion stimulated by IL-6/sIL-6R. Values are means ± SEM (n = 6). *P < 0.05 versus untreated. #P < 0.05 versus IL-6/sIL-6R. (B) THP-1 monocyte migration as determined by chemotaxis assay. IL-6/sIL-6R-induced THP-1 monocyte chemotaxis was inhibited by pretreatment with AG490 (100 μmol·L^{-1}), JAK3 inhibitor II (100 μmol·L^{-1}) and piceatannol (100 μmol·L^{-1}). Fludarabine (100 μmol·L^{-1}) did not inhibit THP-1 monocyte migration enhanced by IL-6/sIL-6R. Bars represent mean ± SEM of three independent experiments. *P < 0.05 versus untreated. #P < 0.05 versus IL-6/sIL-6R.

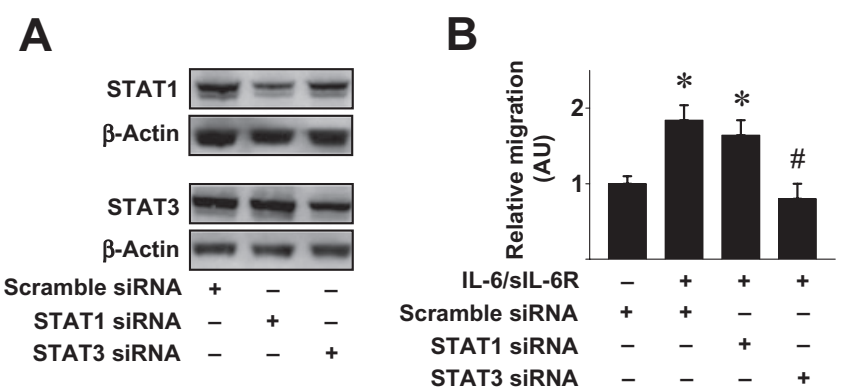

Figure 8 Effect of siRNAs for STAT1 or STAT3, on THP-1 cell migration. (A) Efficacy of transfection of STAT1 siRNA and STAT3 siRNA was confirmed by Western immunoblot analysis. β-Actin served as a loading control. (B) Transfection of STAT3 siRNA, but not STAT1 siRNA, inhibited THP-1 monocyte migration enhanced by IL-6/sIL-6R *P < 0.05 versus untreated. #P < 0.05 versus IL-6/sIL-6R.

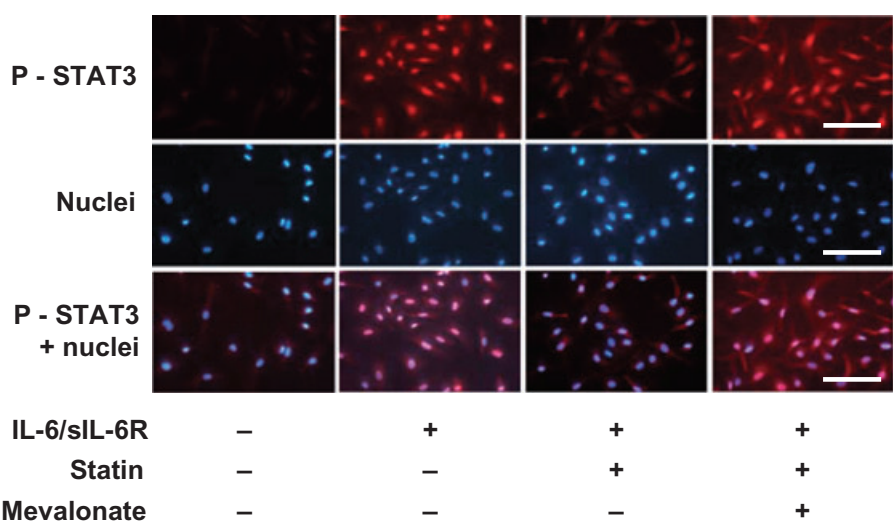

Figure 9 Immunocytochemical staining for phospho-STAT3 in HAECs. HAECs were pre-incubated with statin (fluvastatin, 100 μmol·L^{-1}) or statin plus mevalonate (100 μmol·L^{-1} each) for 24 h, followed by additional incubation with IL-6/sIL-6R (10^{-9} mol·L^{-1}) for 30 min. Red staining indicates the specific Alexa 594 stain for phospho-STAT3, and blue staining indicates the position of the nuclei (Hoechst 33342). Original magnification, ×400. Bar = 50 μm.

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Conflict of interest

The authors state no conflict of interest.

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