Simvastatin Reduces the Expression of Adhesion Molecules in Circulating Monocytes From Hypercholesterolemic Patients

Abdolreza Rezaie-Majd, Gerald W. Prager, Robert A. Bucek, Gerit H. Schernthaner, Thomas Maca, Hans-Georg Kress, Peter Valent, Bernd R. Binder, Erich Minar, Mehrdad Baghestanian

**Objective**—The intercellular adhesion molecule-1 (ICAM-1/CD54) and its ligand, CD11a/CD18, mediate endothelial adhesion of leukocytes and their consecutive transmigration. Anti-inflammatory effects of statins are considered to be exerted in part through inhibition of leukocyte-endothelial interactions. We investigated the in vivo effects of simvastatin treatment in hypercholesterolemic patients and the influence of various statins on expression of cellular adhesion molecules in vitro.

**Methods and Results**—A total number of 107 hypercholesterolemic patients were treated with 20 mg (n=52) or 40 mg (n=55) of simvastatin daily. After 6 weeks of treatment, peripheral blood mononuclear cells (PBMCs) expressed lower amounts of CD54-, CD18-, and CD11a-mRNA compared with pretreatment values. Surface expression of CD54 and CD18/CD11a on CD14+ monocytes also decreased significantly in both groups of patients. Moreover, simvastatin, atorvastatin, and cerivastatin were found to downregulate tumor necrosis factor (TNF)-α-induced expression of CD54 and CD18/CD11a in isolated PBMCs obtained from normal donors as well as TNF-α-dependent expression of these CAMs in cultured human umbilical vein endothelial cells (HUVECs). Furthermore, all three statins were found to reduce the binding of PBMCs to TNF-α–stimulated HUVECs in vitro.

**Conclusions**—Statin-induced inhibition of expression of CD54 and CD18/CD11a in PBMCs and HUVECs with consecutive loss of adhesive function may contribute to the anti-inflammatory effects of these drugs and some of their beneficial clinical activities. (Arterioscler Thromb Vasc Biol. 2003;23:397-403.)

**Key Words:** atherosclerosis ■ cholesterol ■ leukocytes ■ endothelium ■ cell adhesion molecules
that some beneficial effects of statins may result from their ability to directly affect the composition of plaques by mechanisms independent of cholesterol reduction. These activities include an improved endothelial function, enhanced fibrinolysis, and antithrombotic actions.

Recent publications demonstrate the influence of statin treatment on the serum levels of various soluble CAMs, including ICAM-1, vascular cell adhesion molecule, and selectin, but there is little known about the statin-induced effects on membrane-bound CAMs on circulating mononuclear cells of hypercholesterolemic patients (HPs). In this study we investigated further effects of statins on parameters pivotal for endothelium leukocyte interactions.

In the clinical part of our project we studied the effect of simvastatin treatment on expression and production of ICAM-1 and its ligand LFA-1 in circulating PBMCs in HPs. In vitro experiments we investigated the effects of three different statins on the expression and production of endothelial/leukocyte adhesion molecules by TNF-α–treated human umbilical vein endothelial cells (HUVECs) and PBMCs.

### Methods

#### Patients and Control Subjects

Between November 1999 and November 2000, a total number of 107 patients with hypercholesterolemia and at least one manifest form of vascular disease received simvastatin. Simvastatin was prescribed at either 20 mg (n = 52) or 40 mg (n = 55) by mouth daily. The treatment plan and investigations were designed in accordance with the guidelines of the local ethical committee (University of Vienna). Blood was taken from all patients at the first day of treatment (before drug intake), after 6 weeks, and after 6 months of treatment. Previous medication (antihypertensive drugs, acetyl salicylic acid, and antidiabetic drugs as required) did not include lipid-lowering drugs and did not change during statin treatment. No other lipid-lowering drugs were applied throughout the observation period. All patients gave informed consent before blood donation. To investigate the physiological changes in the expression of CAMs on the surface of monocytes, blood was collected from healthy donors within a period of 6 weeks. The control group consisted of 20 healthy normolipemic donors (HNDs) who did not have any major cardiovascular risk factors in their case history or apparent clinical signs of atherosclerosis. Clinical and laboratory findings in HPs and HNDs are shown in the Table.

#### Reagents and Preparation of Statins

The monoclonal antibodies (mAbs) 8.4A6 (CD54), R7.1 (CD11a), 68 to 5A5 (CD18), and fluorescein isothiocyanate (FITC)-labeled CRIS-6 (CD14) were purchased from Biosource Europe (Nivelles, Belgium), FITC-goat antimouse IgG from Ancell (Bayport, Minn), and the PE-labeled mAbs HA58 (CD54), HI111 (CD11a), and 6.7 (CD18) from Becton Dickinson (San Diego, Calif). RPMI 1640 medium and FCS were from Sera Laboratory (Crawley Down, UK), endothelial basal medium from Promo Cell Co (Heidelberg, Germany). Oligonucleotides were from MWG Biotech (Ebersberg, Germany). Simvastatin was obtained from MSD (Linden, NJ), cerivastatin from Bayer (Wuppertal, Germany), and atorvastatin from Pfizer (Karlsruhe, Germany). Mevalonic acid (MVA) was purchased from Sigma Chemical (St. Louis, Mo). Atorvastatin was dissolved in 100% ethanol at 55°C and cerivastatin in 7% ethanol. Simvastatin and MVA (provided in lactone form) were prepared by dissolving in ethanol (7%) and 0.1 N NaOH before dissolving in RPMI-1640 medium.

#### Baseline Characteristics and Laboratory Parameters of Patients and Controls

<table>
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<tr>
<th>Simvastatin Dosage Group</th>
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<th>40 mg/d</th>
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<td>66.5 ± 11.9</td>
<td>56.2 ± 6.3</td>
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<td>Cholesterol, mg/dL</td>
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<td>249 ± 31</td>
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<td>6 Weeks</td>
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<td>97 ± 25</td>
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<td>55 ± 17</td>
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<td>151 ± 70</td>
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</tbody>
</table>

Baseline indicates peripheral arterial disease; CHD, coronary heart disease; CAD, cerebral arterial disease; TG, triglyceride.

There were no significant differences between both dosage groups concerning all stated parameters.

#### Isolation and Culture of PBMCs

Peripheral blood was obtained by venipuncture from HPs (n = 107) and HNDs (n = 20). In HPs, blood was collected before and during statin treatment (at 6 weeks and 6 months). In HNDs, blood was collected at two different time points (6-week interval). PBMCs were separated using Ficoll46 and subjected to RNA extraction and flow cytometry. To evaluate the in vitro effects of statins on expression of CAM-mRNA, normal PBMCs (HNDs, n = 3) were isolated. These cells were then preincubated with statins (simvastatin, cerivastatin, atorvastatin, each 10 μmol/L) in the presence or absence of MVA (100 μmol/L) in RPMI-1640 medium with 10% FCS at 37°C for 6 hours and then exposed to TNF-α (10 ng/mL) for another 6 hours.

#### Isolation and Culture of Primary HUVECs

Umbilical cords were obtained at delivery after informed consent had been given by mothers. HUVECs were isolated using collagenase type IA (Sebak, Austria), and cultured as described.46 To assess the statin effects on expression of adhesion molecules, HUVECs, pas-
sage 2 to 3, were cultured in 6-well plates and incubated with statins (simvastatin, cerivastatin, atorvastatin, each 1 and 10 \( \mu \text{mol/L} \)) in the presence of absence of MVA (100 \( \mu \text{mol/L} \)) in RPMI 1640 medium at 37°C for 2, 6, or 12 hours. Thereafter, TNF-\( \alpha \) (10 ng/mL) was added to HUVECs for additional 6 hours. Cells were then detached with trypsin/EDTA \( ^{17} \) (Sigma) and prepared for flow cytometry or Northern blotting.

**Flow Cytometry**
Expression of cell surface antigens (CD54, CD18, CD11a) on PBMCs and HUVECs was analyzed by flow cytometry. \(^{18} \) To distinguish monocytes from other PBMCs, a FITC-conjugated CD14 mAb was applied. Gating for forward scatter (CD11a-, CD18-, or CD54-PE) and side scatter (CD14-FITC) diagrams was performed. HUVECs were examined by flow cytometry using unlabeled mAbs. Isotype-matched antibodies served as control. Flow cytometry was performed on a FACS-Calibur (Becton Dickinson). At least 5000 cells were analyzed in each sample using CellQuest software (Becton Dickinson).

**RNA Extraction and Northern Blot Analysis**
Total RNA was extracted from PBMCs (HPs before and after treatment, \( n = 20 \); HNDs, \( n = 3 \)) and HUVECs by RNA extraction kit “Purescript” (Genta-System, Minneapolis, Minn) following the manufacturer’s instructions. Northern blot analysis was performed following published techniques. \(^{19} \) Briefly, 10 \( \mu \text{g} \) of RNA was size fractionated on 1.2% agarose gels and transferred to synthetic membranes (Hybond N, Amersham). Hybridization was performed with \(^{32} \text{P}-\text{labeled synthetic specific oligo-nucleotide probes (3’ end). Bound radioactivity was visualized by exposure to XAR-5. As an internal control, \( \beta \)-actin probe was used. The sequences of the oligonucleotide probes were as follows: \( \beta \)-actin (34-mer): 5’-GGCTGGGATTGTGAAAGGTCTCCCAAACATG ATCTCGG-3’; ICAM-1 (30-mer): 5’-CCATACAGGACACGAGCTCCCCTCTGATCTC TG-3’; \(^{19} \) CD18 (27-mer): 5’-GGCTGAAGCCTGAGCTCAGTCTGG-3’; \(^{20} \) and \( \beta \)-actin (27-mer): 5’-TGCTGTCAAG-GAGCAGTGATGGCTTCTC-3’.
Northern blot results were quantified by densitometry as described. \(^{18} \)

**Adhesion Assay**
In adhesion experiments, HUVECs were cultured in 24-well plates in media containing 10% FCS. HUVECs were incubated with statins (simvastatin, cerivastatin, atorvastatin, each 1 and 10 \( \mu \text{mol/L} \)) for 6 hours and then exposed to TNF-\( \alpha \) (10 ng/mL) for additional 6 hours. Cells were then detached with trypsin/EDTA \( ^{17} \) (Sigma) and prepared for flow cytometry or Northern blotting. During Simvastatin Treatment

**Expression of CAM-Specific mRNA in PBMCs**
Influence of Simvastatin on Serum Lipid Parameters
In line with published results, simvastatin treatment led to a significant decrease in plasma cholesterol levels as well as a decrease in LDL (Table). As expected, the lipid-lowering effect was more pronounced in patients receiving 40 mg daily compared with those receiving 20 mg simvastatin per day (\( P<0.001 \)) (Table).

Figure 1. Effect of simvastatin treatment on expression of CAM mRNA in PBMCs. PBMCs were isolated from HPs before (baseline), 6 weeks, and 6 months after treatment with simvastatin. Shown are the Northern blot experiments performed on PBMCs obtained in 5 representative patients (Pat. 1 to Pat. 5). As visible, treatment with simvastatin (20 mg: patients 1 to 2; 40 mg: patients 3 to 5) for 6 weeks and 6 months resulted in a decrease in expression of mRNA specific for CD54, CD18, and CD11a compared with baseline levels. The \( \beta \)-actin control is also shown.

**Results**

**Statistical Analysis**
Statistical analysis was performed by using SPSS 10.0.7 statistical software (SPSS Inc., Chicago, Ill). As a first step numeric values were analyzed for presence of normal distribution. In cases of normal distribution values were stated as mean±standard deviation. The significance of any differences concerning these values was evaluated by a paired \( t \) test. For values, that are not randomly distributed we stated the mean and the interquartile range in parenthesis. The significance of those values was evaluated by the nonparametric Wilcoxon rank sum test and the Mann–Whitney \( U \) test. Differences in proportions were assessed by using the Chi-square statistic. To evaluate the difference of the influence of simvastatin on plasma lipid levels and CAMs and CDs, we calculated the mean relative differences of all values and used the paired \( t \) test. \( P<0.05 \) was considered as significant for all tests.

Figure 1. Effect of simvastatin treatment on expression of CAM mRNA in PBMCs. PBMCs were isolated from HPs before (baseline), 6 weeks, and 6 months after treatment with simvastatin. Shown are the Northern blot experiments performed on PBMCs obtained in 5 representative patients (Pat. 1 to Pat. 5). As visible, treatment with simvastatin (20 mg: patients 1 to 2; 40 mg: patients 3 to 5) for 6 weeks and 6 months resulted in a decrease in expression of mRNA specific for CD54, CD18, and CD11a compared with baseline levels. The \( \beta \)-actin control is also shown.

**Expression of CAM-Specific mRNA in PBMCs During Simvastatin Treatment**
As assessed by Northern blotting, transcripts specific for CD54, CD11a, and CD18 were detectable in circulating PBMCs in HPs (Figure 1). As determined by densitometry, PBMCs obtained after 6 weeks or 6 months of treatment with simvastatin contained significantly lower levels of CD54-, CD18-, and CD11a-mRNA compared with respective pre-treatment levels (\( P<0.05 \); see online Figure I, which can be accessed at http://atvb.ahajournals.org). PBMCs obtained from HNDs expressed low detectable amounts of CAM mRNA in our Northern blotting experiments with no changes in a 6-week period (Figure II).
Simvastatin Treatment Decreases the Expression of Membrane-Bound CAMs on CD14+ Monocytes in Hypercholesterolemic Patients

The change of the amount of CAMs on the surface (ie, change of geometric mean) of CD14+ monocytes was measured by flow cytometry in both treatment groups (Figure 2) and in HND (Figure III). Membrane-bound CAMs (CD54, CD18 and CD11a) were significantly higher in CD14+ monocytes in untreated HPs when compared with HNDs. Treatment with simvastatin decreased the expression of these receptors significantly after 6 weeks as well as after 6 months on the surface of CD14+ monocytes of HP (P<0.001; Figure 2) whereas there were no changes in untreated HNDs within a period of 6 weeks (P>0.05; Figure III). There were no significant differences concerning the change of CAMs between both treatment groups (40 versus 20 mg/day).

The correlation between the mean relative reduction of cholesterol and CAMs is shown in Figure IV. Mean reduction of CD18 was significantly higher at both time points (each P<0.001). However, the mean relative reduction of CD11a and CD54 was significantly higher only after 6 months (each P<0.001) whereas we detected no significant difference compared with the corresponding data of plasma cholesterol after 6 weeks (CD11a: P=0.87, CD54: P=0.8). The change of expression of CAMs on PBMCs of a typical patient treated with 40 mg of simvastatin per day is shown in histograms (Figure V).

In Vitro Effects of Statins on Expression of CD54-, CD11a-, and CD18-mRNA in Cultured Human ECs and PBMCs Obtained From HNDs

As assessed by Northern blotting, unstimulated HUVECs and PBMCs expressed low amounts of mRNA specific for CD54, CD11a, and CD18. Exposure to TNF-α (10 ng/mL) resulted in upregulation of all three CAMs in both HUVECs and PBMCs (Figure 3). Incubation of these cells with simvastatin, atorvastatin, or cerivastatin (each 10 μmol/L) for 8, 12 or 18 hours was found to counteract TNF-α-induced expression of CAMs (Figure 4). There were no differences concerning the modulation of TNF-induced CAMs when the

In Vitro Effects of Statins on ICAM-1 and LFA-1 on the Surface of TNF-α–Activated HUVECs

Unstimulated HUVECs were found to express baseline levels of CD54, CD11a, and CD18 (Figure 4). Stimulation of HUVECs with TNF-α (10 ng/mL) resulted in an upregulation of all three CAMs on HUVECs (Figure 4). Incubation with simvastatin, atorvastatin, or cerivastatin (each 10 μmol/L) for 8, 12 or 18 hours was found to counteract TNF-α-induced expression of CAMs (Figure 4). There were no differences concerning the modulation of TNF-induced CAMs when the
effects of the three statins were compared. The addition of MVA (100 μmol/L) reversed the inhibitory statin effects on TNF-α-stimulated HUVECs (n=3; Figure VI).

Statins Reduce the Adherence of TNF-α-Stimulated HUVECs to PBMCs

HUVECs were exposed to TNF-α with or without statins (1 or 10 μmol/L); subsequently, the adhesion assay was performed. The number of attached PBMCs to HUVECs increased after the stimulation of HUVECs with TNF-α as compared with untreated HUVECs. Statins in both concentrations reduced the number of attached PBMCs to TNF-α-stimulated HUVECs (Figure 5). There were no differences between all statins tested.

Discussion

Multiple clinical studies as well as data from recent experimental studies revealed that statins have additional activity behind their serum cholesterol-lowering effect and that statins alter other biological processes in the vessel wall.23 In the clinical part of our study, we demonstrated for the first time that the membrane-bound adhesion molecules ICAM-1 and its ligand LFA-1 on the surface of monocytes obtained from HPs are increased when compared with HNDs. Simvastatin treatment caused significant reduction of the expression of these molecules on the surface of monocytes in HP after 6 weeks as well as after 6 months.

The earliest events in atherogenesis are activation of vascular endothelial cells, followed by adhesion, and migration of monocytes and T lymphocytes into the subendothelial space.23 Among these, the foam cell conversion of monocyte-derived macrophages by modified LDL is known to play a key role.24,25 LDL upregulation activates vascular cells, such as endothelial cells, macrophages, and smooth muscle cells, and induces several proatherogenic genes, including adhesion molecules, such as ICAM-1.26 In line with these data from in vitro studies, we showed that PBMCs from HPs express higher amounts of ICAM-1 as well as its ligand LFA-1 on both protein and mRNA levels. Statin treatment caused a significant reduction of cholesterol, which alone would cause a decrease of CAMs in circulating PBMCs. We could reveal that the reduction of CAMs, especially CD18, was significantly stronger than the reduction of cholesterol. This effect can be explained by the observation that simvastatin treatment leads to a decrease of serum levels of pro-inflammatory cytokines interleukin-6, interleukin-8, and membrane cofactor protein (MCP)-1,18 which additionally exerts an inactivation of leukocytes. Consequently, in the in vitro experiments we confirmed that statins are capable to directly reduce the membrane bound CAMs in TNF-α-stimulated PBMCs and ECs by the decrease of the mRNA expression of these molecules by statins.

Weitz-Schmidt et al27 have recently shown, that adhesion molecules CD18/CD11a possess a l-binding domain. This domain is the binding site of lovastatin. They revealed that the inhibition of interactions between CD18/CD11a and ICAM-1 by statins correlates with the binding of these drugs to the allosteric site of the LFA-1 and results in decreased leukocyte adhesion to ICAM-1. In our clinical study, we stated that simvastatin treatment of HP results in a decreased mRNA expression of LFA-1 and ICAM-1 in their circulating monocytes. Additionally, data from our in vitro experiments revealed that statins are able to directly reduce the expression of mRNA of CAMs in TNF-α-activated HUVECs and PBMCs. Furthermore, in the in vitro experiments we showed that statins did not influence the amount of surface bound CAMs in unstimulated PBMCs and HUVECs as assessed by flow

![Figure 4](image1.png)  
**Figure 4.** Effect of statins on the expression of CAMs on the surface of TNF-α-stimulated HUVECs. HUVECs were cultured to subconfluency and were then stimulated with/without statins (each 10 μmol/L) and/or TNF-α (10 ng/mL). Incubation with atorvastatin (Ator), cerivastatin (Ceri), and simvastatin (Sim) for 8, 12, and 18 h led to a reduction of expression of CD54, CD11a, and CD18 on the surface of TNF-activated HUVECs at various time points (P<0.05, n=3).

![Figure 5](image2.png)  
**Figure 5.** Statins reduce the adhesion of monocytes to TNF-α-activated HUVECs. HUVECs were stimulated with or without statins (1 or 10 μmol/L) for 12 hours. Untreated PBMCs from healthy volunteers were added to treated HUVECs monolayers and analyzed by adhesion assay. TNF-α stimulation of HUVECs increased the number of adhered PBMCs. Treatment of TNF-α-activated HUVECs with both concentrations of statins led to a decreased adherence of monocytes to HUVECs (P<0.05, statins vs TNF-α-stimulated HUVECs). Data are expressed as the mean±SD (n=3).
cytometry (data not shown). Hence, all these results strengthen the hypothesis that statins might not block activity of LFA-1 merely by attachment of the l-domain but also by reduction of the expression of transcripts of LFA-1 and its ligand CD54. The reduction of mRNA is followed by the decreased amount of these CAMs on the surface of ECs as well as on PBMCs.

TNF-α is a pivotal inflammatory cytokine increased in the serum of patients with hypercholesterolemia.28 In our in vitro studies, we showed that statins are able to inhibit the upregulation of expression of mRNA of ICAM-1 as well as CD11a/CD18 by TNF-α in cultured HUVECs and PBMCs. Statins block the formation of MVA with consecutive depletions of statins vary between 0.5 to 5 μmol/L. Hence, the pharmacokinetic data have shown that serum concentrations used in the in vitro studies are in this range or slightly above these serum concentrations. Finally, our data point to an important novel effect of statins that may contribute to their anti-inflammatory and antiatherosclerotic activities.

Acknowledgments

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References


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Legends of online Figures

**Online Figure I.** Quantitative analysis of Northern blots.

Figure I shows the densitometric evaluation of Northern Blotting results obtained in all patients examined (n=20: 20mg [n=10] vs. 40mg [n=10]). Data are shown in Whiskers box plots. Values obtained after 6 weeks (wk) and 6 months (mo) of treatment are expressed as percentage of baseline levels (=100%). The median values are also shown (horizontal bars).

**Online Figure II.** CAM-mRNA expression in PBMC of HND.

mRNA were isolated from PBMC obtained from HND at two different time points (baseline and 6 weeks) and subjected to Northern blot analysis. As shown in figure II there were only very low amounts of CAM specific mRNA detected in PBMC of healthy donors. There were no visible difference in expression of mRNA at both time points.

**Online Figure III.** Expression of CAMs on the surface of CD14+ monocytes of HND.

PBMC were obtained from healthy donors (n=20) at two different time points (baseline and 6 weeks) and were analyzed by flow cytometry. As shown in figure V There were no significant changes at both time points due to the expression of CAMs on the surface of CD14+ monocytes.
Online Figure IV. Correlation between CAM expression and serum cholesterol levels. Figure V shows the line chart indicating the mean relative plasma cholesterol and the change of the expression of CAMs after simvastatin treatment compared to baseline values. The reduction of CD18 was significantly higher at both time points (each p<0.001). However, the mean relative reduction of CD11a and CD54 was significantly higher only after 6 months (each p<0.001).

Online Figure V. Change of expression of CAMs on the surface of PBMC of a representative patient. This figure shows histograms of a typical patient treated with 40mg/d before, 6 weeks and 6 months after therapy.

Online Figure VI. MVA reverses the effect of statins on CAMs expression. HUVEC were stimulated with/without statins (each 10μM) and/or TNF-α (10ng/ml) for 12 hours. Statin treatment led to a reduction of CAM expression. Addition of mevalonate (100μM) reversed this inhibitory effect of statins on TNF-α stimulated cells as assessed by flow cytometry (p<0.05). Data is expressed as the mean±SD (n=3).
Online Figure I
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CD18

Percent of baseline

baseline

6 wk

6 mo

20 mg

40 mg

6 wk

6 mo
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Online Figure II
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Online Figure III
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Online Figure V