Statins inhibit Ox-LDL-induced macrophage growth

Statins suppress oxidized low-density lipoprotein-induced macrophage proliferation by inactivation of small G protein-p38 MAPK pathway

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Summary

Inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (statins) ameliorate atherosclerotic diseases. Macrophages play an important role in the development and subsequent stability of atherosclerotic plaques. We previously reported that oxidized low-density lipoprotein (Ox-LDL) induced macrophage proliferation through the secretion of granulocyte/macrophage colony-stimulating factor (GM-CSF) and consequent activation of p38 MAPK. The present study was designed to elucidate the mechanism of the inhibitory effect of statins on macrophage proliferation. Mouse peritoneal macrophages were used in our study. Cerivastatin and simvastatin each inhibited Ox-LDL-induced $[^3]$H]thymidine incorporation into macrophages. Statins did not inhibit Ox-LDL-induced GM-CSF production, detected by ELISA and real-time RT-PCR, but inhibited GM-CSF-induced p38 MAPK activation, detected by Western blot. Farnesyl transferase inhibitor (FTI) and geranylgeranyl transferase inhibitor (GGTI) inhibited GM-CSF-induced macrophage proliferation, and farnesylpyrophosphate (FPP) and geranylgeranylpolyphosphate (GGPP) prevented the effect of statins. GM-CSF-induced p38 MAPK phosphorylation was also inhibited by FTI or GGTI, and FPP and GGPP prevented the suppression of GM-CSF-induced p38 MAPK phosphorylation by statins. Furthermore, we found that statin significantly inhibited the membrane translocation of small G protein family, Ras and Rho. GM-CSF-induced p38 MAPK activation and macrophage proliferation was partially inhibited by overexpression of dominant negative Ras and completely by that of RhoA. In conclusion, statins inhibited GM-CSF-induced Ras- or RhoA-p38 MAPK signal cascades, thereby suppressed Ox-LDL-induced macrophage proliferation. The significant inhibition of macrophage proliferation by statins may also explain, at least in part, their anti-atherogenic action.

(Word count = 232)
**Introduction**

Immunohistochemical and pathological studies have established that foam cells observed in the early phase of atherosclerotic lesions are derived from blood monocytes/macrophages (1-3). Macrophages take up oxidized low-density lipoprotein (Ox-LDL) through the scavenger receptor pathways and transform into foam cells *in vitro* (1). These cells in turn produce various bioactive molecules, such as growth factors and cytokines, which play an important role in the progression of early-stage atherosclerotic process (4). Previous *in vivo* studies reported that macrophages and macrophage-derived foam cells proliferated in atherosclerotic lesions (1-3). On the other hand, macrophages also play an important role in the development and subsequent stability of atherosclerotic plaques (5). In this regard, Aikawa et al. (6) demonstrated the proliferation and activation of macrophages in the atherosclerotic plaque of Watanabe heritable hyperlipidemic rabbits. Therefore, the proliferation of macrophages may be involved in both the development of early-phase of atherosclerotic lesions and the stability of atherosclerotic plaques.

We (7, 8) and other groups (9, 10) have shown that Ox-LDL enhances macrophage proliferation and survival *in vitro*. In addition, our previous study revealed that Ox-LDL could induce a rise in intracellular calcium concentration and subsequent activation of protein kinase C (PKC) in mouse peritoneal macrophages (8). PKC activation mediates production of granulocyte/macrophage colony stimulating factor (GM-CSF), which plays a priming role in the Ox-LDL-induced macrophage proliferation (11). Furthermore, p38 mitogen activated protein kinase (p38 MAPK)-dependent phosphatidylinositol-3 kinase (PI-3K) activation is involved, at least in part, downstream in the signaling pathway(s) after GM-CSF production (12). However, the mechanism(s) of p38 MAPK activation through GM-CSF receptor is not clearly understood.
Inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, commonly known as statins, are generally used for the treatment of hyperlipidemia. Their use is associated with significant reduction of adverse coronary events, including myocardial infarction and a marginal regression of plaque size (13, 14). Furthermore, recent studies, both in vitro and in vivo, have suggested that the beneficial effects of statins may extend to mechanisms beyond cholesterol reduction (14-17). The pleiotropic effects of statins include improvement or restoration of endothelial function, inhibition of proliferation and migration of smooth muscle cells, reduction of vascular inflammation, and importantly, stabilization of atherosclerotic plaques (15, 16). These pleiotropic effects of statins are based on blocking the synthesis of isoprenoid intermediates, which serve as lipid attachments for a variety of intracellular signaling molecules, especially Rho-family small GTP binding proteins, whose proper membrane localization and function are dependent on isoprenylation (15, 16, 18).

Although our previous study revealed that statins suppressed Ox-LDL-induced macrophage proliferation (19), the mechanism of the inhibitory action is not still fully understood. The purpose of the present study was to determine the mechanisms of the inhibitory effects of statins, cerivastatin and simvastatin, on Ox-LDL-induced macrophage proliferation, as well as identify the molecular mechanisms of p38 MAPK activation during macrophage proliferation in the presence of Ox-LDL. Our results demonstrated that RhoA, a member of the small G protein super family, is involved in Ox-LDL-induced macrophage proliferation in the signaling pathway downstream of the GM-CSF receptor. While statins did not inhibit Ox-LDL-induced GM-CSF production, they inhibited RhoA-dependent p38 MAPK activation, thereby inhibiting macrophage proliferation.
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Experimental Procedures

Materials

Simvastatin in lactone form was a generous gift from BANYU Pharmaceutical (Tokyo, Japan). Simvastatin in lactone form (94.2 mg) was dissolved in 1.8 ml of ethanol. The solution was added to 19 ml of 0.1 M NaOH and then incubated for 40 min at 40°C to yield the active form, followed by the addition of 0.1 M HCl to adjust the pH to 7.4. Cerivastatin was a generous gift from Bayer Pharmaceutical (Osaka, Japan). Farnesylpyrophosphate (FPP), geranylgeranylpyrophosphate (GGPP), and L-mevalonate were purchased from Sigma Chemical Co. (St. Louis, MO). FTI-276, GGTT-286 and Y-27632 were obtained from Calbiochem (San Diego, CA). Recombinant mouse GM-CSF was purchased from R&D systems, Inc. (Minneapolis, MN). [3H]Thymidine (80 Ci/mmol) was from Amersham Biosciences Corp. (Piscataway, NJ). Rabbit polyclonal anti-phospho ERK1/2, anti-ERK1/2, anti-phospho p38 MAPK, and anti-p38 MAPK antibody were from Cell Signaling Technology, Inc. (Beverly, MA). Rabbit polyclonal anti-pan Ras and RhoA antibody was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Other chemicals were of the best grade available from commercial sources.

Lipoprotein Preparation

Human LDL (d=1.019 to 1.063 g/ml) was isolated by sequential ultracentrifugation from the plasma of consented normolipidemic subjects obtained after overnight fasting (20). LDL was dialyzed against 0.15 M NaCl and 1 mM ethylenediaminetetraacetic acid (EDTA), pH 7.4. Ox-LDL was prepared by incubation of LDL with 5 μM CuSO4 for 20 h at 37°C followed by the addition of 1 mM EDTA and cooling (21). The concentration of proteins was determined by BCA protein assay reagent (Pierce Chemical Co., Rockford, IL). Endotoxin level of
Ox-LDL was < 1 pg/mg protein measured by a commercially available kit (Toxicolor System, Seikagaku Corp., Japan). We confirmed that proliferation and viability of mouse resident macrophages was not affected by endotoxin at a concentration < 1 ng/ml under our experimental conditions.

Cell Culture
The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Kumamoto University. Peritoneal macrophages were collected from anesthetized male C3H/He mice (25-30 g) by peritoneal lavage with 8 ml of ice-cold PBS, centrifuged at 200 × g for 5 min and suspended in RPMI 1640 medium (Nissui Seiyaku Co., Tokyo) supplemented with 10% heat-inactivated fetal calf serum (Life Technologies, Inc., Rockville, MD), streptomycin (0.1 mg/ml), and penicillin (100 units/ml) (medium A) (22). Cell suspensions were incubated in appropriate tissue culture plates for 90 min with medium A. More than 98% of adherent cells were considered to be macrophages based on four criteria, as described previously (23, 24).

Adenoviral Vectors
Mouse peritoneal macrophages were infected with a recombinant replication-deficient adenovirus containing each of the genes of dominant negative Ras (Asn^{17}-H-Ras), dominant negative RhoA (N^{19}-RhoA) and dominant negative p38 MAPK at a multiplicity of infection (MOI) of approximately 50, as described previously(25-27), and allowed to recover in medium A for 3 h. This condition conferred expression of LacZ as a marker gene in nearly 100% of transfected cells (25).
Tritiated Thymidine Incorporation and Cell-counting Assays

Macrophage monolayers (2 × 10^6 cells/well) were cultured in 24-well tissue culture plates (15.5 mm in diameter, Corning Glass works, Corning, NY) in the presence of the indicated effectors for 6 days. For thymidine incorporation assay, 18 h before the termination of the experiments, 1 μCi/ml [3H]thymidine was added to each well and incubated. Tritiated thymidine incorporation assay was performed as described previously (11). For cell-counting assay, macrophage monolayers (5 × 10^3 cells/well) were incubated in 96-well tissue culture plates with the indicated effectors. After incubation for 5 days, cells were lysed in 1% (w/v) Triton X-100, and naphthol blue-black-stained nuclei were counted in a hemocytometer, as described previously (11).

Enzyme-Linked Immunosorbent Assay (ELISA) for GM-CSF

Macrophage monolayers (2 × 10^6 cells/well in 6-well plate, Nunc) were cultured in 2 ml of medium A with or without the indicated effectors. After incubation for 4 h, 300 μL of the medium were collected and immediately centrifuged at 10,000 × g for 1 min to remove any particulate material. The concentration of GM-CSF protein was determined as described previously (11).

Quantitative PCR Analysis for GM-CSF mRNA

Macrophages (2 × 10^6 cells/well) were incubated with or without the indicated effectors. Total RNA was extracted with TRIzol (Life Technologies, Inc.). The first strand cDNA synthesis containing 1 μg of total RNA was primed with oligo dT. To quantify GM-CSF transcripts, the LightCycler System (Roche Molecular Biochemicals, Indianapolis, IN) was used (28). PCRs were performed using SYBR Green I master mix and specific primers for mouse GM-CSF and mouse β-actin, which were designed as described previously (11, 29).
Quantitative results of GM-CSF mRNA were normalized for the levels of β-actin mRNA. To assess the specificity of the amplified PCR products, after the last cycle, a melting curve analysis was performed.

**Western Blot Analysis**

Macrophages (2 × 10^6 cells/well in 6-well plate, Nunc) were lysed by the lysis buffer, and centrifuged (20,000 × g at 4°C for 10 min). Supernatants were used as sample proteins. Protein concentrations were determined by the Micro BCA Protein Assay Reagent (Pierce), according to the protocol recommended by the manufacturer. Samples were applied to 10% sodium dodecyl sulfate (SDS) gels and transferred to nitrocellulose membranes (Bio-Rad, Richmond, CA) by using semi-dry blotting. Membranes were incubated with the indicated antibodies at a dilution of 1:1000 for 2 h. After washing, the membranes were stained with horseradish peroxidase (HRP)-conjugated goat anti-rabbit or anti-mouse antibodies (Santa Cruz, Inc.). Antigen detection was performed with ECL plus kit (Amersham Biosciences Corp.). Immunoreactive bands were quantified by the NIH Image analysis software.

**Membrane Fraction Extraction**

Macrophages (2 × 10^6 cells/well in 6-well plate, Nunc) were suspended in 1 ml of sample preparation buffer (5 mM EDTA, 10 mM ethylene glycol bis (beta-aminoethyl ether)-N,N,N',N'-tetraacetic acid [EGTA], 50 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonylfluoride [PMSF], 10 mM benzamidine, and 50 mM Tris-HCl, pH 7.5) and sonicated for 30 sec at 4°C by Sonifier (Branson Sonic Power Co., Danbury CT). Homogenates were centrifuged at 20,000 × g for 1 h at 4°C. The supernatant was discarded and the precipitates were resuspended in 1 ml of buffer and used as the membrane fractions.
Statistical Analysis

All data were expressed as mean ± SD. Differences between groups were examined for statistical significance using the one-factor ANOVA procedure. A $p$ value less than 0.05 denoted the presence of a statistically significant difference.
Results

Effect of statins on Ox-LDL-induced proliferation and GM-CSF production by macrophages

Consistent with our previous report with simvastatin (19), cerivastatin as well as simvastatin reduced Ox-LDL-induced $[^3]$Hthymidine incorporation in a dose-dependent manner (Fig. 1A). Cerivastatin was more potent than simvastatin in inhibiting Ox-LDL-induced macrophage proliferation; the concentration of cerivastatin required for 50% inhibition was 5 nM with a maximum effect observed at 100 nM, whereas 100 nM and 1 μM were required, respectively, for simvastatin (Fig. 1A). Under these conditions, cerivastatin or simvastatin alone had no effect on $[^3]$Hthymidine incorporation up to a concentration of 10 μM. The cell counting assay also showed that the Ox-LDL-induced increase in cell number was inhibited by 88% or 84% when cells were incubated with 100 nM of cerivastatin or 10 μM of simvastatin, respectively (Table 1). In the presence of 100 nM cerivastatin or 10 μM simvastatin, more than 95% of the macrophages were viable as determined by both the cell counting assay using the trypan blue staining and the release of lactic dehydrogenase (LDH) from these cells (data not shown), indicating that statins at a dose less than 10 μM are not cytotoxic to macrophages.

Since we focused on the role of GM-CSF in the Ox-LDL-induced macrophage proliferation (11), we next examined the effect of cerivastatin on Ox-LDL-induced GM-CSF production. Our results showed that GM-CSF production was not affected by pre-treatment with statins both at mRNA (Fig. 1B) and protein levels (Fig. 1C). Cerivastatin or simvastatin alone had no effect on the expression of GM-CSF mRNA and protein in these conditions (data not shown).
Effect of statins on GM-CSF-induced macrophage proliferation

GM-CSF-induced $[^3H]$thymidine incorporation was also reduced by both simvastatin and cerivastatin in a dose-dependent manner. Cerivastatin showed stronger effect than simvastatin, and their IC$_{50}$ were 80 nM and 700 nM, respectively (Fig. 2A). On the other hand, cerivastatin and simvastatin alone had no effect on $[^3H]$thymidine incorporation at the concentration up to 10 µM. Concordant with the result of $[^3H]$thymidine incorporation assay, GM-CSF-induced increase in cell number was significantly inhibited by 80% and 86.4% when macrophages were incubated with either cerivastatin or simvastatin, respectively (Table 1). Furthermore, these statins had no effect when used alone (Table 1).

Roles of isoprenoids in GM-CSF-induced macrophage proliferation and inhibition by statins

To clarify the roles of metabolites of mevalonate pathway on GM-CSF-induced macrophage proliferation, we examined the effect of mevalonate and its metabolites. As shown in figure 2B, cerivastatin- or simvastatin-mediated inhibition of GM-CSF-induced $[^3H]$thymidine incorporation was completely prevented by co-incubation with mevalonate, whereas squalene failed to reverse the effect of statins. Cerivastatin- or simvastatin-mediated inhibition of GM-CSF-induced $[^3H]$thymidine incorporation was completely prevented by co-incubation with geranylgeranyl pyrophosphate (GGPP) and partially by farnesyl pyrophosphate (FPP). On the other hand, farnesyl transferase inhibitor, FTI-276, inhibited GM-CSF-induced $[^3H]$thymidine incorporation by 46.5%. Geranylgeranyl transferase inhibitor, GGTI-286, also inhibited GM-CSF-induced $[^3H]$thymidine incorporation by 77.5%, which was similar to the inhibitory effect of statins.

Effect of statins on GM-CSF-induced MAPK activation in macrophages
We previously reported the involvement of p38 MAPK activation in GM-CSF-induced macrophage proliferation (12). We next examined the effect of statins on GM-CSF-induced ERK1/2 or p38 MAPK phosphorylation. Simvastatin, cerivastatin and isoprenyl transferase inhibitors (FTI and GGTI) had no effect on GM-CSF-induced ERK1/2 phosphorylation (Fig. 3A and C), but significantly decreased GM-CSF-induced p38 MAPK phosphorylation (Fig. 3B and D). To determine whether statin-induced inhibition of p38 MAPK phosphorylation was mediated by metabolites of mevalonate pathway, we examined the restorative effect of isoprenyl pyrophostates on the suppression of p38 MAPK phosphorylation by simvastatin. As shown in figure 3E, simvastatin-mediated reduction of p38 MAPK phosphorylation induced by GM-CSF was significantly rescued by co-incubation with FPP or GGPP.

**Effect of statins on membrane localization of Ras and RhoA**

To determine whether statins regulate the activity of small G proteins, which are upstream components of MAPK cascades, in macrophages, we next examined the membrane localization of Ras and RhoA. Stimulation with GM-CSF (15 pM) increased the level of membrane-bound Ras (Fig. 4A) and RhoA (Fig. 4B), and simvastatin significantly prevented GM-CSF-induced Ras and RhoA membrane localization without affecting the total amount of these proteins in macrophages (Fig. 4A and B). The inhibitory effect of simvastatin on Ras membrane localization was completely reversed by FPP and partially reversed by GGPP (Fig. 4A). In contrast, co-treatment with GGPP completely reversed the inhibitory effect of simvastatin on RhoA membrane localization, whereas only partially with FPP (Fig. 4A).

**Involvement of small G proteins in GM-CSF-induced macrophage proliferation through the activation of p38 MAPK**
We next performed a series of experiments to clarify whether the small G proteins mediate the activation of MAPKs in the signaling pathways of macrophage proliferation induced by GM-CSF. For this purpose, we introduced adenoviruses that expressed dominant negative forms of Ras and Rho. GM-CSF-induced ERK1/2 phosphorylation was increased in the cells infected with dominant negative form of Rho, but was significantly decreased in the cells infected with dominant negative form of Ras (Fig. 4C). On the other hand, GM-CSF-induced p38 MAPK phosphorylation was significantly decreased in the cells infected with dominant negative form of Ras or Rho, with much stronger effect of DN-Rho (Fig. 4D). Adenoviral control vector has no effect on ERK1/2 phosphorylation or p38 MAPK phosphorylation (Fig. 4C and D). In addition, pre-treatment with Rho kinase inhibitor, Y-27632, significantly inhibited GM-CSF-induced p38 MAPK phosphorylation at concentrations ≥10 μM (Fig. 4E).

On the other hand, infection with dominant negative Ras and Rho partially but significantly inhibited GM-CSF-induced [3H]thymidine incorporation into macrophages by 21.1% and 55.5%, respectively (Fig. 5). GM-CSF-induced macrophage proliferation was also partially but significantly inhibited by pre-treatment with 10 and 100 μM of Y-27632 by 59.2% and 62.8 %, respectively (Fig. 5). In addition, infection with the dominant negative form of p38 MAPK also inhibited GM-CSF-induced macrophage proliferation by 57.4% (Fig. 5).
Discussion

Macrophage proliferation in atherosclerotic lesions has been demonstrated by several groups (1-3), and the proliferation was also reported to be reduced by statin treatment (6). Regarding the effect of statins, we demonstrated in a previous study that Ox-LDL-induced macrophage proliferation was inhibited by simvastatin and pravastatin (19). In the present study, we further demonstrated the impacts of Ras and Rho on Ox-LDL-induced macrophage proliferation, and provided evidences that statins inhibit the proliferation by suppressing p38 MAPK through the inhibition of Ras and Rho in the signaling pathways downstream of GM-CSF receptor, without affecting GM-CSF production.

Based on our previous studies, Ox-LDL-induced GM-CSF production is mainly involved in macrophage proliferation (11). Thus, the mechanisms of Ox-LDL-induced macrophage proliferation can be divided into two parts; (i) intracellular signaling pathway before GM-CSF release, and (ii) proliferation signaling pathway through GM-CSF receptor. Moreover, we reported that ERK1/2 and p38 MAPK play pivotal role in each stage of signaling pathways (12). In the present study, although cerivastatin as well as simvastatin inhibited the Ox-LDL-induced macrophage proliferation, these two statins had no effect on GM-CSF production either at mRNA or protein levels. On the other hand, these statins showed significant inhibition on recombinant GM-CSF-induced p38 MAPK phosphorylation and macrophage proliferation. Therefore, statins inhibited macrophage proliferation by altering the signals from GM-CSF receptor to p38 MAPK.

We demonstrated in the present study that the inhibitory effect of statins on Ox-LDL- or GM-CSF-induced macrophage proliferation was prevented completely by mevalonate, but not by squalene. In addition, we found that GGTI showed more potent inhibitory effect than FTI on Ox-LDL- or GM-CSF-induced macrophage proliferation.
Moreover, statin-mediated decrease in Ox-LDL- or GM-CSF-induced macrophage proliferation was reversed completely by GGPP and partially by FPP, and these phenomena were also observed in GM-CSF-induced p38 MAPK activation. These results suggest that the geranylgeranylation of protein(s) plays a major role in Ox-LDL-induced macrophage proliferation, and the growth inhibitory effects of statins mainly depend on the suppression of protein geranylgeranylation.

Several isoprenylated proteins have been reported to be potential targets for statin-induced inhibition of atherogenic activities in vascular cells (16, 30). Although several reports focused on the role of Ras in mediating cell proliferation (31-33), a number of recent reports demonstrated that a proper processing of not only Ras but also Rho (34-37) or Rac (38) proteins by mevalonate-derived products served as a crucial step in atherogenic changes in vascular cells, suggesting that the suppression of cellular activity by statins is mediated through inhibition of Rho or Rac function. Especially, regarding macrophage proliferation, Aikawa et al. (6) demonstrated that M-CSF-induced cell proliferation was suppressed by cerivastatin in human monocyte-derived macrophages, and the inhibition was reversed by mevalonate, FPP and GGPP, suggesting that inhibition of protein farnesylation or geranylgeranylation by cerivastatin was a critical point in statin-mediated inhibition of macrophage proliferation. However, the distinct roles of farnesylated or geranylgeranylated proteins in macrophage proliferation have not been fully clarified.

In our series of experiments by overexpression of dominant negative forms of small G proteins, Ras and RhoA, GM-CSF-induced p38 MAPK phosphorylation and macrophage proliferation was inhibited by dominant negative RhoA and to a less extent by dominant negative Ras. In addition, Rho kinase inhibitor, Y-27632, inhibited GM-CSF-induced p38 MAPK phosphorylation and macrophage proliferation, similar to the effect of overexpression...
of dominant negative RhoA. These results suggest that GM-CSF-induced p38 MAPK phosphorylation is mainly mediated by signals through RhoA.

The activation of ERK is widely known to be mediated through the Ras-Raf-MEK cascade (39). In the present study, it was of note that simvastatin completely inhibited GM-CSF-induced membrane translocation of Ras, but had only a small effect on the phosphorylation of ERK. These results suggest that in macrophages, GM-CSF-induced ERK1/2 activation is mediated through some other signaling pathways than Ras-Raf-MEK. It is generally accepted that the targets of protein farnesylation and geranylgeranylation are members of Ras and Rho protein family, respectively. However, the effect of simvastatin on Ras membrane translocation was reversed by co-incubation with not only FPP but also with GGPP, although the latter effect was partial. This is possibly explained by alternative modification of small G proteins. Indeed, K-Ras is normally farnesylated but it can be alternatively geranylgeranylated in the absence of FPP (40). Similarly, RhoB can be either geranylgeranylated or farnesylated by geranylgeranyl transferase I (41, 42). Thus, it is suggested that Ras, which is usually modified by FPP, was prenylated by GGPP under our experimental conditions. However, our data also indicated that farnesyl transferase inhibitor and dominant negative Ras still suppressed p38 MAPK phosphorylation and macrophage proliferation. These results suggest that even it is only partial, Ras mediates the GM-CSF-induced p38 MAPK phosphorylation, which results in macrophage proliferation.

Recent studies reported that small G-protein Rho and its effector, Rho kinase/ROK/ROCK is involved in accelerating atherosclerotic process. Indeed, Miyata et al. reported the involvement of Rho-kinase in macrophage-mediated formation of coronary vascular lesions, coronary constrictive remodeling and vasospastic responses in the in vivo porcine model (43, 44). Mallat et al. (45) reported that inhibition of Rho kinase significantly limited early atherosclerotic plaque development in the LDL receptor knock-out mice. These
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studies identified Rho kinase inhibitors as potential candidates for the treatment of atherosclerosis. We demonstrated in the present study that statins also have an inhibitory effect on Ox-LDL-induced RhoA activation in macrophages. Therefore, statin may act as an inhibitor of Rho-dependent signaling pathway, thereby preventing the acceleration of atherosclerosis.

Recently, Schaefer et al. reported that statins also prevented Ox-LDL-induced human umbilical vein endothelial cell proliferation by suppressing the mevalonate pathway independent of the regulation of reactive oxygen species or nitric oxide (46). Therefore, the inhibition of small G protein-p38 MAPK pathway by statins, which we proposed in the present study, may be involved in the suppression of endothelial cell proliferation.

In conclusion, we have demonstrated in the present study the role of small G proteins in Ox-LDL-induced macrophage proliferation and identified the mechanisms of statin-mediated inhibition of Ox-LDL-induced macrophage proliferation. RhoA plays a pivotal role in p38 MAPK activation and macrophage proliferation while Ras has only partial effect, and statins inhibit macrophage proliferation by suppressing both Ras and RhoA activation, which mainly mediates GM-CSF-induced p38 MAPK phosphorylation (summarized in figure 6). This action may contribute to the beneficial effect of statins in the prevention of cardiovascular disease.

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Abbreviations

Abbreviations used are:

FPP, Farnesylpyrophosphate;
FTI, farnesyl transferase inhibitor;
ERK1/2, extracellular-signal regulated kinase 1/2;
GGPP, geranylgeranylpyrophosphate;
GGTI, geranylgeranyl transferase inhibitor;
G protein, GTP binding protein;
GM-CSF, granulocyte/macrophage colony-stimulating factor;
HMG-CoA, 3-Hydroxy-3-methylglutaryl coenzyme A;
MAPK, mitogen-activated protein kinase;
MOI, multiplicity of infection;
Ox-LDL, oxidized low-density lipoprotein;
PI-3K, phosphatidylinositol 3-kinase;
PKC, protein kinase C;
SMCs, smooth muscle cells.
Figure Legends

**Fig. 1. Effect of statins on Ox-LDL-induced macrophage proliferation and GM-CSF production.** A, Macrophages (5 × 10⁴) were pre-incubated with the indicated concentrations of cerivastatin (Ceriva) and simvastatin (Simva) for 1 h, and then cells were incubated for 6 days with 20 μg/ml Ox-LDL. During the last 18 h of incubation, [³H]thymidine was added, and the radioactivity was determined as described in “Experimental Procedures.” Data represent six experiments with different cell preparations. B, Macrophages were pre-incubated for 1 h with 100 nM cerivastatin (Ceriva) or 10 μM simvastatin (Simva), then cells were incubated with or without 20 μg/ml Ox-LDL for 1 h. Total RNA was extracted, and the expression of mRNA for GM-CSF or β-actin was evaluated by a real-time RT-PCR as described in “Experimental Procedures.” Data represent the mean ± SD of four separate experiments. *, p<0.01, compared with non-load. C, Macrophages were pre-incubated for 1 h with 100 nM cerivastatin (Ceriva) or 10 μM simvastatin (Simva), then cells were incubated with or without 20 μg/ml Ox-LDL for 4 h. The level of GM-CSF in collected culture media was determined by ELISA as described in “Experimental Procedures.” Data represent the mean ± SD of four separate experiments. *, p<0.01, compared with non-load.

**Fig. 2. Statins inhibit GM-CSF-induced macrophage proliferation through the reduction of mevalonate pathway.** Macrophages were pre-incubated with the indicated concentrations of cerivastatin (Ceriva) and simvastatin (Simva) for 1 h (A), or pre-incubated with 100 nM cerivastatin, 10 μM simvastatin, 10 μM FTI or 10 μM GGTI with or without 50 μM mevalonate, 50 μM squalene, 5 μM FPP or 5 μM GGPP for 1 h (B), then cells were incubated for 5 days with 15 pM of GM-CSF. During the last 18 h of incubation, [³H]thymidine was added, and the radioactivity was determined as described in “Experimental Procedures.” Data
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represent the mean ± SD of four separate experiments. *, \( p < 0.01 \), compared with non-load. †, \( p < 0.01 \), compared with cells incubated with GM-CSF alone. ‡, \( p < 0.01 \), compared with cells incubated with GM-CSF plus cerivastatin or simvastatin.

Fig. 3. Statins inhibit GM-CSF-induced MAPK phosphorylation through the reduction of mevalonate pathway. Macrophages were pre-incubated with 100 nM cerivastatin (Ceriva), 10 \( \mu \)M simvastatin (Simva) (A and B), 10 \( \mu \)M FTI or 10 \( \mu \)M GGTI (C and D) for 1 h, or pre-incubated with 10 \( \mu \)M simvastatin with or without 5 \( \mu \)M FPP or 5 \( \mu \)M GGPP for 1 h (E), then cells were incubated with 15 pM of GM-CSF for 10 min. Protein samples were immunoblotted for phosphorylated ERK1/2 or p38 MAPK as described in “Experimental Procedures.” Data represent the mean ± SD of three separate experiments. *, \( p < 0.01 \), compared with non-load. †, \( p < 0.01 \), compared with cells incubated with GM-CSF alone. ‡, \( p < 0.01 \), compared with cells incubated with GM-CSF and simvastatin.

Fig. 4. Roles of Ras and RhoA on statin-mediated suppression of MAPK phosphorylation. A and B, Macrophages were pre-incubated with 10 \( \mu \)M simvastatin with or without 5 \( \mu \)M farnesyl pyrophosphate (FPP) or geranylgeranyl pyrophosphate (GGPP) for 1 h. After the incubation with 15 pM recombinant GM-CSF for 10 min, whole cell lysate (whole) and membrane fraction (membrane) of the macrophages were extracted and immunoblotted for pan Ras (A) and RhoA (B). C and D, After the infection with adenoviral vectors containing LacZ, dominant negative forms of Ras (Ad-DN-Ras) or RhoA (Ad-DN-RhoA), macrophages were incubated for 10 min with 15 pM recombinant GM-CSF. Protein samples were immunoblotted for phosphorylated ERK1/2 (C) or p38 MAPK (D). E, Macrophages were pre-incubated with the indicated concentrations (1, 10 or 100 \( \mu \)M) of Y-27632 for 1 h, then cells were incubated for 10 min with 15 pM recombinant GM-CSF. Protein samples
were immunoblotted for phosphorylated p38 MAPK. Data represent the mean ± SD of three separate experiments. *, \( p<0.01 \), compared with non-load. †, \( p<0.01 \), compared with cells incubated with GM-CSF alone. ‡, \( p<0.01 \), compared with cells incubated with GM-CSF and simvastatin. #, \( p<0.01 \), compared with Ad-LacZ-infected cells. §, \( p<0.01 \), compared with Ad-LacZ-infected cells incubated with GM-CSF.

**Fig. 5. GM-CSF-induced macrophage proliferation is mediated by Ras and RhoA.**

After infection with adenoviral vectors containing LacZ, dominant negative forms of Ras (Ad-DN-Ras), RhoA (Ad-DN-RhoA) or p38 MAPK (Ad-DN-p38), or pre-treatment with the indicated concentration (1, 10 or 100 \( \mu \)M) of Y-27632, macrophages were pre-incubated with 10 \( \mu \)M simvastatin for 1 h and then incubated with 15 pM of GM-CSF for 5 days. During the last 18 h of incubation, \([3H] \)thymidine was added, and the radioactivity was determined as described in “Experimental Procedures.” Data represent the mean ± SD of four separate experiments. *, \( p<0.01 \), compared with non-load. †, \( p<0.01 \), compared with cells incubated with GM-CSF alone. ‡, \( p<0.01 \), compared with cells incubated with GM-CSF and simvastatin. #, \( p<0.01 \), compared with Ad-LacZ-infected cells. §, \( p<0.01 \), compared with Ad-LacZ-infected cells incubated with GM-CSF.

**Fig. 6. Schematic representation of the inhibitory effects of statins on Ox-LDL-induced macrophage proliferation.** The results of the present and our previous studies(8,11,12) demonstrated the following scheme regarding the signaling pathways of Ox-LDL-induced macrophage proliferation. Ox-LDL-induced stimulation is first transmitted into cells via an unidentified pertussis toxin-sensitive G protein-coupled receptor, which activates PKC by stimulating calcium release from the endoplasmic reticulum (ER)(8). Then, activation of ERK1/2 induces the expression of GM-CSF and its release into the medium(12). Binding of
GM-CSF to its receptor in an autocrine or paracrine fashion leads to proliferation of macrophages via Ras and Rho-p38 MAPK pathway. Statins suppress FPP and GGPP production by inhibiting mevalonate pathway, thereby inhibiting geranylgeranylation and farnesylation, and subsequent translocation of Ras and RhoA. The suppression of Ras and RhoA translocation inhibits GM-CSF-induced activation of Ras and Rho, thereby inhibits macrophage proliferation via suppression of p38 MAPK. Ox-LDL, oxidized low density lipoprotein; G protein, GTP binding protein; PKC, protein kinase C; GM-CSF, granulocyte/macrophage colony-stimulating factor; ERK1/2, extracellular-signal regulated kinase 1/2; MAPK, mitogen-activated protein kinase; FPP, farnesylpyrophosphate; GGPP, geranylgeranylpyrophosphate; HMG-CoA, 3-Hydroxy-3-methylglutaryl coenzyme A; GM-CSFR, GM-CSF receptor.
<table>
<thead>
<tr>
<th></th>
<th>Cell number $\times 10^4$ cells/well</th>
<th>(% of medium alone)</th>
</tr>
</thead>
<tbody>
<tr>
<td>medium alone</td>
<td>3.4 ± 0.3</td>
<td>(100 %)</td>
</tr>
<tr>
<td>Ox-LDL (20 µg/ml)</td>
<td>7.6 ± 0.7</td>
<td>(224 %)*</td>
</tr>
<tr>
<td>GM-CSF (15 pM)</td>
<td>7.8 ± 0.6</td>
<td>(229 %)*</td>
</tr>
<tr>
<td>cerivastatin (100 nM)</td>
<td>3.5 ± 0.4</td>
<td>(103 %)</td>
</tr>
<tr>
<td>simvastatin (10 µM)</td>
<td>3.4 ± 0.3</td>
<td>(100 %)</td>
</tr>
<tr>
<td>Ox-LDL + cerivastatin</td>
<td>3.9 ± 0.4</td>
<td>(115%)†</td>
</tr>
<tr>
<td>GM-CSF + cerivastatin</td>
<td>4.0 ± 0.4</td>
<td>(118 %)‡</td>
</tr>
<tr>
<td>Ox-LDL + simvastatin</td>
<td>4.1 ± 0.3</td>
<td>(121%)†</td>
</tr>
<tr>
<td>GM-CSF + simvastatin</td>
<td>4.3 ± 0.5</td>
<td>(126%)‡</td>
</tr>
</tbody>
</table>

Peritoneal macrophages were incubated with 20 µg/ml Ox-LDL or 15 pM GM-CSF in the presence of cerivastatin or simvastatin for 7 days. On day 7, the number of cells was counted as described in “Experimental Procedures.” Each experiment was performed in triplicate. Data are expressed as mean ± SD of three separate experiments. Percentages of control value (medium alone) are expressed in parentheses. *, $p < 0.01$, compared to medium alone; †, $p < 0.01$, compared with cells incubated with Ox-LDL alone; ‡, $p < 0.01$, compared with cells incubated with GM-CSF alone.
Figure 1

A

![Graph showing [3H]Thymidine Incorporation](#)

- Ceriva
- Simva
- Ox-LDL+Ceriva
- Ox-LDL+Simva

Concentrations (M)

0 10^{-9} 10^{-8} 10^{-7} 10^{-6} 10^{-5} 10^{-4}

[3H]Thymidine Incorporation (x10^3 cpm/well)

B

![Bar chart showing GM-CSF mRNA levels](#)

C

![Bar chart showing GM-CSF levels](#)
Figure 2

A

![Graph showing concentration versus [H]Thymidine Incorporation (x10^3 cpm/well) for different groups: Ceriva, Simva, GM-CSF+Ceriva, and GM-CSF+Simva.](image)

B

![Bar graph showing [H]Thymidine Incorporation (x10^3 cpm/well) for Ceriva and Simva with various treatments: GM-CSF, Statins, Mev, Squalene, FPP, GGPP, FTI, and GGTI.](image)
Figure 3

A

p-ERK1/2

Phosphorylated ERK1/2 (fold basal)

GM-CSF
Simva
Ceriva

B

p-p38 MAPK

Phosphorylated p38 MAPK (fold basal)

GM-CSF
Simva
Ceriva

C

p-ERK1/2

Phosphorylated ERK1/2 (fold basal)

GM-CSF
FTI
GGTI

D

p-p38 MAPK

Phosphorylated p38 MAPK (fold basal)

GM-CSF
FTI
GGTI

E

p-p38 MAPK

Phosphorylated p38 MAPK (fold basal)

GM-CSF
Simva
FPP
GGPP

Note: GM-CSF, Simva, Ceriva, FTI, GGTI, FPP, and GGPP are listed in columns, with negative (-) or positive (+) symbols indicating their presence or absence.
Figure 4

A

Ras

membrane

total cell

B

RhoA

membrane

total cell

C

p-ERK1/2

Phosphorylated
ERK1/2

fold basal

D

p-p38 MAPK

Phosphorylated
p38 MAPK

fold basal

E

p-p38 MAPK

Phosphorylated
p38 MAPK

fold basal

GM-CSF
Simva
FPP
GGPP

GM-CSF
Simva
FPP
GGPP

GM-CSF
Ad-LacZ
Ad-DN-Ras
Ad-DN-Rho

GM-CSF
Ad-LacZ
Ad-DN-Ras
Ad-DN-Rho

GM-CSF
Y-27632

pan-Ras

RhoA

fold basal

GM-CSF
Simva
FPP
GGPP

GM-CSF
Simva
FPP
GGPP

p-ERK1/2

Phosphorylated
ERK1/2

fold basal

p38 MAPK

Phosphorylated
p38 MAPK

fold basal

Y-27632

−

+ 100

−

100

GM-CSF

pan-Ras

RhoA

fold basal
Figure 5

[Graph showing [3H]Thymidine Incorporation (x 10^3 cpm/well) for various treatments.]

- GM-CSF
- Simva
- Ad-LacZ
- Ad-DN-Ras
- Ad-DN-Rho
- Y-27632
- Ad-DN-p38

Legend:
- *: +
- #: +
- $: +
- †: +

Values on the y-axis represent [3H]Thymidine Incorporation (x 10^3 cpm/well) with error bars indicating standard deviation.
Statins suppress oxidized low-density lipoprotein-induced macrophage proliferation by inactivation of small G protein-p38 MAPK pathway

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Nishikawa, Shokei Kim-Mitsuyama, Yoh Takuwa and Eiichi Araki

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