HMG-CoA Reductase Inhibitor Modulates Monocyte–Endothelial Cell Interaction Under Physiological Flow Conditions In Vitro

Involvement of Rho GTPase–Dependent Mechanism

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Abstract—3-Hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, or statins, have been reported to exert actions independent of their lipid-lowering effects. To critically assess the effects of statins on monocyte–endothelial cell interactions, we used an in vitro model that mimicked physiological flow conditions. Monocytic U937 cells were incubated in the presence of cerivastatin for 48 hours. Adhesive interactions of statin-treated U937 cells were then analyzed by use of activated (interleukin-1β 10 U/mL, 4 hours) human umbilical vein endothelial cells in an in vitro flow apparatus. Flow cytometric analysis of adhesion molecules and measurement of F-actin content in U937 cells were performed before and after statin treatment. Preincubation with cerivastatin significantly decreased U937 firm adhesion to activated human umbilical vein endothelial cells, whereas U937 rolling was not decreased. Fluorescence-activated cell sorter analysis revealed downregulation of U937 surface expression of CD11a, CD18, and VLA4 after statin treatment. Cerivastatin significantly reduced F-actin content in U937 cells and inhibited RhoA translocation, whereas preincubation with C3 exoenzyme reduced U937 adhesion under flow. Cerivastatin reduces monocyte adhesion to vascular endothelium under physiological flow conditions via downregulation of integrin adhesion molecules and inhibition of actin polymerization via RhoA inactivation. Our findings have important implications for the lipid-independent effects of statins. (Arterioscler Thromb Vasc Biol. 2001;21:1165-1171.)

Key Words: HMG-CoA reductase inhibitor □ adhesion molecules □ monocytes □ atherosclerosis

Atherosclerosis is a complex disease process that is associated with vascular wall dysfunction.1 An increasingly large body of evidence points to a crucial role of leukocyte–endothelial cell interactions in atherosclerotic plaque formation. The adhesion of circulating monocytes to the intimal endothelial cell monolayer is thought to be one of the earliest events in naturally occurring human and experimental animal models of atherosclerosis.1 Uregulation of the number and/or affinity of numerous adhesion receptors and counterreceptors expressed on both endothelial cells and leukocytes appears to be involved in atherosclerosis, including selectins, intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1, and β1- and β2-integrins2 in atherosclerosis. Moreover, administration of antibodies to adhesion molecules has been found to decrease atherosclerotic lesion formation in experimental animal models, suggesting a potential therapeutic role for inhibition of leukocyte adhesion.3,4

Recently, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins), which lower the plasma level of LDL cholesterol,5 have been shown to decrease the incidence of myocardial infarction and other ischemic vascular events in hyperlipidemic and atherosclerotic individuals. Interestingly, several clinical trials have suggested that there might be additional beneficial effects of statins that are independent of their cholesterol-lowering actions.6 In fact, these compounds have recently been reported to restore endothelial function via stimulating endothelial constitutive nitric oxide synthase activity in addition to lowering serum cholesterol levels.7 Moreover, HMG-CoA reductase inhibitors have been reported to be effective in blunting the hyperadhesiveness of leukocytes to endothelium in vitro8 and in vivo,9 implicating a potential effect of statin therapy on the expression and function of adhesion molecule(s). No direct studies, however, have been performed on the effects of statins on monocyte–endothelial cell adhesion
under physiological flow conditions. Thus, the purpose of this study was to observe the effect of cerivastatin on monocyte–endothelial cell adhesive interactions in an in vitro model that mimicked physiological flow conditions and to examine the cellular mechanisms involved in modulating monocyte adhesion.

Methods

Cell Culture and Reagents

Human umbilical vein endothelial cells (HUVECs) were isolated from normal-term umbilical veins and cultured on 0.1% gelatin–coated tissue culture dishes as described previously10,11 in RPMI-1640 with 20% FCS (Life Technologies Oriental Inc), endothelial growth factor (25 µg/mL, Funakoshi Co Ltd), and porcine intestinal heparin (50 µg/mL, Sigma-Aldrich Japan, KK), along with penicillin and streptomycin as antibiotics. The U937 and THP-1 cell lines were obtained from the American Type Culture Collection and cultured in RPMI-1640 containing 10% FCS. For use in the flow-chamber apparatus, HUVECs (passages 2 and 3) were plated onto 22-mm fibronectin-coated glass coverslips as previously described.12,13 Re-combinant human interleukin (IL)-1β was obtained from Genzyme. BCECF-AM, FITC-phalloidin, and BODIPY-FL-GTP-γS were purchased from Molecular Probes. Cerivastatin sodium, (+)-3R,5S-sodium-erythro-(C)-7-[(4-fluorophenyl)-2,6-disopropyl-3-yl]-3,5-dihydroxyhept-6-enoate, was a gift from Bayer AG (Leverkusen, Germany) and was stored as a 10-mmol/L stock solution in DMSO. Monoclonal antibodies (mAbs) used in this study were as follows: mouse anti-CD11a (clone 38, Ancell Corp), mouse anti-CD11b (clone 44, YLEM), mouse anti-CD18 (clone MEM48, Southern Biotechnology Associates Inc), mouse anti-MLA4 (clone A4-PUJ1, Upstate Biotechnology), and mouse anti-SLx (clone KM93, Serotec Ltd). Mouse anti-RhoA mAb was purchased from Santa Cruz Biotechnology, Inc. Wild-type and dominant negative mutant (Asn19RhoA) forms of RhoA cDNA constructs were kindly provided by Dr Shinya Kuroda (Nara Institute of Technology, Nara, Japan).

Adhesion Assay Under Laminar Flow

Apparatus Design

The parallel-plate flow chamber used in the present study was previously described in detail.14,15 Briefly, the chamber was composed of 2 aluminum steel plates separated by a 200-µm-thick Silastic gasket, and the flow channel was formed by removal of a 20-mm rectangular section from the gasket. Defined levels of flow were applied to the HUVEC monolayer by drawing the perfusion medium (D-PBS containing 0.2% human serum albumin) through the channel with a syringe pump (model 44 Harvard Apparatus). A plastic heating plate (Tokai Hit Co) was mounted on the stage of an inverted microscope (IX50, Olympus) to maintain the temperature at 37°C. The channel flow could then be approximated as a 2D fully developed laminar flow with a simple parabolic velocity profile.

Experimental Application

Endothelial monolayers on coverslips were stimulated with IL-1β (10 U/mL) for 4 hours and positioned in the flow chamber, which was mounted on an inverted microscope. The monolayers were perfused for 5 minutes with perfusion medium and then examined carefully to verify the monolayer as confluent. Then, U937 cells pretreated with cerivastatin were diluted in the perfusion medium to 10⁶ cells/mL. The U937 cells were drawn through the channel at controlled flow rates to generate calculated wall shear stresses of 1.0 and 2.0 dyne/cm² for 10 minutes. The entire period of perfusion was recorded on videotape with a digital video recorder containing a time length of 485 nm and an emission wavelength of 535 nm.

Flow Cytometric Analysis

U937 cells were first incubated with the indicated primary antibodies for 45 minutes on ice and washed twice with RPMI 1640/5% FCS, then incubated with an FITC-tagged goat anti-mouse antibody (Caltag). Fluorescence was analyzed with a FACS Calibur (Becton-Dickinson).

Quantitation of F-Actin in U937 Cells

The filamentous (F) actin content of U937 cells after cerivastatin treatment was quantitated as described previously.16 In brief, after treatment with cerivastatin (1.0 µmol/L for 48 hours), U937 cells were washed 3 times with RPMI/1% FCS and then fixed with 3.7% formaldehyde in D-PBS for 5 minutes at 20°C. The cells were washed 3 times and then made permeable with a buffer containing 1.4% formaldehyde in D-PBS and 0.1% NP-40 for 90 seconds at 0°C, which was followed by staining with FITC/phalloidin (1:20 dilution in D-PBS) for 40 minutes at 20°C. After 3 additional washes, 100 µL methanol was added and the F-actin content was measured with a fluorescence plate reader with an excitation wavelength of 485 nm and an emission wavelength of 535 nm.

Assays for Rho GTPase Activity

The activity of Rho GTPase was determined by 2 independent assays.

Rho Translocation Assay

A Rho translocation assay was performed as previously described.17,18 Briefly, 1×10⁶ U937 cells were incubated in a lysis buffer containing 50 mmol/L HEPES (pH 7.4), 50 mmol/L NaCl, 1 mmol/L MgCl₂, 2 mmol/L EDTA, 1 mmol/L PMSF, 10 mg/mL leupeptin, 1 mmol/L NaVO₃, and 0.1% Triton X-100 for 5 minutes on ice. The cell lysates were centrifuged at 15000 rpm for 15 minutes. After the supernatant had been collected as the cytosol fraction, the pellet was resuspended in 1% Triton X-100 in the lysis buffer and centrifuged at 15 000 rpm for 15 minutes. The supernatant was then collected as the membrane fraction. Equal amounts (10 µg) of protein from each fraction were subjected to SDS-polyacrylamide gel electrophoresis followed by immunoblotting with anti-RhoA mAb. The immunoreactive RhoA proteins were detected by an enhanced chemiluminescence kit (Amersham Pharmacia Biotech Inc) according to the manufacturer’s protocol.

RhoA GTP Binding Assay

The membrane and cytosol proteins were isolated as described above. Protein samples (30 µg) were suspended in a mixture consisting of 1 µmol/L of a nonhydrolyzable fluorescence-labeled GTP-γS⁰ (BODIPY FL GTP-γS, Molecular Probes), 20 mmol/L Tris, 5 mmol/L MgCl₂, 1 mmol/L EDTA, and 1 mmol/L DTT, pH 8.0, in a total volume of 100 µL. A GTP-binding reaction was carried out for 30 minutes at 22°C. Samples were then suspended in 300 µL of immunoprecipitation buffer containing 100 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 5 mmol/L EDTA, 1 mmol/L PMSF, 10 mg/mL leupeptin, 1 mmol/L NaVO₃, and 1% Triton X-100. Anti-RhoA mAb (5 µg) was then added to the mixture before incubation for 16 hours at 4°C. The antibody-RhoA complexes were then incubated with 50 µL of goat anti-murine IgG–coupled Sepharose beads (Cappel) for 2 hours at 4°C. The immune complexes were collected by centrifugation at 12 000 g for 15 minutes. The resulting pellets containing the immunoprecipitated fluorescent GTP-γS-labeled RhoA were measured by a fluorescence reader.

Overexpression of RhoA

Three expression constructs, pEF-BOS-HA-WT-RhoA, pEF-BOS-HA-DN-RhoA, and pEF-BOS, were used in the following experiment. All vectors used a promoter of human elongation factor-1 (EF-1) to obtain a high level of expression.20 The vector contains an amino-terminal hemaglutinine epitope (HA) adjacent to the cloning sites for detection of the target protein. For transient transfection,
Reduction of U937 adhesion was observed with as little as 0.1 μmol/L cerivastatin (CS), 1.0 μmol/L cerivastatin + 10 μmol/L mevalonic acid (CS+MV), or in parallel with the medium alone (Control) were perfused over activated (IL-1β 10 U/mL, 4 hours) HUVEC monolayers at a flow rate of 2.0 to 1.0 dyne/cm² as described in Methods. Mean±SD, 10 HPFs. Data are representative of the results of 5 separate experiments. *P<0.005 vs Control-U937. **P<0.0005 vs CS-U937.

Figure 1. Cerivastatin treatment significantly reduces U937 cell adhesion to activated HUVEC monolayers under physiological flow. U937 cells treated with 1.0 μmol/L cerivastatin (CS), 1.0 μmol/L cerivastatin + 10 μmol/L mevalonic acid (CS+MV), or in parallel with the medium alone (Control) were perfused over activated (IL-1β 10 U/mL, 4 hours) HUVEC monolayers at a flow rate of 2.0 to 1.0 dyne/cm² as described in Methods. Mean±SD, 10 HPFs. Data are representative of the results of 5 separate experiments. *P<0.005 vs Control-U937. **P<0.0005 vs CS-U937.

THP-1 cells instead of U937 cells were used, because of their higher transfection efficiency, by a method that used a cationic liposome (Lipofectin, Gibco-BRL). THP-1 cells (2×10⁵ per well) were transfected with 20 μg of pEF-BOS-HA-WT-RhoA, pEF-BOS-HA-DNRhoA, or pEF-BOS without an insert according to the manufacturer’s protocol. The transfected THP-1 cells were harvested 48 hours after transfection, and RhoA expression was determined by immunoblot with an mAb against the HA tag.

Statistics
Data are presented as mean±SD as indicated. Two-tailed Student’s t tests were performed with Microsoft Excel. Probability values represent the results of these t tests, and values of P<0.05 were considered statistically significant.

Results

HMG-CoA Reductase Inhibitor Reduces U937 Adhesion to Vascular Endothelium Under Physiological Flow Conditions

The effect of statin was evaluated under physiological flow conditions in vitro. In preliminary experiments, U937 cells were incubated in the presence of various concentrations (0, 0.1, 1.0, and 2.0 μmol/L) of cerivastatin for 48 hours. Significant reduction of U937 adhesion to both resting and cytokine-activated (IL-1β, 10 U/mL, 4 hours) HUVECs was observed under static (no-flow) conditions (data not shown). Reduction of U937 adhesion was observed with as little as 0.1 μmol/L cerivastatin (data not shown). On the basis of this experiment, we chose to treat U937 cells with cerivastatin at a concentration of 1.0 μmol/L in the following experiments. Because our preliminary experiments suggested that few interactions would occur at flow rates of >2.0 dyne/cm², we chose 2 flow rates: 1.0 dyne/cm² (low shear) and 2.0 dyne/cm² (high shear). Essentially, no U937 interactions were observed when unactivated HUVECs were used (data not shown). As shown in Figure 1, activated HUVECs supported rolling (low shear, 4.6±0.5 cells per high-power field [HPF]; high shear, 4.33±1.1 cells/HPF) as well as stable (low shear, 40.3±12.9 cells/HPF; high shear, 15.6±4.3 cells/HPF) adhesion of U937 cells under both flow levels. When U937 cells were pretreated with cerivastatin before adhesion, adhesion was significantly reduced (P<0.003 versus control U937). In contrast, the numbers of rolling U937 cells were not significantly changed by cerivastatin pretreatment. The addition of mevalonic acid to metabolically bypass the effect of cerivastatin completely reversed this reduced adhesion of U937 cells.

HMG-CoA Reductase Inhibitor Alters Integrin Expression of U937 Cells

To measure the cell-surface expression profiles of adhesion molecules after cerivastatin treatment, flow cytometric analysis of U937 cells was carried out. As shown in Figure 2, downregulation of CD11a, CD18, and VLA-4 was observed after cerivastatin treatment. In contrast, the surface expression of CD11b was not affected by cerivastatin treatment. Expression levels of sLx, a carbohydrate structure that binds to the selectin family of adhesion molecules, was not changed by cerivastatin treatment. Furthermore, the attenuated expression of integrins (CD11a, CD18, and VLA-4) was reversed by addition of mevalonic acid.

HMG-CoA Reductase Inhibitor Inhibits Actin Polymerization

It is known that HMG-CoA reductase is important in synthesizing isoprenyl moieties; therefore, the statin group of drugs is thought to be an effective means of inactivating signaling molecules that require isoprenylation of CAAX
sequences at their C-termini, such as small GTP-binding proteins. We also investigated the possible involvement of Rho, a small GTP-binding protein critically important for cell motility and cytoskeleton organization. First, we measured the F-actin content after cerivastatin treatment of U937 cells. U937 cells were incubated in the presence of 1.0 μmol/L cerivastatin for 48 hours, and then fixed, made permeable, and incubated with FITC-phalloidin, after which fluorescence intensity was measured with a fluorescence reader. Data are representative of the results of 3 separate experiments. *P<0.005 vs Control U937. **P<0.05 vs CS-U937.

Cerivastatin Inhibits Membrane Translocation of Rho GTPase in U937 Cells
Because membrane translocation of Rho GTPases from the cytosol is necessary for their proper function, we also studied the effect of cerivastatin on membrane translocation of Rho GTPases in U937 cells. Western blotting analyses of cytosol and membrane fractions prepared from U937 cells revealed that cerivastatin treatment (1.0 μmol/L, 48 hours) significantly decreased the amount of membrane-associated RhoA proteins compared with the basal condition (Figure 4A). Addition of mevalonic acid restored the distribution of cytosol- and membrane-associated immunoreactive RhoA proteins to basal levels. The level of G3PDH did not change after statin treatment, validating the specificity of the inhibitory effect of statin in translocation of RhoA.

Effect of Cerivastatin on RhoA GTP-Binding Activity
To examine whether cerivastatin treatment affects RhoA activity, the GTP-binding capacity of RhoA was measured in U937 cells. Fluorescent GTP-γS–bound RhoA was immunoprecipitated from the membrane and the cytosol from U937 cells was treated with cerivastatin (1.0 μmol/L) in the presence or absence of mevalonic acid. As shown in Figure 4B, RhoA activity under basal conditions was 957±156 RFU in the membrane fraction and 176.3±64.5 RFU in the cytosol fraction. Cerivastatin treatment reduced membrane-associated GTP binding activity significantly, by 46% (P<0.02), which was canceled by cotreatment with mevalonic acid. GTP-binding activities of cytosol RhoA were not significantly affected by statin treatment.

Overexpression of Dominant Negative RhoA Reduces THP-1 Adhesion to Activated HUVECs
To further confirm the potential role(s) of RhoA in leukocyte adhesion, mononcytic THP-1 cells with a transient expression of wild-type (WT-RhoA) or dominant negative (DN-RhoA) RhoA were prepared. Western blotting analysis with anti-HA mAb revealed comparable levels of RhoA expression in both WT-RhoA– and DN-RhoA–transfected THP-1 cells (Figure 5A). F-actin formation was significantly inhibited in THP-1 cells transfected with DN-RhoA, but not in those with WT-RhoA (WT-RhoA, 54±6 RFU; DN-RhoA, 20±7.2 RFU, P<0.0005, Figure 5B). Moreover, transfection of DN-RhoA significantly reduced THP-1 adhesion to activated HUVECs compared with WT-RhoA (WT-RhoA, 15.16±2.4% adhesion; DN-RhoA, 9±3.48%, P<0.03, Figure 5C).
to those treated with 1.0 μmol/L cerivastatin ($P<0.003$ versus control U937) under flow conditions (shear stress = 1.0 dyne/cm$^2$).

**Discussion**

Monocyte accumulation to vascular endothelium has been defined as one of the earliest manifestations of atherosclerosis.$^1$ Previous pathological studies have clearly demonstrated the existence of monocyte accumulation in aortic segments, even in the absence of lipid deposition.$^{21}$ This monocyte accumulation often coincides with the expression of adhesion molecules, such as VCAM-1 and selectins, suggesting their role in the early stage of atherosclerosis.$^{22}$ A previous study that used an in vitro adhesion assay system under physiological flow conditions reported involvement of the sequential action of selectins and integrins in monocyte recruitment to vascular endothelium.$^{15}$ Modulation of monocyte adhesion to vascular endothelium is thought to be a potential therapeutic approach for atherosclerosis.$^{3,4}$ HMG-CoA reductase inhibitors, or statins, have been used to treat hyperlipidemia, a major risk factor of atherosclerosis, because of their effects toward blocking cholesterol synthesis by inhibiting the mevalonate pathway in the liver. Recent observations, however, have revealed that statins might exert lipid-independent effects in atherosclerosis.

In the present study, we demonstrated that treatment of a monocytic cell line, U937, with an HMG-CoA reductase inhibitor, cerivastatin, significantly reduced adhesion to resting and activated HUVECs under physiological flow conditions. This effect of cerivastatin on monocyte adhesion was concentration-dependent and was abrogated by simultaneous treatment with mevalonic acid. Interestingly, statin treatment did not significantly change the fraction of rolling U937 cells under flow. One possible explanation is that although U937 cells are able to roll on a monolayer, they subsequently detach from the monolayer because of reduced adhesion, rather than remain rolling on the monolayer. This suggests that the primary target of statin action is the stable adhesion step that follows initial rolling on activated endothelium. Flow cytometric analysis of U937 cells after statin treatment clearly revealed downregulation of certain cell surface integrins (CD11a, CD18, and VLA-4), which are heterodimeric counterreceptors known to bind to ICAM-1 and VCAM-1 on endothelial cells. These results suggest that the mechanisms by which statins reduce U937 adhesion involve, at least in part, the downregulation of integrin expression in U937 cells. To what extent these integrins are responsible for this observed U937 cell adhesion to HUVECs, however, remains to be examined. These inhibitory effects of statins on integrin expression are consistent with the previous findings of another group.$^8$ Interestingly, Prufer et al.$^{23}$ recently reported that administration of a statin significantly decreased P-selectin–dependent leukocyte–endothelial cell interactions in rat mesenteric arteries in vivo, suggesting the potential anti-inflammatory effects of statins. Our data, obtained from an in vitro flow-chamber system, also suggest anti-inflammatory effects. In addition to confirming previous observations showing an inhibitory effect of statins on leukocyte–endothelial cell adhesion under static assay conditions,$^6$ our experiments were the first to examine the component step in the leukocyte–endothelial cell adhesion cascade under physiological flow conditions.$^{15}$ Although the amounts of shear stress used in our study were much lower than average unidirectional arterial shear stress, they are similar to the mean wall shear stress that has been determined for atherosclerosis-prone human carotid artery bifurcation (between +4 and −4 dyne/cm$^2$).$^{24}$ Because integrin-dependent leukocyte adhesion is also modulated by receptor affinity and/or cytoskeletal organization,$^{25,26}$ we also investigated the
possible involvement of Rho family proteins. RhoA GTPase is thought to be one of the most important molecules involved in regulation of the cytoskeleton network. A recent study demonstrated that statin pretreatment of U937 cells directly modulated integrin affinity via inhibition of geranylgeranylation of RhoA protein. In the present study, we were able to document that actin cytoskeleton organization, as reflected by filamentous actin content, as well as the activity of RhoA, judged from membrane translocation and GTP-ys binding, were significantly altered after statin treatment. In addition, overexpression of DN-RhoA, but not WT-RhoA, in THP-1 cells reduced F-actin formation and attenuated their adhesion to HUVECs, suggesting a crucial role for RhoA in this process.

It has been reported that protein geranylgeranylation is required for integrin-dependent adhesion in leukocytes; thus, it is conceivable that statin treatment may affect integrin-dependent leukocyte adhesion via inhibition of the geranylgeranylation of RhoA. Although inhibition of RhoA by its specific inhibitor, C3 toxin, did not significantly alter the expression levels of integrins in U937 cells (data not shown), RhoA may modulate the affinity of integrins without changing their expression levels. Wojciak-Stothard et al25 recently reported that RhoA is required for the clustering of adhesion molecules in endothelial cells when monocytes adhere to endothelial cells. It may be possible that statin treatment directly inhibits Rho activation and disrupts actin polymerization, which leads to failure of integrin clustering, eventually resulting in reduced adhesion to endothelial cells.

Our new finding that the initial rolling step appears to be unaltered after statin treatment is consistent with the lack of any observable effect on U937 cell-surface expression of SLx, an oligosaccharide ligand of E-selectin. Statin effects on integrin expression and function may also influence the interaction of the migrating leukocyte with the subendothelial extracellular matrix, and thus leukocyte retention in the vessel wall. Although higher than those obtained in plasma, the concentrations of cerivastatin used in this study were within the range of expected tissue levels derived from prescribed pharmacological dosages.28 It remains unclear, however, whether these findings obtained from in vitro assay can be extrapolated to an in vivo situation.

Increasing evidence from controlled clinical studies indicates that statin therapy can exert a beneficial effect in various pathological conditions, including stroke, organ transplantation, and osteoporosis, independent of its lipid-lowering action.

The present study demonstrated that statin treatment of a human monocytic cell significantly reduced its adhesion, but not rolling, on activated human endothelial cells under physiological flow conditions. The mechanisms by which statin reduces U937 adhesion involve reduced expression of integrins, inhibition of Rho GTPase activity, and disruption of F-actin organization. These findings provide further mechanistic insights into the added benefits of this class of lipid-lowering agents for the treatment of atherosclerotic vascular disease.

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References


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