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. 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. Upregulation 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-integrins 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.

Recently, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins), which lower the plasma level of LDL cholesterol, 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. 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. Moreover, HMG-CoA reductase inhibitors have been reported to be effective in blunting the hyperadhesiveness of leukocytes to endothelium in vitro and in vivo, 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.

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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 previously.

Adhesion Assay Under Laminar Flow

The parallel-plate flow chamber used in the present study was previously described in detail.

Quantitation of F-Actin in U937 Cells

The filamentous (F) actin content of U937 cells after cerivastatin treatment was quantitated as described previously.

Assays for Rho GTPase Activity

1.4% formaldehyde in D-PBS and 0.1% NP-40 for 90 seconds at room temperature. The cells were washed 3 times and then made permeable with a buffer containing 0.5% Triton X-100. The cell lysates were centrifuged at 15 000 rpm for 15 minutes. The supernatant was 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.

Overexpression of RhoA

Three expression constructs, pEF-BOS-HA-WT-RhoA, pEF-BOS-HA-DNRhoA, and pEF-BOS-HA, 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. The vector contains an amino-terminal hemaglutinine epitope (HA) adjacent to the cloning site for detection of the target protein. For transient transfection,
Reduction of U937 adhesion was observed with as little as 0.1 mmol/L cerivastatin (CS), 1.0 mmol/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.

Figure 2. Flow cytometric analysis of U937 cell adhesion molecule expression after cerivastatin treatment. U937 cells were incubated in the presence of medium alone (shaded area), 1.0 μmol/L cerivastatin (gray line), or 1.0 μmol/L cerivastatin and 10 μmol/L mevalonic acid (hatched line) for 48 hours. The cells were then analyzed by flow cytometry with mAbs for CD11a, CD11b, CD18, VLA-4, and sLx, with 10,000 cells used for each condition. Data are representative of the results of 3 separate experiments.
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 cytoso 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 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, monocytic 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).

C3 Exoenzyme, a Specific Inhibitor of Rho GTPase, Reduces U937 Adhesion
To further determine the potential role of Rho GTPase in this phenomenon, we treated U937 cells with the bacterial exoenzyme Clostridium botulinum C3 ADP-ribosyltransferase for 48 hours at a concentration of 30 μg/mL. This enzyme has been shown specifically to ADP-ribosylate, and thus inactivate, Rho proteins. As shown in Figure 6, treatment with C3 exoenzyme for 48 hours reduced the number of adhered U937 cells (P<0.01 versus control U937), which was comparable
to those treated with 1.0 \( \mu \)mol/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.\(^2\) 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.\(^3\) 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.\(^4\) 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.\(^5\) Interestingly, Pruefer 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.\(^2\) 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-\(\gamma\)S 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\(^2\); 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 al\(^2\) 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 S\(\text{Lex}\), 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.\(^2\)\(^8\) 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,\(^2\)\(^9\) organ transplantation,\(^3\)\(^0\)\(^3\)\(^1\) and osteoporosis,\(^3\)\(^2\) 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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