An HMG-CoA Reductase Inhibitor, Cerivastatin, Suppresses Growth of Macrophages Expressing Matrix Metalloproteinases and Tissue Factor In Vivo and In Vitro

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Background—Unstable atherosclerotic plaques that cause acute coronary events usually contain abundant macrophages expressing matrix metalloproteinases (MMPs) and tissue factor (TF), molecules that probably contribute to plaque rupture and subsequent thrombus formation. Lipid lowering with HMG-CoA reductase inhibitors reduces acute coronary events.

Methods and Results—To test whether lipid lowering with an HMG-CoA reductase inhibitor retards macrophage accumulation in rabbit atheroma, we administered cerivastatin to immature Watanabe heritable hyperlipidemic rabbits (cerivastatin group, n = 10, cerivastatin 0.6 mg · kg⁻¹ · d⁻¹; control group, n = 9, saline 0.6 mL · kg⁻¹ · d⁻¹) for 32 weeks and measured macrophage accumulation and expression of MMPs and TF. Serum cholesterol levels after 32 weeks were 809±40 mg/dL (control group) and 481±24 mg/dL (treated group). Cerivastatin diminished accumulation of macrophages in aortic atheroma. Macrophage expression of MMP-1, MMP-3, MMP-9, and TF also decreased with cerivastatin treatment. Cerivastatin reduced the number of macrophages expressing histone mRNA (a sensitive marker of cell proliferation) detected by in situ hybridization but did not alter macrophages bearing a marker of death (TUNEL staining). Cerivastatin treatment (≥0.01 μmol/L) also reduced growth, proteolytic activity due to MMP-9, and TF expression in cultured human monocyte/macrophages.

Conclusions—These results suggest that lipid lowering with HMG-CoA reductase inhibitors alters plaque biology by reducing proliferation and activation of macrophages, prominent sources of molecules responsible for plaque instability and thrombogenicity. (Circulation. 2001;103:276-283.)

Key Words: atherosclerosis ■ inflammation ■ thrombosis ■ hypercholesterolemia ■ metalloproteinases

Atherosclerotic plaques prone to rupture, thrombus formation, and consequent acute coronary events frequently contain a prominent accumulation of inflammatory cells, including macrophages.¹⁻³ Macrophages in atheroma overexpress matrix metalloproteinases (MMPs), such as MMP-1 (collagenase-1), MMP-3 (stromelysin-1), and MMP-9 (gelatinase-B).⁴⁻⁶ Lesional macrophages also express tissue factor (TF), a strong activator of blood coagulation, as well as its inducer CD40 ligand (CD40L or CD154).⁷⁻⁸ Overexpression of such matrix-degrading enzymes and prothrombotic molecules most likely contributes to plaque instability and thrombogenicity.⁹,¹⁰ Thus, the macrophage content of an atheroma can critically influence its clinical consequences.

Several mechanisms regulate macrophage accumulation in plaques. Endothelial cells in atheroma overexpress molecules that contribute to monocyte recruitment.¹¹ Survival factors such as macrophage-colony—stimulating factor (M-CSF) promote persistence of these cells in plaques.¹² Macrophage proliferation may also participate in the formation of vulnerable atheroma rich in this cell type.¹³⁻¹⁵ M-CSF, granulocyte macrophage-CSF (GM-CSF), and oxidized LDL, each of which accumulates in atheroma, can induce macrophage proliferation in vitro.¹⁶⁻¹⁸ Cholesterol lowering with inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase inhibitors) reduces the incidence of acute coronary events in patients, probably by functional changes of atheroma ("stabilization").¹⁹⁻²¹ We have recently demonstrated that dietary lipid lowering in cholesterol-fed rabbits reduces expression and activity of MMPs and TF in established atheroma by reducing macrophage number and, in turn, ameliorating...
smooth muscle cell (SMC) activation. These experiments, although informative, used a model of exogenous hypercholesterolemia and an intervention that produced a greater degree of lipid lowering than clinically practicable. Also, these experiments evaluated the effects of lipid lowering on existing lesions. Moreover, many recent studies have involved possible effects of the HMG-CoA reductase inhibitor beyond their lipid-lowering actions. We therefore tested cerivastatin, a potent novel HMG-CoA reductase inhibitor, on new lesion development in Watanabe heritable hyperlipidemic (WHHL) rabbits that have endogenous hypercholesterolemia due to LDL receptor deficiency. Shiomi et al demonstrated that cerivastatin retards progression of atherosclerosis in terms of plaque size and macrophage accumulation in WHHL rabbits. We report here an extension of this study on WHHL rabbits designed to test the hypothesis that cerivastatin can prevent macrophage expression of molecules responsible for plaque disruption and thrombus formation. We also determined whether cerivastatin reduces macrophage growth both in vivo and in vitro as a potential mechanism of reduced macrophage accumulation.

Methods

Animal Experiment
The protocol of this experiment has been described in detail. Nineteen WHHL rabbits at 2 months of age were housed individually in metal cages and were fed a regular laboratory diet. Cerivastatin solution (Bayer Yakuhin) was administered to the animals of the cerivastatin group (n=10, 0.6 mg·kg\(^{-1}\)·d\(^{-1}\) SC) for 32 weeks. The animals of the control group received saline (n=9, 0.6 mL·kg\(^{-1}\)·d\(^{-1}\) SC) for 32 weeks. All animal experiments were performed in accordance with the Guidelines for Animal Experimentation of the Kobe University School of Medicine.

Tissue Sampling
The aortic tissues were excised 2 mm above the ligamentum arteriosum and snap-frozen or fixed with 4% paraformaldehyde. Surgical specimens of human carotid plaques were obtained in accordance with a protocol approved by the Human Investigation Review Committee at Brigham and Women’s Hospital.

Histochemical Assays
Immunohistochemistry was performed by the ABC method (Vector). Antibodies included mouse monoclonal antibodies against rabbit CD11b (Spring Valley), human α-smooth muscle actin (1A4, Dako), human MMP-1, rabbit MMP-3, human MMP-9 (Calbiochem), rabbit TF (American Diagnostica), and a rat monoclonal antibody for mouse CD40L (a gift from Immunex; Seattle, Wash). For a negative control, nonimmune IgG was applied in place of antibodies. Picrosirius red polarization was performed to detect interstitial collagen content according to Junqueira’s method as modified by us. Nonisotopic in situ hybridization for histone mRNA was performed with the Hyb-Probe Detection System (Shandon/Lipshaw) as previously described. Briefly, sections of rabbit and human atheroma and cultured human macrophages (day 7) were fixed briefly with 4% paraformaldehyde and hybridized with fluorescein-labeled oligonucleotide cocktail for histone mRNAs and random oligomers (negative control). mRNA signals were detected by alkaline phosphatase–conjugated antibody against FITC. Immunohistochemistry for CD11b was then performed. Quantitative analysis of immunohistochemistry and picrosirius red staining used a computer-assisted color image analysis system. For in situ hybridization and TUNEL staining, positive macrophages in the intima were counted manually by 2 independent investigators.

In Vitro Experiments
Human monocytes were obtained from human peripheral blood by density gradient centrifugation and adherence. Monocytes were plated at 5×10^6 cells/well on 6-well plates in M199 medium containing 2% human serum and 200 U/mL M-CSF (a gift from Genetics Institute; Cambridge, Mass) and cultured for 10 days with and without cerivastatin. The cell numbers were measured by both manual counting and MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay (Promega). M-CSF–induced replication was determined on day 10 macrophages with an ELISA for bromodeoxyuridine (BrdU, Roche) incorporation. Immunocytochemistry using peroxidase-conjugated anti-BrdU antibody detected BrdU uptake by macrophages. Monocyte/macrophages at days 1 and 10 were used for gel electrophoresis. The amount of culture medium applied for the gel electrophoresis was adjusted on the basis of the cell number. FITC-conjugated mouse monoclonal anti–human TF antibody (American Diagnostica) was used for fluorescence-activated cell sorter (FACS) analysis.

Statistical Analysis
Statistical testing for quantification of histochemical assays used the Mann-Whitney U test. One-way ANOVA followed by the post hoc test was used for in vitro assays.

Results

Lipid Profile
Plasma lipid analysis on these animals has already been reported. Briefly, the mean total cholesterol, triglyceride, and phospholipid levels (mg/dL) of the 2 groups were similar at the beginning of the experiment. At 32 weeks, we observed a statistically significant reduction in total cholesterol levels in the cerivastatin group (481±24 mg/dL) versus the control group (809±40 mg/dL). Phospholipid levels also decreased (447±22 and 318±24 mg/dL, respectively), but there was no significant difference in triglyceride levels (228±23 and 183±2 mg/dL, respectively).

Effects of Cerivastatin on MMP and TF in Atheroma
Atheroma of WHHL rabbits treated with saline for 32 weeks contained many macrophages (CD11b-positive cells) (Figure 1A). Immunoreactive MMP-1, MMP-3, and MMP-9 colocalized with macrophages. However, the areas immunopositive for CD11b (mm^2) decreased in the cerivastatin group, determined by quantitative color image analysis (Figures 1B and 2A). We further quantified MMP-positive areas within macrophage-containing regions (Figures 2B, 2C, and 2D). The data suggest decreased macrophage expression of MMP-1, MMP-3, or MMP-9 by cerivastatin treatment. Picrosirius red polarization was performed to determine whether increased accumulation of interstitial collagen paralleled reduced matrix-degrading enzymes. Aortas from the control group showed intense staining in the adventitia and relatively weak staining in the intima and the media (Figure 3A). However, the aortic intimas from the cerivastatin group showed more intense...
staining than those from the control group (Figure 3A). Quantitative analysis showed a 1.7-fold increase in picrosirius red–positive intimal areas in the cerivastatin group compared with the control group (Figure 3B). The intima of the control group showed TF expression that colocalized with its inducer, CD40L (Figure 4A). Intimal TF expression of the cerivastatin group was lower than that of the control group, in association with reduced expression of CD40L (Figure 4A). Quantitative analysis demonstrated that cerivastatin significantly reduced the percentage of TF-positive macrophages in the intima (Figure 4B). Negative control applying nonimmune IgG in place of the antibodies abrogated the staining (data not shown).

Effects of Cerivastatin on Proliferation and Death of Macrophages in Atheroma

To address the potential mechanisms of reduced macrophage number, we performed in situ hybridization for histone mRNA (a sensitive marker for cell proliferation) and TUNEL staining for cell death. Based on the expression of histone mRNA, 9.0% of macrophages showed this sign of replication in atheroma of the control group. However, only 4.3% of macrophages stained positively for histone mRNA in animals treated with cerivastatin (Figures 5 and 7). No signal was detected with random oligomers used as negative control (data not shown). In contrast, TUNEL staining demonstrated that macrophages in both groups displayed similar levels of DNA fragmentation characteristic of apoptotic cell death (Figures 6 and 7). No positive signal was seen on the nuclei without TdT reagent (data not shown).

Effects of Cerivastatin on Macrophage Survival and Proliferation In Vitro

In situ hybridization for histone mRNA detected macrophage proliferation in human atheroma and verified the utility of this technique for detection of proliferation of human macrophages as well (Figure 8). After 10-day culture with 200 U/mL of M-CSF and 2% human serum, human monocyte–derived macrophages expressed histone mRNA and incorporated BrdU in their nuclei (Figure 9). By either cell counting or MTS assay, cerivastatin treatment (0.01 to 0.5 μmol/L) for 10 days significantly reduced numbers of cultured human macrophages (P<0.01) (Figure 10A and 10B). BrdU incorporation by macrophages was inhibited by 0.05 μmol/L of cerivastatin, an effect reversed by coincubation with meval-
donate, farnesyl pyrophosphate, or geranylgeranyl pyrophosphate, suggesting that suppression of macrophage growth by cerivastatin was achieved by inhibition of the mevalonate pathway, not by cell injury or toxicity (Figure 10C).

Effects of Cerivastatin on MMP and TF In Vitro
Gelatin zymography and FACS analysis tested whether cerivastatin treatment affects macrophage production of MMP and TF, 2 functions inhibited by cerivastatin treatment in vivo. Gelatinolytic activity at 92 kDa ascribable to MMP-9 rises in human macrophages at day 10 compared with day 1 macrophages. However, cerivastatin treatment (0.05 µmol/L) prevented this increase, yielding levels similar to those of macrophages at day 1 (Figure 11A).

Discussion
Macrophages proliferate in human and hypercholesterolemic rabbit atheroma.13–15 Proliferation probably plays an important role in formation of macrophage-rich vulnerable plaques. Thus, inhibition of macrophage proliferation may promote stabilization of atheroma. M-CSF and GM-CSF induce macrophage survival and proliferation in vitro, and oxidized LDL enhances their action.12,16–18 Reduced oxidized LDL accumulation and GM-CSF and M-CSF expression in atheroma due to lowered LDL levels might suppress macrophage proliferation.30 The present study used in situ hybridization for histone mRNA28,31 to measure proliferation of macrophages in atheroma of WHHL rabbits. Histone mRNAs increase (∼15-fold) in cells in S phase and decrease immediately after cells leave S phase because they lack polyA tails.29,32 Lipid lowering by cerivastatin in this study (40.5% reduction in total cholesterol) reduced the number of macrophages expressing histone mRNAs in atheroma of WHHL rabbits. We recently found that dietary lipid lowering reduces oxidized
LDL accumulation in atheroma of cholesterol-fed rabbits (M.A., et al, manuscript submitted). Such improved oxidative stress might be one potential mechanism by which cerivastatin treatment suppressed macrophage proliferation in rabbit atheroma in the present study.

We recently demonstrated that lipid lowering by diet alone can improve features typical of so-called unstable atheromatous plaques in rabbits.22–24 Thus, lipid lowering by dietary manipulation alone can ameliorate features of plaques associated with instability and thrombogenicity. Reaching target cholesterol levels in patients, however, often requires drug treatment in addition to diet. HMG-CoA reductase inhibitors decrease conversion of HMG-CoA into mevalonate, a precursor for endogenous cholesterol. The decreased cholesterol pool in cells in turn augments LDL-receptor levels on the cell surface, which lowers blood cholesterol levels. Thus, LDL-receptor deficiency limits this mechanism of cholesterol reduction by HMG-CoA reductase inhibitors. In this study of LDL receptor–deficient WHHL rabbits, the mean blood cholesterol levels of cerivastatin-treated animals still by far exceeded the normal range, unlike the dietary manipulation used in our previous rabbit studies.22–24 Nonetheless, cerivastatin treatment significantly reduced macrophage accumulation, despite persistent hypercholesterolemia. Cerivastatin suppresses SMC proliferation independently of its lipid-lowering effect.33,34 We therefore conjectured that cerivastatin has an antiproliferative effect on macrophages in addition to its lipid-lowering effect. To address this hypothesis, we determined the effects of cerivastatin on human monocyte–derived macrophages cultured with M-CSF. Cerivastatin (0.01 to 0.5 μmol/L) reduced macrophage number in a dose-dependent manner, suggesting that cerivastatin may suppress M-CSF–induced macrophage survival. Many previous in vitro demonstrations of the cellular effects of HMG-

![Figure 5](image1.png)  
**Figure 5.** Cerivastatin reduced macrophage proliferation in atheroma of WHHL rabbits. Top, In situ hybridization for histone mRNA in intima of control and cerivastatin groups shows decreased number of histone-positive cells with cerivastatin treatment. Bottom left, Double staining for in situ hybridization for histone mRNA (blue) and α-smooth muscle actin (red). Monocytic cells stained positively for histone but not for α-actin. Bottom right, Double staining for histone (blue) and CD11b for macrophages (red) indicates that histone-positive cells are macrophages. Arrowheads indicate tunica media. Bar=50 μm. Magnification ×400.

![Figure 6](image2.png)  
**Figure 6.** Cerivastatin treatment did not affect macrophage death in atheroma of WHHL rabbits. Double staining for TUNEL and CD11b. Relatively small numbers of intimal macrophages were stained positively by TUNEL technique in aortic intima from both control and cerivastatin groups. Arrowheads indicate tunica media. Bar=50 μm. Magnification ×400.

![Figure 7](image3.png)  
**Figure 7.** Quantitative analysis of in situ hybridization for histone mRNA and TUNEL staining. Data are reported as percent of CD11b-positive macrophages also expressing histone mRNA or exhibiting a positive signal for TUNEL staining. Bars represent SEM.
CoA reductase inhibitors have used doses that most likely exceed those encountered in patients. In the present study, 0.6 mg kg\(^{-1}\) d\(^{-1}\) of cerivastatin was administered to significantly decrease cholesterol levels of WHHL rabbits with an LDL-receptor deficiency. This dose was much higher than those used for humans. This is customary in animal studies, because much higher doses are required for statin effects than in humans. However, the level of cerivastatin effective in vitro (0.01 to 0.05 \(\mu\)mol/L) is close to peak plasma concentrations achieved in humans after a single oral administration of 300 to 800 \(\mu\)g of cerivastatin.\(^{35,36}\) Furthermore, effective doses of cerivastatin are lower than those of simvastatin and pravastatin required to retard growth of murine peritoneal macrophages induced by oxidized LDL (IC\(_{50}\) values, 0.7 and 70 \(\mu\)mol/L, respectively).\(^{37}\)

The balance between proliferation and death in part determines the accumulation of cells in atheroma. This study demonstrates no significant difference in the number of macrophages bearing a marker of DNA fragmentation in the 2 groups, suggesting that cerivastatin treatment does not reduce macrophage accumulation by cell injury or apoptotic death. HMG-CoA reductase inhibitors can induce the death of cultured SMCs.\(^{38}\) Induction of death of SMCs in the fibrous cap by high doses of HMG-CoA reductase inhibitors may not be beneficial in terms of plaque stability, because SMCs synthesize extracellular matrix constituents, such as collagen, which strengthen plaques. Shiomi et al.\(^{27}\) interestingly, demonstrated that cerivastatin treatment reduced accumulation of macrophages, but not of SMCs, in atheroma of the same animals as used in this present study. Taken together, these results raise the possibility that certain doses of HMG-CoA reductase inhibitors can reduce accumulation of collagenolytic and prothrombotic macrophages in atheroma without inhibiting SMCs in the fibrous cap.

We show here that cerivastatin treatment reduced expression of MMPs and TF in atheroma of WHHL rabbits. A decrease in the percentage of macrophages expressing MMPs

Figure 8. Macrophage (\(\text{Mo}\)) proliferation in human atheroma. In situ hybridization detected histone mRNA expression in intima of 2 samples of human carotid arteries. mRNA signals colocalized with macrophages detected by immunohistochemistry for CD68 on serial sections. Bar=50 \(\mu\)m. Magnification \(\times 400\).

Figure 9. Macrophage proliferation in human monocyte–derived macrophages cultured with 200 U/mL M-CSF for 10 days. Left, In situ hybridization detected histone mRNA expression by human macrophages. Right, Immunocytochemistry for BrdU showed that BrdU had been incorporated by human macrophages.

Figure 10. Cerivastatin treatment reduced survival and proliferation of human macrophages cultured with 200 U/mL M-CSF for 10 days. In both cell counting (A) and MTS assay (B), cerivastatin treatment (>0.01 \(\mu\)mol/L) produced statistically significant reduction (\(P<0.05\)) in cell numbers. BrdU incorporation (C) by macrophages was inhibited by 0.05 \(\mu\)mol/L of cerivastatin (Ceriva; \(P<0.01\) vs M-CSF–treated macrophages), and this suppression was reversed by addition of mevalonate (100 \(\mu\)mol/L), famesyl pyrophosphate (F-PP, 10 \(\mu\)mol/L), or geranylgeranyl pyrophosphate (GG-PP, 10 \(\mu\)mol/L). Bars represent SEM.
and TF in the cerivastatin group suggests that reduced expression of such proteolytic or prothrombotic molecules probably results from not only a diminished number of macrophages but also a reduced macrophage activation (Figure 2). We studied these macrophage functions further in vitro. Coincubation with cerivastatin (0.05 μmol/L) for 10 days reduced proteolytic activity ascribable to MMP-9 and TF expression. Bellosta et al. and Colli et al. demonstrated similar results on human macrophages in vitro with regard to reduced MMP-9 activity with fluvastatin treatment (5 to 100 μmol/L) and decreased TF expression with fluvastatin (2.5 to 5 μmol/L) or simvastatin (2.5 μmol/L). These results suggest that HMG-CoA reductase inhibitors can suppress not only proliferation but also activation of macrophages.

Lipid lowering by HMG-CoA reductase inhibitors may alter the biology of atherosclerotic lesion formation. Among all vascular cell types, macrophages in particular are involved in all phases of atherosclerosis from initiation through progression and finally plaque rupture and thrombosis. This study demonstrated that cerivastatin can suppress the growth of macrophages that express proteolytic enzymes and a thrombogenic factor in atheroma of animals with endogenous hypercholesterolemia. Cerivastatin, in a concentration that can be achieved in patients, also suppresses proliferation and activation of macrophages in culture. This study provides new evidence for an effect of HMG-CoA reductase inhibitors on macrophage functions beyond lipid lowering and sheds new light on the mechanisms of plaque stabilization and reduced thrombotic complications in patients treated with HMG-CoA reductase inhibitors.

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