Simvastatin Reduces Expression of Cytokines Interleukin-6, Interleukin-8, and Monocyte Chemoattractant Protein-1 in Circulating Monocytes From Hypercholesterolemic Patients

Abdolreza Rezaie-Majd, Thomas Maca, Robert A. Bucek, Peter Valent, Michael R. Müller, Peter Husslein, Ahmad Kashanipour, Erich Minar, Mehrdad Baghestanian

Objective—A number of studies have shown that statins decrease morbidity and mortality in patients with cardiovascular diseases. The anti-inflammatory effects of statins have recently been implicated in the clinical benefit that can be obtained in the treatment of atherosclerosis. Little is known about the mechanisms by which statins counteract inflammation.

Methods and Results—In this study, we asked whether simvastatin can influence in vitro and in vivo production of the proinflammatory cytokines interleukin (IL)-6, IL-8, and monocyte chemoattractant protein-1. A total of 107 hypercholesterolemic patients were treated with simvastatin. As measured by ELISA, serum levels of cytokines significantly decreased after 6 weeks of treatment ($P<0.05$). Furthermore, simvastatin decreased the expression of IL-6, IL-8, and monocyte chemoattractant protein-1 mRNA in peripheral blood mononuclear cells. Similar results were obtained in vitro by using cultured human umbilical vein endothelial cells and peripheral blood mononuclear cells from healthy normolipemic donors. Exposure to simvastatin, atorvastatin, or cerivastatin caused downregulation of the expression of cytokine mRNA in a time- and dose-dependent manner. Furthermore, all statins tested were able to reduce the concentrations of cytokines in cellular and extracellular fractions of human umbilical vein endothelial cells ($P<0.05$).

Conclusions—Our data show that simvastatin is anti-inflammatory through the downregulation of cytokines in the endothelium and leukocytes. These effects may explain some of the clinical benefits of these drugs in the treatment of atherosclerosis. (Arterioscler Thromb Vasc Biol. 2002;22:1194-1199.)

Key Words: atherosclerosis ■ inflammation ■ statins ■ chemokine ■ hypercholesterolemia

Statins are potent inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A reductase. These drugs are capable of lowering the serum levels of cholesterol and are successfully used to treat hypercholesterolemia and atherosclerosis.$^1,2$ Moreover, the ability of statins to reduce the mortality and morbidity of cardiovascular diseases has been ascribed not only to their cholesterol-lowering activities but also to a number of additional effects, including improved endothelial cell function,$^3$ enhanced fibrinolysis,$^4$ and antithrombotic activity.$^5$ In addition, a number of important anti-inflammatory effects of statins have been reported.$^6$

Recent studies have shown that atherosclerosis shares a number of features with inflammatory responses of the (micro)vasculature.$^7$ The recruitment and accumulation of leukocytes, which are common in inflammatory reactions and in atherosclerosis, require a gradient of chemotactic factors, including plaque.$^8$ Chemokines are a family of structurally related chemotactic cytokines that are involved in leukocyte trafficking and activation and that modulate granulocyte and endothelial cell functions and smooth muscle cell (SMC) proliferation.$^9,10$ An important mediator of monocyte recruitment into the vascular wall is monocyte chemoattractant protein (MCP)-1, which is secreted by vascular cells and activated leukocytes. A number of studies have shown that atherosclerotic lesions (including plaques) contain increased amounts of MCP-1, suggesting a potential role for this molecule in the pathogenesis of atherosclerosis.$^10$ Interleukin (IL)-8 is a well-known regulator of the migration of neutrophils and vascular endothelial cells. Recent studies have shown that increased levels of IL-8 are expressed in atherosclerotic plaques.$^11$ IL-6 is a pleiotropic cytokine and a central mediator of the acute-phase response, with a broad range of effects on diverse immune cells,$^12$ which is produced by vascular endothelial cells, SMCs, and leukocytes. IL-6 is also
Simvastatin and Serum Levels of Cytokines

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Simvastatin Dosage-Group

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Risk factors of atherosclerosis, n

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Tg indicates triglyceride; PAOD, peripheral arterial obstructive disease; CHD, coronary heart disease; CAOD, cerebral arterial obstructive disease.

There were no significant differences between both groups according to the patients' characteristics.

expressed in human atherosclerotic lesions and is elevated in patients with cardiovascular disease.

In patients treated with statins, a marked decrease in inflammation-associated parameters such as the C-reactive protein and adhesion molecules has been described. Recent publications have shown the inhibitory effects of statins on chemokine production in vitro and in vivo, but little is known about the effect of statins on proinflammatory cytokines in hypercholesterolemic patients. In the present study, we investigated the influence of simvastatin on serum levels and mRNA expression of MCP-1, IL-8, and IL-6 in blood monocytes of hypercholesterolemic patients. Furthermore, we studied the in vitro effects of the statins on cytokine production in cultured human endothelial cells and isolated leukocytes.

Methods

Patients

Between November 1999 and November 2000, a total of 107 hypercholesterolemic patients received routine simvastatin treatment (20 mg/d PO, n=52; 40 mg/d PO, n=55). The dose of simvastatin was adjusted to the actual cholesterol level. In all patients, blood samples were taken the first day of treatment and after 6 weeks and 6 months of treatment with simvastatin. All patients gave informed consent before blood donation. The patients' baseline characteristics and their laboratory parameters are shown in the Table. The medication for each individual was not changed during the investigation period of 6 months.

Reagents

The phycoerythrin-conjugated monoclonal antibody (mAb) 5D3-F7 (IgG1) against MCP-1, anti–IL-8 mAb G265-8 (IgG2a), and anti–IL-6 mAb MQ2-6A3 (IgG2b) were purchased from Becton Dickinson.

RPMI 1640 medium, gentamycin, amphotericin B, and FCS were purchased from Sera Laboratory; glutamine, penicillin, and streptomycin were from GIBCO Life Technologies; collagenase type IA and Ficol were from Sebak; trypsin/EDTA and gelatin were from Sigma Chemical Co; endothelial basal medium and recombinant endothelial growth factor were from PromoCell Co; and oligonucleotides were from MWG Biotech. Simvastatin was kindly provided by Merck, Sharp & Dohme; cerivastatin, by Bayer (Wuppertal, Germany); and atorvastatin, by Pfizer (Karlsruhe, Germany). Mevalonic acid (MVA) was purchased from Sigma.

Measurement of Cytokine Concentration by ELISA

Serum was separated by centrifugation of the blood samples from hypercholesterolemic patients at week 0 (baseline) and at 6 weeks and 6 months after the start of therapy with simvastatin and was quantified by ELISA. Commercial ELISA assays detecting MCP-1, IL-8 (both R&D Systems), and IL-6 (Becton Dickinson) were applied. All serum samples for each individual were stored at −70°C and were measured at the same time by the same ELISA to avoid variation of assay conditions. Detection limits of ELISA assays were as follows: MCP-1, 5 to 2000 pg/mL; IL-8, 1 to 5000 pg/mL; and IL-6, 0.15 to 10 pg/mL.

Isolation of Peripheral Blood Monocytes From Hypercholesterolemic Patients and Healthy Volunteers

Peripheral blood mononuclear cells (PBMCs) from hypercholesterolemic patients (n=107) obtained before and after 6 weeks and 6 months of treatment were separated by Ficoll gradient centrifugation as described. Cell viability was always >95%, as measured by trypan blue exclusion. To investigate the change of transcripts of proinflammatory cytokines in monocytes of patients treated with simvastatin, mRNA was extracted from PBMCs from hypercholesterolemic patients immediately after Ficoll isolation, as described below. For in vitro studies, PBMCs from healthy volunteers were processed as described above.

Isolation and Culture of Primary HUVECs

Umbilical cords were obtained at delivery after informed consent had been given by mothers. Human umbilical vascular endothelial cells (HUVECs) were isolated from umbilical veins by using collagenase type IA according to a standard protocol. HUVECs were cultured to confluence in endothelial basal medium containing 10% FCS and passed 2 times. To assess chemokine/cytokine release, HUVECs were cultured in 6-well plates and incubated with various concentrations of statins (0.1 to 50 μmol/L) in RPMI 1640 medium for 12 or 24 hours. Conditioned media were collected and stored at −70°C. To evaluate the effect of statins on MCP-1, IL-8, and IL-6 mRNA expression, HUVECs were incubated for 4, 8, 12, and 24 hours in medium in the presence or absence of statins (10 μmol/L).

Isolation and Culture of PBMCs for In Vitro Studies

To evaluate the influence of statins on MCP-1, IL-8, and IL-6 mRNA expression, monocytes from 3 healthy volunteers were isolated by Ficoll gradient centrifugation as described before. Isolated PBMCs (2×10⁶ cells per milliliter) were resuspended in conditioned medium and cultured in 75-cm² flasks for 24 hours. Thereafter, statins (10 μmol/L) were added for 6 hours, followed by tumor necrosis factor (TNF)-α (10 ng/mL) stimulation for an additional 6 hours before being subjected to RNA isolation.

RNA Extraction and Northern Blot Analysis

Total RNA was extracted from PBMCs obtained from hypercholesterolemic patients before and after treatment (n=20), from PBMCs from healthy normolipemic donors, and from cultured treated and untreated HUVECs with the use of an RNA extraction kit (Purescript, Gentra-System) according to the manufacturer's instructions.
Northern blot analysis was carried out according to published techniques with the use of \(^{32}P\)-labeled synthetic specific oligonucleotide probes (3' end).\(^{16}\) The sequences of the oligonucleotide probes were as follows: β-actin (34-mer), 5'-GGCTGGGTGTTGAGGTCTCAA CATGATCTGG-3'; MCP-1 (27-mer), 5'-GGGTTGCTTGTCCAGGGT- GGTCCAT GG-3'; IL-8 (27-mer), 5'-GGGGTGGAAAGTGTTGAGATGATGTCCT-3'; and IL-6 (30-mer), 5'-GGACTTTGATCATTCTGCACAGCTCCTGG-3'.

Flow Cytometry Staining for Intracellular Antigens

HUVECs were incubated in the absence or presence of statins (1 or 10 μmol/L) and were then harvested by using trypsin/EDTA. Intracellular staining was performed by using a Cytofix/Cytoperm Plus kit (PharMingen) and mAbs against MCP-1, IL-8, and IL-6. In the first step, HUVECs were permeabilized by adding 250 μL Cytofix/Cytoperm solution. After 20 minutes of incubation at 4°C, cells were washed with Perm/Wash solution (PharMingen). Permeabilized cells were resuspended in 50 μL of Perm/Wash solution containing fluorochrome-conjugated anti-cytokine antibodies or isotype-matched control antibodies and incubated for 25 minutes at 4°C. HUVECs were then washed with 1× Perm/Wash solution and resuspended in 250 μL staining buffer before flow cytometry analysis was performed.

Statistical Analysis

Statistical analysis was performed by using SPSS 10.0.7 statistical software (SPSS Inc). As a first step, numeric values were analyzed for the presence of normal distribution. In cases of normal distribution, values are stated as mean ± SD. The significance of any differences concerning these values was evaluated by paired \(t\) test. For values that are not randomly distributed, we stated mean, with the interquartile range (IQR) in parentheses. Significance of those values was evaluated by a nonparametric Wilcoxon rank test and Mann-Whitney \(U\) test. Differences in proportions were assessed by the \( \chi^2 \) statistic. To evaluate the difference of the influence of simvastatin on plasma lipid levels and cytokines and chemokines, we calculated the mean relative differences of all values and used the paired \(t\) test. A value of \(P < 0.05\) was considered significant for all tests.

Results

Influence of Simvastatin on Serum Lipid Parameters

In line with published data, simvastatin treatment led to a significant reduction of plasma cholesterol levels (baseline, 254.8±34.8 mg/dL; 6 weeks, 191.1±38.7 mg/dL; and 6 months, 167.7±37.5 mg/dL; \(P < 0.001\) for both periods). As expected, the relative plasma cholesterol reduction was significantly stronger in the 40-mg group compared with the 20-mg group (\(P < 0.001\)).

Simvastatin Treatment Decreases Serum Levels of Proinflammatory Cytokines MCP-1, IL-8, and IL-6 in Hypercholesterolemic Patients

There were no significant differences between the 2 simvastatin treatment groups concerning the baseline serum levels of all 3 peptide cytokines. Treatment of patients (n = 107) with simvastatin led to a significant decrease of IL-6 (baseline, median 8.1 pg/mL [IQR 9.0]; 6 weeks, 5.2 pg/mL [IQR 5.0]; and 6 months, 3.8 pg/mL [IQR 5.4]; \(P < 0.001\)). Corresponding results were observed concerning IL-8 (baseline, median 11.0 pg/mL [IQR 7.0]; 6 weeks, 3.5 pg/mL [IQR 4.6]; and 6 months, 2.4 pg/mL [IQR 2.4]; \(P < 0.001\)) and MCP-1 (baseline, median 550.4 pg/mL [IQR 292.9]; 6 weeks, 379.2 pg/mL [IQR 98.4]; and 6 months, 278.2 pg/mL [IQR 63.6]; \(P < 0.001\)). Figure 1A shows the change of serum levels of MCP-1, IL-8, and IL-6 in the 20-mg and 40-mg treatment groups. Subgroup analysis revealed that there was no significant difference between both groups concerning the change of measured parameters after 6 weeks and after 6 months. The mean relative reduction of IL-6, IL-8, and MCP-1 after 6 weeks and 6 months was significantly higher (all \(P < 0.001\)) compared with the relative reduction of plasma cholesterol for the whole study population. For results in detail, see Figure 1B.

Effect of Simvastatin Therapy on Cytokine Transcript Levels in PBMCs of Hypercholesterolemic Patients

The expression of mRNA specific for IL-6, IL-8, and MCP-1 in PBMCs from 20 randomly selected patients (10 from each group) was analyzed by Northern blotting and quantified by densitometry. Signals were normalized to β-actin. In line with previous data, significant levels of transcripts for MCP-1, IL-8, and IL-6 were detectable in circulating PBMCs from hypercholesterolemic patients. Figure 2A shows results from 5 typical patients of both groups. Compared with pretreatment levels, PBMCs obtained after 6 weeks or 6 months of treatment with simvastatin were found to contain markedly lower levels of mRNA for MCP-1 (baseline, 100%; 6 weeks, 74.5±12.6%; and 6 months, 29.8±13.3%), IL-8 (baseline, 100%; 6 weeks, 49.9±19.3%; and 6 months, 29.3±9.8%), and IL-6 (baseline, 100%; 6 weeks, 15.4±2.9; and 6 months, 11.3±3.4%) as determined by densitometry with β-actin–normalized signals obtained from 20 patients (Figure 2B). The data indicate that simvastatin treatment for...
6 hours inhibits the production of proinflammatory cytokines in PBMCs of hypercholesterolemic patients.

**In Vitro Effects of Statins on Expression of MCP-1, IL-8, and IL-6 mRNA in Cultured HUVECs and PBMCs Obtained From Healthy Donors**

Northern blot analyses were carried out by using HUVECs as well as PBMCs from healthy normolipemic donors. HUVECs were exposed to simvastatin, atorvastatin, and cerivastatin (each 10 μmol/L). The exposure of HUVECs to statins resulted in a time-dependent decrease in cytokine mRNA expression (Figure 3). Maximum inhibition occurred after 12 hours of incubation. There was no significant difference when the effects of the various statins on cytokine production in HUVECs were compared. Unstimulated PBMCs contained only low baseline levels of MCP-1, IL-8, and IL-6 mRNA (please see online Figure I, available at http://atvb.ahajournals.org). When PBMCs were stimulated with TNF-α (10 ng/mL) for 6 hours, however, significant amounts of cytokine mRNA was detectable by Northern blotting (please see online Figure I). The addition of simvastatin, atorvastatin, or cerivastatin (each 10 μmol/L) to these cells was found to suppress the TNF-α–induced transcription of cytokine mRNA in PBMCs. Again, no significant differences were found when the inhibitory effects of the various statins were compared. These data show that various statins are capable of inhibiting the production of proinflammatory/proatherogenic cytokines in HUVECs and PBMCs. To confirm the specificity of the effects of statin, experiments using MVA were conducted. As expected, the addition of MVA (100 μmol/L) reversed the inhibitory effects of the statins in unstimulated HUVECs as well as in TNF-α–activated PBMCs (please see online Figure I). These data also confirmed that the statin-induced effects are regulated through signal transduction pathways involving the isoprenoid/MVA pathway.

**In Vitro Effect of Statins on Intracellular MCP-1, IL-8, and IL-6 in HUVECs**

To study the effects of the statins on cytokine expression at the protein level, cytokine protein levels were measured in HUVECs by ELISA (supernatants) and flow cytometry (cytoplasmic staining). HUVECs were found to express and secrete substantial amounts of MCP-1, IL-8, and IL-6 (please see online Figure II, available at http://atvb.ahajournals.org). Incubation of HUVECs with simvastatin, atorvastatin, or cerivastatin for 12 or 24 hours was followed by a significant decrease in the expression and secretion of all 3 cytokines examined. In fact, all statins tested reduced the levels of MCP-1, IL-8, and IL-6 in the conditioned media of HUVECs.
in a dose-dependent manner at 12 and 24 hours. Maximum inhibition occurred at a dose of 50 μmol/L (please see online Figure II). There was no significant difference when the effects of the various statins were compared.

As assessed by flow cytometry, MCP-1, IL-8, and IL-6 were expressed in untreated HUVECs. However, when the HUVECs were exposed to simvastatin, atorvastatin, or cerivastatin (1 or 10 μmol/L) for 16 hours, a significant decrease in cytokine content could be detected by flow cytometry. As shown in Figure III (available at http://atvb.ahajournals.org), all 3 statins showed a similar inhibitory effect on cytokine expression, with no significant differences between 1 and 10 μmol/L.

Discussion

Recent data suggest that apart from their lipid-lowering effects, statins can act as anti-inflammatory agents. Little is known about the mechanism(s) that underlies the anti-inflammatory effects of statins. In the present study, we demonstrated that simvastatin treatment for 6 weeks resulted in a significant decrease of proinflammatory cytokines MCP-1, IL-8, and IL-6 measured in the sera of hypercholesterolemic patients. In the same patients, simvastatin treatment resulted in a decreased expression of MCP-1, IL-8, and IL-6 mRNA in circulating PBMCs compared with pretreatment values. There were no significant differences between the 2 treatment groups (20 versus 40 mg) on cytokine production. Pharmacokinetic data have shown that serum concentrations of statins vary between 0.5 to 5 μmol/L. Hence, the concentrations used in the in vitro studies are in this range or slightly above these serum concentrations. In all, our data point to an important novel pharmacological effect of statins that may contribute to their anti-inflammatory and antiatherosclerotic activity.

An interesting aspect of the present study was that cytokine levels decreased after 6 weeks of treatment with simvastatin and that they showed an additional decrease after 6 months compared with 6 weeks. By contrast, cholesterol levels decreased after 6 weeks and then showed only a slight additional decrease at 6 months. The reason for this difference in the time kinetics of cytokine versus cholesterol levels is not known. An explanation could be that molecular mechanisms underlying the statin-induced inhibition of cytokine production differ from those responsible for statin-induced inhibition of cholesterol synthesis. In fact, statins may influence cell functions through diverse receptors and multiple pathways. An alternative explanation would be that the cytokines analyzed were expressed only in distinct types of cells (ie, endothelial cells or blood leukocytes) and, therefore, were more sensitive to long-term inhibitory effects of statins compared with hepatic cholesterol synthesis.

Statins are 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors that block the formation of MVA with consequential depletion of isoprenoid compounds, including major regulators of cell growth and cellular signaling. In the present study, the statin-induced downregulation of cytokine production in HUVECs and PBMCs was reversed by coinubcation with MVA. This observation points to a specific statin effect and a role of the MVA-isoprenoid pathway in cytokine production. Recent data obtained with SMCs suggest that statins inhibit the activation of nuclear factor-κB, a major nuclear factor that regulates the expression of diverse cytokines, including MCP-1, IL-8, and IL-6. On the basis of the above findings, it is also tempting to speculate that the statin-induced downregulation of cytokine production in HUVECs and PBMCs may involve, in part, a loss of functional nuclear factor-κB. However, this hypothesis was not formally proven in the present study. Statins may act through multiple receptors and pathways in their target cells.

A number of recent studies suggest that statins act on diverse types of mesenchymal cells, including blood leukocytes, SMCs, and vascular endothelial cells. In the present study, we have analyzed the in vitro effects of statins on isolated PBMCs from healthy normolipemic donors and HUVECs. In both types of cells, the statins were found to inhibit the production of peptide cytokines. In particular, the statins inhibited the constitutive expression of MCP-1, IL-8, and IL-6 mRNA in HUVECs as well as the TNF-α-induced expression of MCP-1, IL-8, and IL-6 mRNA in PBMCs. These data suggest that the in vivo effects of simvastatin on cytokine levels in our hypercholesterolemic patients were caused by downregulation of cytokine production in vascular cells and leukocytes.

In the present study, we have applied 3 different lipophilic statins and compared their effects on in vitro cytokine production in HUVECs and leukocytes. However, the 3 statins showed comparable effects on cytokine mRNA and protein expression in HUVEC and also comparable effects on cytokine mRNA expression in PBMCs. From the data, it seems reasonable to speculate that the anti-inflammatory effects of various statins in vivo are exerted at least in part through inhibition of the production of MCP-1, IL-8, and IL-6 in leukocytes and endothelial cells.

A number of factors, including plasma lipid and local factors in the vascular wall, contribute to the pathophysiology and development of atherosclerosis. A number of studies have shown that the atherosclerotic syndrome has several features in common with inflammatory tissue reactions. With regard to chemotactic and proinflammatory cytokines, a number of chemokines and interleukins, including MCP-1, IL-8, and IL-6, have been detected in human atherosclerotic lesions. In addition, increased levels of cytokines have been detected in sera and circulating blood leukocytes in hypercholesterolemic patients compared with healthy subjects. Correspondingly, in the present study, significant amounts of MCP-1, IL-8, and IL-6 mRNA were detected in PBMCs from hypercholesterolemic patients, whereas only low amounts of mRNA were found in PBMCs from healthy subjects. Therefore, the notion that statins are capable of downregulating the production and expression of proinflammatory and chemotactic cytokines in hypercholesterolemic patients as well as in vitro in vascular cells and blood leukocytes may be of pathophysiological and clinical significance. Likewise, it is tempting to speculate that statins can inhibit the chemokine-dependent accumulation of macrophages (and other cells, ie, fibroblasts/lymphocytes) in areas of atherosclerosis by blocking the synthesis of respective chemokines (MCP-1 and IL-8) in vascular and perivascular tissues.
cells, thus accomplishing their lipid-lowering activities. In line with this concept, several clinical studies have demonstrated that statins are able to reduce the incidence of coronary heart and cerebrovascular diseases.\textsuperscript{1,2}

In the present study, we have shown that simvastatin decreases the serum level of IL-6 in hypercholesterolemic patients. Additionally, in vitro, statins are able to reduce the transcript level of IL-6 in unstimulated HUVECs and TNF-\(\alpha\)-stimulated PBMCs and to diminish the amount of IL-6 in the supernatant of HUVECs. IL-6 is known to have profound effects on bone metabolism by regulating osteoclast and osteoblast development and function.\textsuperscript{12} Recent data from in vivo experiments have shown that the osteoclast-activating effect of this cytokine (F. Parhami, unpublished data, 2002). More recently, Scheidt-Nave et al\textsuperscript{26} revealed that the serum level of IL-6 was the single most important predictor of postmenopausal bone loss and might also be a predictor of fracture risk. The results of the present study might provide an additional explanation for the antiosteooporotic/pro-osteogenetic activity of statins in clinical trials.

Together, our data show that simvastatin treatment leads to a decreased expression of mRNA of peptide cytokines in circulating PBMCs and of serum MCP-1, IL-8, and IL-6 levels in hypercholesterolemic patients. The inhibition of cytokine production in vascular endothelial cells, as shown in vitro, may also ascribe to these statin effects. Statin-induced inhibition of cytokine production may play an important role in the pharmacological and clinical effects of statins seen in cardiovascular diseases.

Acknowledgments

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References

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