

Atorvastatin Decreases Vascular Endothelial Growth Factor in Patients With Coronary Artery Disease

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OBJECTIVES	The aim of this study was to test a possible influence of atorvastatin on the production of vascular endothelial growth factor (VEGF) in patients with coronary artery disease (CAD) and in vitro.
BACKGROUND	Vascular endothelial growth factor is suggested to be involved in the growth of atherosclerotic plaque by inducing its neovascularization. Hepatic hydroxymethyl glutaryl-coenzyme A reductase inhibitors (statins) are known to have atheroprotective effects beyond lipid lowering.
METHODS	Blood was collected from 14 male hypercholesterolemic patients with angiographically confirmed CAD at baseline and after two months of atorvastatin therapy (20 mg/d) and from eight male control patients. In an ex vivo assay, human coronary artery smooth muscle cells (HCASMC) were incubated with the patient plasma collected before and after atorvastatin therapy. To test the direct effect of atorvastatin on VEGF synthesis in vitro, HCASMC were treated with atorvastatin (1, 3 and 10 μ M). The VEGF concentration was measured by enzyme-linked immunosorbent assay.
RESULTS	Atorvastatin therapy reduced VEGF plasma levels in CAD patients (from 31.1 ± 6.1 to 19.0 ± 3.6 pg/ml; $p < 0.05$). The VEGF plasma concentration tended to be higher in CAD patients before treatment compared to control patients (31.1 ± 6.1 vs. 23.4 ± 3.6 pg/ml; $p = \text{NS}$). Plasma collected before therapy induced significantly more VEGF in HCASMC compared to the plasma collected after treatment and compared to control cells. In vitro, atorvastatin decreased both the basal and the interleukin-1 β -induced VEGF release in HCASMC.
CONCLUSIONS	These data suggest that atorvastatin may lower the plasma level of VEGF in CAD patients, which could represent a novel beneficial effect of this and perhaps other statins. (J Am Coll Cardiol 2002;39:1951-5) © 2002 by the American College of Cardiology Foundation

Chronic inflammation, a key component of atherogenesis (1), requires the recruitment of circulating leukocytes into atherosclerotic plaques. This leukocytic infiltration is probably enhanced by plaque neovascularization (2,3). With regard to this potentially harmful effect of neoangiogenesis, the main angiogenic growth factor, vascular endothelial growth factor (VEGF), has been investigated in several studies.

In accordance with the supposed proatherogenic effect of VEGF, the administration of VEGF to both apo-E/apo-B100 deficient mice and hypercholesterolemic rabbits enhanced plaque growth (4). Additionally, inhibition of angiogenesis resulted in the abrogation of plaque formation in transgenic apo-E knockout mice (5). To our knowledge, such effects have not been examined so far in human studies with VEGF protein or gene therapy; however, it is well documented that VEGF is strongly expressed in human atherosclerotic vessels, but not in healthy arteries (6).

The clinical benefit of hepatic hydroxymethyl glutaryl-coenzyme A reductase inhibitors (statins) has been proven

in several large clinical trials (7-10). It is believed that the reduction in cardiovascular morbidity and mortality is partially due to non-lipid-lowering properties of statins including antithrombotic, antiproliferative and anti-inflammatory effects (11,12). So far, however, there are only a few studies on the possible influence of statins on VEGF production. It has been shown that lovastatin decreased VEGF synthesis in Ha-ras transformed human fibroblasts (13) and that this antiangiogenic mechanism might be responsible for its antitumor effect. In addition, mevastatin was demonstrated to influence VEGF production in rat aortic endothelial cells (14). Recently, fluvastatin has been shown to lower VEGF serum levels in patients with hypercholesterolemia or with hypertension (15).

Regarding the well established benefit of statins and the potential significance of modulating VEGF levels in patients with coronary artery disease (CAD), we investigated the effect of atorvastatin on VEGF plasma concentration in hypercholesterolemic patients with angiographically confirmed CAD. In addition, we examined the effect of atorvastatin on VEGF production of human coronary artery smooth muscle cells (HCASMC) in vitro.

METHODS

Patients. Written informed consent was obtained from all patients. The study complies with the declaration of Helsinki. Ethylenediamine-tetraacetic acid (EDTA) anticoag-

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Manuscript received October 31, 2001; revised manuscript received March 4, 2002, accepted March 15, 2002.

Abbreviations and Acronyms

CAD	= coronary artery disease
DMSO	= dimethyl sulfoxide
EDTA	= ethylenediamine-tetraacetic acid
ELISA	= enzyme-linked immunosorbent assay
FCS	= fetal calf serum
HCASMC	= human coronary artery smooth muscle cells
IL	= interleukin
LDH	= lactate dehydrogenase
LDL	= low-density lipoprotein
VEGF	= vascular endothelial growth factor

ulated blood was collected from 14 male patients (age 52.9 ± 2.4 years) with angiographically confirmed CAD (defined as $>30\%$ lumen stenosis in at least one major coronary artery branch) and with hypercholesterolemia requiring lipid-lowering therapy according to the National Cholesterol Education Program guidelines (16). Additional risk factors were hypertension ($n = 4$) and cigarette smoking ($n = 3$) or both ($n = 3$). Smoking status did not change during the study period. Exclusion criteria included diabetes mellitus, prior myocardial infarction (<1 month before inclusion), known tumor disease, peripheral arterial occlusive disease, impaired left ventricular function (ejection fraction $<45\%$) and chronic obstructive pulmonary disease. Blood was collected before and after a lipid-lowering therapy with atorvastatin (20 mg/d) for two months. Furthermore, EDTA plasma was collected from eight control patients (age 56.5 ± 1.3 years) who underwent coronary angiography because of chest pain, which revealed smooth coronary arteries. Blood was centrifuged for 15 min at 500 g. Plasma was stored below -30°C .

Reagents. Atorvastatin calcium was a gift from Parke Davis (Groton, Connecticut). Interleukin (IL)- 1β was purchased from Sigma (St. Louis, Missouri); VEGF enzyme-linked immunosorbent assay (ELISA) kit for determination of human VEGF was from R&D Systems (Minneapolis, Minnesota). Lactate dehydrogenase (LDH) release assay was from Promega (Madison, Wisconsin). Cy-Chrome anti-human CD3 antibody was purchased from PharMingen (San Diego, California) (Cat. No. 555334) and Lymphoprep was obtained from Nycomed (Oslo, Norway).

Cells. Human coronary artery smooth muscle cells were obtained from Clonetics (CellSystems, St. Katharine, Germany) and cultured till confluence in SmBM with 5% fetal calf serum (FCS).

Experimental protocols in vitro. Confluent HCASMC were put on starving medium (0.5% FCS) for 24 h. Thereafter, cells were treated for 24 h with different concentrations of atorvastatin (1 to 10 μM) diluted in dimethyl sulfoxide (DMSO) and finally dissolved in the culture media. The concentration of DMSO never exceeded 0.1% and the control cells were treated with the same amount of diluent. Some cells were additionally treated with IL- 1β (10 ng/ml). After 24 h, the medium was collected

and used for determination of VEGF concentration and LDH release. The VEGF levels were determined in at least three independent experiments performed in duplicate or triplicate.

Experimental protocols ex vivo. In another set of experiments, HCASMC were treated with 10 μl of filter-sterilized plasma collected from 6 randomly selected patients of the 14 investigated CAD patients before and after two months of atorvastatin therapy. Cells were cultured for 24 h and VEGF concentration was determined in the conditioned media.

VEGF determination. Human VEGF was detected in the patients' plasma with Human Quantikine VEGF ELISA. The VEGF measurements in the samples collected from CAD patients before and after two months of therapy were performed at the same time by an investigator blinded to the kind of treatment applied to the patients. The VEGF release by HCASMC was determined by the same kit.

Other measurements. Plasma lipid concentrations and white blood cells were determined by the central laboratory of the University Clinic of Innsbruck. The LDH release assay was used to investigate whether atorvastatin exerts any cytotoxic effect on HCASMC. T-lymphocytes were determined using FACScan (Becton Dickinson, Belgium) after incubation of Lymphoprep (Nycomed) separated leukocytes with 10 μl of anti-CD3 antibodies for 30 min at room temperature.

Statistical analysis. Values are expressed as mean \pm SEM. To determine normal distribution of variables, we used the Kolmogorov-Smirnov test. According to this result, Wilcoxon-Signed Ranks test and Mann-Whitney *U*-test were used for nonparametric comparison. When appropriate, one-way analysis of variance with post hoc Scheffe test was performed for multiple comparisons. A *p* value <0.05 was considered to be statistically significant.

RESULTS

The effect of atorvastatin therapy on lipid levels.

Atorvastatin significantly decreased both total cholesterol and low-density lipoprotein (LDL) cholesterol levels in CAD patients (Table 1). Total cholesterol level was reduced by $29.3 \pm 2.7\%$ (from 262.3 ± 13.0 to 189.9 ± 8.9 mg/dl; $p < 0.001$), while LDL cholesterol level decreased by $37.1 \pm 4.5\%$ (from 174.4 ± 9.8 to 109.6 ± 9.1 mg/dl; $p < 0.001$). There was no significant difference in triglycerides (151.2 ± 16.9 vs. 116.5 ± 8.0 mg/dl; not significant [NS]) and high-density lipoprotein cholesterol levels (59.4 ± 9.7 vs. 49.9 ± 3.0 mg/dl; NS) after treatment with atorvastatin.

The effect of atorvastatin treatment on blood cells. No significant differences in leukocyte ($6,714 \pm 360$ vs. $6,050 \pm 431$ cells/ μl ; NS), lymphocyte ($1,985 \pm 149$ vs. $1,889 \pm 151$ cells/ μl ; NS), monocyte (566 ± 42 vs. 514 ± 46 cells/ μl ; NS) and thrombocyte counts ($234,929 \pm 12,558$ vs. $216,857 \pm 12,832$ cells/ μl ; NS) were observed in CAD patients after atorvastatin treatment (Table 1). Furthermore,

Table 1. Lipid Levels and Blood Cells in Patients With Coronary Artery Disease and Control Subjects

Variables	Baseline (n = 14)	Atorvastatin (n = 14)	Controls (n = 8)
Total cholesterol (mg/dl)	262.3 ± 13.0	189.9 ± 8.9†	241.4 ± 11.2‡
LDL cholesterol (mg/dl)	174.4 ± 9.8	109.6 ± 9.1†	170.4 ± 9.7‡
HDL cholesterol (mg/dl)	59.4 ± 9.7	49.9 ± 3.0	53.9 ± 3.2
Triglycerides (mg/dl)	151.2 ± 16.9	116.5 ± 8.0	119.8 ± 22.4
Platelets (μl)	234,929 ± 12,558	216,857 ± 12,832	197,500 ± 10,742
Monocytes (μl)	566 ± 42	514 ± 46	456 ± 39
Leukocytes (μl)	6,714 ± 360	6,050 ± 431	6,688 ± 601
Lymphocytes (μl)	1,985 ± 149	1,889 ± 151	1,971 ± 254
Fibrinogen (mg/dl)	375.6 ± 31.3	319.4 ± 16.2	370.4 ± 21.4
VEGF (pg/ml)	31.1 ± 6.1	19.0 ± 3.6*	23.4 ± 3.6

*p < 0.05 vs. baseline. †p < 0.001 vs. baseline. ‡p < 0.01 vs. atorvastatin.

LDL = low-density lipoprotein; HDL = high-density lipoprotein; VEGF = vascular endothelial growth factor.

there was no difference in blood cells between control and CAD patients at any time.

VEGF levels in and ex vivo. In CAD patients, VEGF plasma concentration was significantly decreased after two months of atorvastatin therapy (31.1 ± 6.1 pg/ml compared to 19.0 ± 3.6 pg/ml; p < 0.05; Fig. 1). There was a tendency toward lower VEGF plasma levels in control patients compared to CAD patients before atorvastatin treatment (23.4 ± 3.6 vs. 31.1 ± 6.1; p = NS). There was no significant difference in VEGF plasma concentration between control subjects and CAD patients after atorvastatin therapy.

When tested ex vivo, plasma collected before therapy induced significantly more VEGF in HCASMC compared to the plasma collected after treatment and compared to control (i.e., nontreated) cells (225.3 ± 27.9% of control cells vs. 137.0 ± 21.9% of control cells; p < 0.03). However, plasma collected after atorvastatin induced VEGF production to a level that is not significantly different from the basal VEGF generation of nontreated HCASMC (Fig. 2).

VEGF levels in vitro. Atorvastatin (1, 3 and 10 μM) treatment resulted in a significant decrease of VEGF release in HCASMC compared to untreated control cells (59.0 ± 10.4%, 58.4 ± 7.3%, 64.9 ± 5.3% of control cells; p < 0.0005; Fig. 3). No inhibition of VEGF synthesis has been observed with lower concentrations of atorvastatin (data not shown). In addition, atorvastatin also reversed the IL-1β-induced synthesis of VEGF (Fig. 3).

DISCUSSION

This study demonstrates that atorvastatin leads to a decrease in the production of VEGF in vitro and in vivo. This is shown in three ways: 1) VEGF plasma levels of CAD patients decreased after two months of atorvastatin therapy; 2) plasma collected from patients before atorvastatin treatment increased the VEGF production in HCASMC to a significantly higher level than the plasma collected after

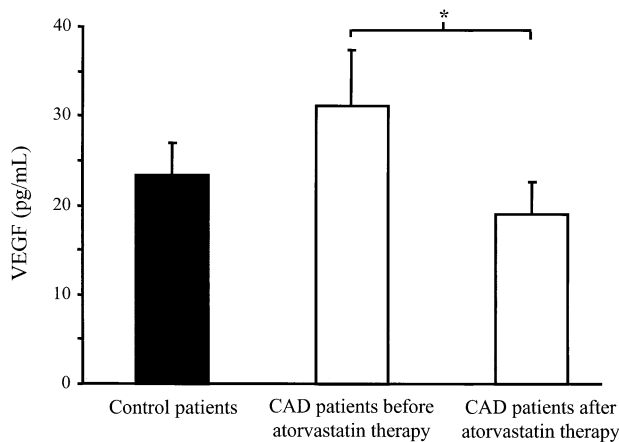


Figure 1. The effect of atorvastatin therapy on vascular endothelial growth factor (VEGF) plasma levels. Two months of atorvastatin therapy (20 mg/d) decreased VEGF plasma levels (pg/ml) in patients with coronary artery disease (CAD) (white bars, *p < 0.05). No significant difference in VEGF plasma concentration between control patients (black bar) and CAD patients after treatment with atorvastatin could be observed. The VEGF concentrations in CAD patients tended to be higher compared to those in control patients (p = NS).

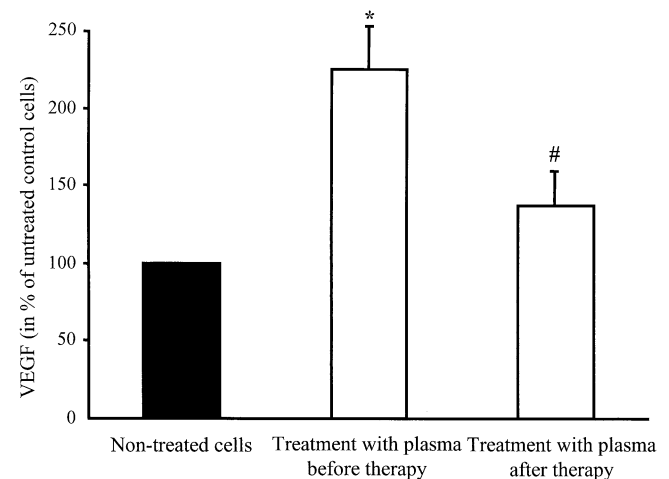


Figure 2. The effect of patient plasma collected before and after therapy with atorvastatin on vascular endothelial growth factor (VEGF) synthesis by human coronary artery smooth muscle cells (HCASMC). The VEGF synthesis in HCASMC (in % of untreated cells, black bar) incubated with the plasma of patients with coronary artery disease (white bars) collected before atorvastatin therapy was increased (*p < 0.003 vs. untreated cells). The plasma collected after atorvastatin treatment reversed this stimulatory effect (#p < 0.03 vs. the plasma collected before therapy).

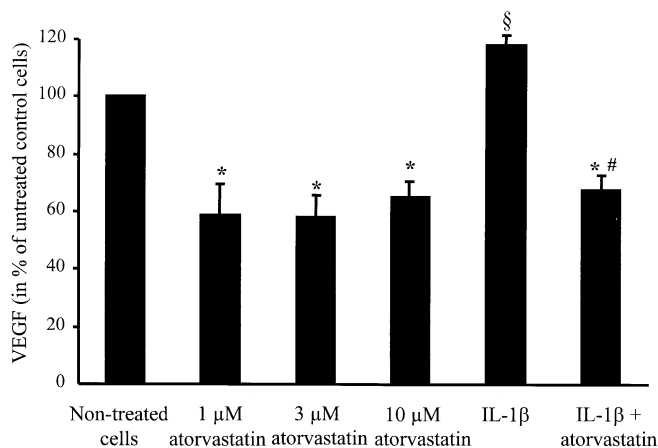


Figure 3. The effect of atorvastatin on vascular endothelial growth factor (VEGF) synthesis by human coronary artery smooth muscle cells (HCASMC) in vitro. The VEGF synthesis in HCASMC (in % of untreated cells). Atorvastatin (1, 3 and 10 μmol/l) decreased basal (*p < 0.003 vs. untreated cells) VEGF production in HCASMC. Interleukin (IL)-1β (10 ng/ml) induced VEGF synthesis (§p < 0.003 vs. untreated cells) in these cells. Incubation of IL-1β-treated HCASMC with atorvastatin again decreased the VEGF production (#p < 0.0001 vs. IL-1β-treated cells).

therapy; and 3) atorvastatin decreased VEGF synthesis in HCASMC in vitro.

Vascular endothelial growth factor is considered the most potent angiogenic growth factor for the stimulation of collateral vessel growth in peripheral (17) and myocardial (18) ischemia. However, there is accumulating evidence that VEGF is strongly expressed in atherosclerotic vessels (6,19) and can enhance neoangiogenesis in atherosclerotic plaque (4). Celletti et al. (4) have shown that injection of recombinant VEGF enhanced atherosclerosis in apo-E/B100 knockout mice and in hypercholesterolemic rabbits, thereby supporting the possible role of VEGF in plaque progression. In addition, inhibition of endothelial cell proliferation by endostatin and TNP-470 in apo-E knockout mice was shown to attenuate lesion formation (5). Taken together, these animal studies strongly suggest that VEGF may aggravate plaque growth. Although adverse events attributable to plaque progression or destabilization in VEGF-treated patients have not been reported in clinical trials so far (to our knowledge), the potential proatherogenic effect of VEGF cannot be fully excluded. While the clinical settings in which VEGF may be harmful are not evident, a prudent approach would be to avoid VEGF gene therapy in stages that are indicative of progressive rather than stable disease.

If one extrapolates the putative proatherogenic effect of VEGF to human atherosclerosis, the reduction of VEGF by atorvastatin could represent another atheroprotective property of this statin (12).

In accordance with our data, Blann et al. (20) recently demonstrated that lipid-lowering therapy with fluvastatin or fenofibrate decreased the VEGF plasma levels in hypercholesterolemic patients with/without peripheral or carotid

atherosclerosis. Our data extend these observations to patients with CAD and give additional insight into the possible mechanisms of statins on VEGF production. In contrast, Vasa et al. (21) have shown that atorvastatin therapy (40 mg) for four weeks did not change VEGF levels in the serum of patients with stable CAD. Potential explanations for the latter study compared to ours and the study by Blann et al. (20) partially include methodologic differences. First, distinct statins (fluvastatin vs. atorvastatin) and different concentrations of statins (40 mg vs. 20 mg of atorvastatin) were used. Second, the duration of treatment is different. After four weeks of statin therapy, no effect on VEGF levels was observed by Vasa et al. (21). However, 8 weeks (our data) and 12 weeks (20) of statin treatment resulted in decreased VEGF concentrations. Third, the VEGF concentration was determined in different kinds of blood samples (serum by Vasa et al. [21] vs. plasma in ours and in Blann et al. [20]). The fact that the VEGF plasma levels in the control patients tended to be lower than in the CAD patients before treatment is in accordance with a study by Fleisch et al. (22) that showed a trend toward higher VEGF levels in patients with more advanced CAD. The lack of statistical significance is probably due to the small number of control patients.

The influence of atorvastatin on VEGF production can be attributed to both a direct or indirect effect of statins. Recently, we have demonstrated that oxidized LDL enhanced VEGF expression and generation in vascular smooth muscle cells (23). Therefore, hypercholesterolemia may induce VEGF production in different cells including smooth muscle cells (23), macrophages (24) or endothelial cells (25). Statin treatment, by decreasing cholesterol level, probably interferes with a lipid-dependent enhancement of VEGF. In our ex vivo experiments (Fig. 2), we cannot exclude that the decreased production of VEGF in HCASMC is only an epiphenomenon of the lowered cholesterol content in the plasma of treated CAD patients. As the total LDL cholesterol amounts in the plasma collected before and after therapy are only slightly different and as oxidized LDL is known to stimulate VEGF synthesis (23), the observed reduction of VEGF may be due to an antioxidative effect of atorvastatin on LDL cholesterol (26).

Statins are known to exert effects independent of their lipid-lowering activity. This can be related to anti-inflammatory properties, as evidenced by an inhibition of the activity of transcription factors (27) and the synthesis of inflammatory cytokines (28). Our results demonstrate that atorvastatin reduces both constitutive and cytokine-induced VEGF synthesis in cell culture (29), a setting in which cholesterol content is constant (Fig. 3). Therefore, we can assume that this effect of atorvastatin also represents a non-lipid-dependent activity.

The clinical significance of the observed decrease in VEGF plasma levels in CAD patients certainly cannot be derived from in vitro data. Because we cannot elucidate the source of the measured VEGF plasma concentration, we

tried to exclude possible confounding factors. First, we used EDTA plasma instead of serum. It has been shown previously that during clotting of blood in a serum tube, VEGF is released by platelets (30). Second, we determined the number of monocytes and T-lymphocytes, which are known sources of VEGF (31,32), and found no difference before and after therapy with atorvastatin. Third, we excluded all patients with acute inflammatory diseases, diabetes mellitus (33), cancer or other chronic diseases in which VEGF is thought to play a role (34).

In conclusion, we show a decrease in VEGF plasma levels in CAD patients after two months of atorvastatin therapy. The results could be confirmed in two different *in vitro* experiments. In accordance with the proposed detrimental role of VEGF-induced neovascularization in atherosclerotic plaque growth, these observations are likely to represent another beneficial effect of atorvastatin and perhaps other statins (35).

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