Fluvastatin Inhibits Matrix Metalloproteinase-1 Expression in Human Vascular Endothelial Cells

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Abstract—Matrix metalloproteinase-1 (MMP-1), also called interstitial collagenase, may play an important role in the pathogenesis of atherosclerosis and atherosclerotic plaque rupture. We investigated the effects of fluvastatin on MMP-1 expression in human vascular endothelial cells (ECs). The addition of fluvastatin decreased the basal MMP-1 levels in the culture media of ECs in a time-dependent (0 to 48 hours) and dose-dependent (10^{-8} to 10^{-5} mol/L) manner. On the other hand, fluvastatin did not affect tissue inhibitor of metalloproteinase-1 levels. Collagenolytic activity in conditioned media of ECs was also dose-dependently reduced by fluvastatin. The effect of fluvastatin on MMP-1 expression was completely reversed in the presence of mevalonate or geranylgeranyl-pyrophosphate, but not in the presence of squalene. Inhibition of Rho by C3 exoenzyme also significantly decreased MMP-1 expression in ECs. Our findings revealed that fluvastatin decreases MMP-1 expression in human vascular ECs through inhibition of Rho. (Hypertension. 2000;36:325-329.)

Key Words: atherosclerosis ■ nitric oxide ■ extracellular matrix ■ collagen

Unstable atherosclerotic plaque rupture is an important event that triggers acute coronary syndrome. Plaque rupture is frequently correlated with loss of the extracellular matrix at certain locations, often in the shoulder areas of the plaque. Focal destruction of the extracellular matrix renders the plaques less resistant to the mechanical stresses imposed during systole and therefore vulnerable to rupture.1–3 Recent studies have suggested that matrix metalloproteinases (MMPs) may contribute to the vulnerability of atherosclerotic plaques by degrading the components of the fibrous cap: collagens, elastin, fibronectin, and proteoglycans.4 Immuno-cytochemistry studies have demonstrated that MMP-1, MMP-9, and MMP-3 are expressed by cells present in atheromas, including luminal and neovascular endothelial cells, macrophages, and smooth muscle cells, but not by cells present in the walls of normal arteries.5 Studies including in situ zymography and enzymatic activity assays showed a significantly enhanced collagenase activity in atherosclerotic plaques.6–8 The expression of MMP-1, also called interstitial collagenase, in atherosclerotic lesions warrants special attention because this enzyme is involved in the initial cleavage of collagens, mainly type I collagen. Type I collagen is the predominant protein in atherosclerotic plaques that confers strength to the fibrous cap. MMP-1 is also the only enzyme able to initiate the degradation of collagen at neutral pH.

Hydroxymethylglutaralyl coenzyme A (HMG-CoA) reductase inhibitors (statins) have been widely used for treatment of hyperlipidemia. They may also directly interfere with the major processes of atherogenesis occurring in the arterial wall.9 Smooth muscle cell migration and proliferation are inhibited by HMG-CoA reductase inhibitors,10,11 and cholesterol accumulation is prevented in macrophages by reducing modified-LDL endocytosis.12 All of these cellular effects are mediated by inhibition of the isoprenoid pathway. If HMG-CoA reductase inhibitors affect MMP activity, they could influence plaque stability and disease progression of coronary artery diseases. Recently, Bellosta et al13 reported that HMG-CoA reductase inhibitors reduced MMP-9 secretion by macrophages, but their effects on vascular endothelial cells (ECs) have not been determined. We thus investigated whether the HMG-CoA reductase inhibitor fluvastatin modulates MMP-1 expression in normal cultured human vascular ECs.

Methods

Reagents

Fluvastatin, pravastatin, and lovastatin were kindly provided by Tanabe Pharmaceutical Co, Sankyo Co, and Banyu Pharmaceutical Co, respectively. Stock solutions of fluvastatin and pravastatin were made in water. Stock solution of lovastatin was made in ethanol. Before use, the lactone ring of lovastatin was hydrolyzed by a 45-minute incubation in 0.1 mol/L NaOH at 40°C, followed by neutralization with HCl. Recombinant human interleukin-1β (IL-1β) was provided by Otsuka Pharmaceutical Co, Mevalonate, squalene, farnesyl-pyrophosphate (FPP) and geranylgeranyl-pyrophosphate (GGPP) were obtained from Sigma. Clostridium botulinum C3 exoenzyme was purchased from List Biological Laboratory, Inc.
Human EC Culture
Primary ECs were harvested from human umbilical cord veins treated as described elsewhere. Confluent ECs between passages 2 through 4 in serum-free, 0.1% BSA-containing medium were used for the experiments.

Assay for MMP-1 and Tissue Inhibitor of Metalloproteinase-1 Levels
The MMP-1 and tissue inhibitor of metalloproteinase-1 (TIMP-1) concentrations of the culture media were determined with the use of respective ELISA kits according to the manufacturer’s instructions (Amersham International plc). The ELISA assay recognized latent and activated MMP-1. There was no cross-reactivity or interference with MMP-2, -3, and -9. The lower limits of detection of MMP-1 and TIMP-1 were 6.25 ng/mL and 3.13 ng/mL, respectively. MMP-1 and TIMP-1 levels were corrected by protein measurement, and data are shown as micrograms per milligram of protein.

Assay for Collagenolytic Activity
MMP-1 activity was estimated by fluorescent-labeled collagen digestion (Yagai Co). Briefly, fluorescent-labeled collagen tubes (50 μg/50 μL), after addition of 50 μL neutralized fluids (pH 7.5), were mixed with 100-μL samples. Samples were then incubated at 37°C for 3 hours with 10 μL of aminophenyl mercuric acetate (2.4 mg/mL). The reaction was stopped by adding 200 μL of stop solution, and the fluorescence in the supernatants was measured at 520 nm with excitation at 495 nm with a fluorescence spectrometer (Nihon Bunko Corp). Collagenolytic activity was calculated according to the manufacturer’s method.

Statistical Analysis
All values are expressed as mean±SEM of 4 samples, which represented at least 3 separate experiments. The significance of differences was determined with 1-way ANOVA combined with Scheffe’s test. Differences of P<0.05 were considered significant.

Results
Effects of Fluvastatin on MMP-1 and TIMP-1 Levels
We initially investigated MMP-1 levels in conditioned media of ECs incubated with or without fluvastatin (10⁻⁵ mol/L) for 48 hours. As shown in Figure 1A, the basal MMP-1 levels increased in a time-dependent manner, and addition of fluvastatin significantly decreased MMP-1 levels of ECs.

We then investigated the dose-response effect of fluvastatin on MMP-1 levels in conditioned media of ECs. Incubation of ECs with fluvastatin for 48 hours dose-dependently decreased the basal MMP-1 levels (Figure 1B). Addition of IL-1β (10 ng/mL) significantly increased MMP-1 levels of ECs, and fluvastatin also dose-dependently decreased MMP-1 levels in IL-1β-stimulated ECs.

The ECs expressed low levels of TIMP-1, and addition of IL-1β slightly increased TIMP-1 levels (Figure 2). Fluvastatin affected neither basal nor IL-1β-induced TIMP-1 production.

We then investigated the effect of fluvastatin on collagenolytic activity in conditioned media of ECs. Collagenolytic activity reflects mainly MMP-1 activity as well as MMP-8 and MMP-13 activities. As shown in Figure 3, addition of IL-1β for 48 hours significantly increased collagenolytic activity. The addition of fluvastatin dose-dependently reduced both basal and IL-1β-induced collagenolytic activity.

Effects of Mevalonate and Isoprenoids on Action of Fluvastatin
Treatment with HMG-CoA reductase inhibitors causes mevalonate starvation within the cells. Mevalonate metabolism yields a series of isoprenoid compounds, including squalene, FPP, and GGPP. We postulated that the inhibitory effect of fluvastatin might be due to deprivation of these isoprenoid compounds caused by the drug. To test this hypothesis, we incubated ECs with mevalonate or isoprenoid compounds in the presence of fluvastatin. Addition of mevalonate (10⁻⁵ mol/L) or GGPP (15 μmol/L), which is involved in geranylgeranylation of proteins, completely reversed the effect of fluvastatin. Similarly, FPP (15 μmol/L), which is involved in farnesylation of proteins, partially blocked the action of fluvastatin.
Effects of C3 Exoenzyme on MMP-1 Expression

Rho is an important geranylgeranylated protein. To determine whether inhibition of Rho is related to downregulation of MMP-1 expression by fluvastatin, we incubated cells with C3 exoenzyme, which ADP-ribosylates and inactivates Rho. As shown in Figure 5, the addition of C3 exoenzyme decreased the basal MMP-1 levels in ECs in a dose-dependent manner. Neither mevalonate nor GGPP prevented this inhibitory effect of C3 exoenzyme (data not shown).

Effects of Other Statins on MMP-1 Expression

To determine whether the inhibitory effect we observed was specific to fluvastatin, we examined the effect of other HMG-CoA reductase inhibitors, lovastatin and pravastatin. As shown in Figure 6A, incubation of ECs with increasing concentrations of lovastatin (10^-8 to 10^-5 mol/L) also caused a significant decrease of MMP-1 level in conditioned media of ECs. On the other hand, the addition of pravastatin showed no significant effect on MMP-1 levels (Figure 6B).

Discussion

Our findings revealed that fluvastatin decreases MMP-1 expression in human vascular ECs. Some previous studies suggested that MMP activity contributes to the destruction of connective tissues in atherosclerotic lesions leading to surface

Figure 2. Dose-dependent effects of fluvastatin on TIMP-1 levels in ECs. Cells were incubated in 24-well plates in serum-free, 0.1% BSA-containing medium for 48 hours with fluvastatin (10^-8 to 10^-5 mol/L) in presence (diagonal bars) or absence (stippled bars) of IL-1β (10 ng/mL); TIMP-1 levels in culture media were determined by ELISA as described in Methods section. Each value represents mean±SEM of 4 samples.

Figure 3. Dose-dependent effects of fluvastatin on collagenolytic activity in ECs. Cells were incubated in 24-well plates in serum-free, 0.1% BSA-containing medium for 48 hours with fluvastatin (10^-8 to 10^-5 mol/L) in presence (diagonal bars) or absence (stippled bars) of IL-1β (10 ng/mL); collagenolytic activity in culture media was determined by fluorescent collagen-labeled digestion as described in Methods section. Each value represents mean±SEM of 4 samples. *P<0.05 compared control cells indicated as (-).

Figure 4. Effects of mevalonate and isoprenoids on effect of fluvastatin on MMP-1 expression. Cells were incubated in 24-well plates in serum-free, 0.1% BSA-containing medium for 48 hours with mevalonate (10^-4 mol/L), squalene (10^-5 mol/L), farnesyl-pyrophosphate (FPP; 15 μmol/L), or geranylgeranyl-pyrophosphate (GGPP; 15 μmol/L), MMP-1 levels in culture media were determined by ELISA as described in Methods section. Each value represents mean±SEM of 4 samples. *P<0.05 compared control cells.

Figure 5. Effects of C3 exoenzyme on MMP-1 levels in ECs. Cells were incubated in 24-well plates in serum-free, 0.1% BSA-containing medium for 48 hours with C3 exoenzyme (1 to 25 μg/mL); MMP-1 levels in culture media were determined by ELISA as described in Methods section. Each value represents mean±SEM of 4 samples. *P<0.05 compared with control cells indicated as (-).
Several lines of evidence presented in this report suggest the involvement of mevalonate metabolites in reduced MMP-1 expression by HMG-CoA reductase inhibitors (fluvastatin and lovastatin). HMG-CoA reductase catalyzes the conversion of HMG-CoA to mevalonate. Treatment with fluvastatin may cause mevalonate starvation within ECs. This seemed to cause the inhibition of MMP-1 expression, because coincubation with mevalonate completely blocked the fluvastatin-induced reduction of MMP-1 expression. Mevalonate metabolism yields a series of isoprenoid compounds, including squalene, FPP, and GGPP. We also observed that GGPP completely reversed the effect of fluvastatin on MMP-1 expression. Interestingly, mevalonate or GGPP alone did not produce any change in MMP-1 expression (data not shown), indicating that basal intracellular mevalonate and GGPP levels may be sufficient to maximally stimulate MMP-1 expression. Previously, lovastatin was reported to inhibit geranylgeranylated proteins, and this effect was associated with some effects of lovastatin, including regulation of endothelial nitric oxide synthase expression. Among geranylgeranylated proteins, Rho may be the most important and linked to the control of the contractile mechanism in vascular smooth muscle cells. It is unique, small GTP-binding proteins that can be inhibited by C3 exoenzyme. In the present study, inhibition of Rho by C3 exoenzyme significantly decreased the basal MMP-1 levels. These observations suggest that inhibition of Rho secondary to depletion of mevalonate and GGPP may be related to the inhibitory effect of fluvastatin on MMP-1 expression.

In contrast to fluvastatin, pravastatin does not inhibit MMP-1 expression in ECs. It has been demonstrated that fluvastatin and pravastatin exert different effects on smooth muscle proliferation. Fluvastatin inhibits the proliferation of vascular smooth muscle cells in vitro and in vivo, but pravastatin does not. Furthermore, fluvastatin has been shown to inhibit intimal thickening after catheterization-induced injury, an effect that has been attributed to reduced migration and proliferation of smooth muscle cells rather than a serum lipid-lowering action. Pravastatin did not show the same inhibitory effect. Pravastatin inhibits HMG-CoA reductase in the liver to a greater extent than in other organs, and it readily permeates hepatocytes. If fluvastatin, which is more lipophilic than pravastatin, is more permeable through EC membranes, this may explain the different effects of the two HMG-CoA reductase inhibitors on MMP-1 expression. We thus tested the effects of another lipophilic HMG-CoA reductase, lovastatin. Our results indicated that lovastatin also inhibits MMP-1 expression in ECs similarly to fluvastatin.

This observation may be explained by the differences in binding capacities of lipophilic fluvastatin and lovastatin and hydrophilic pravastatin to specific binding sites on ECs. Indeed, van Vliet et al reported that the drug concentration at which 50% inhibition of the sterol synthesis in human ECs (IC_{50} value) was 100-fold higher in pravastatin (8520±3673 nmol/L) than in lovastatin (79.3±12.8 nmol/L) or simvastatin (56.1±35.4 nmol/L). Osamah et al reported that fluvastatin and lovastatin share similar platelet binding sites and inhibit platelet aggregation, whereas pravastatin neither binds to platelets nor inhibits platelet aggregation.

### Figure 6. Effects ofLovastatin and Pravastatin on MMP-1 Levels in ECs.

Cells were incubated in 24-well plates in serum-free, 0.1% BSA-containing medium for 48 hours with (A) lovastatin (10^{-8} to 10^{-5} mol/L) or (B) pravastatin; MMP-1 levels in culture media were determined by ELISA as described in Methods section. Each value represents mean±SEM of 4 samples. *P<0.05 compared with control cells indicated as (-).
In atherosclerotic lesions, collagen is the major component of extracellular matrix, comprising up to 40% of the total protein. The accumulation of collagen is influenced by its de novo synthesis and deposition and by degradation of existing collagen by MMP-1. We found that fluvastatin decreases MMP-1 expression in human vascular ECs at concentrations of $10^{-7}$ to $10^{-5}$ mol/L. The peak concentration ($C_{\text{max}}$) of fluvastatin in the plasma after administration of multiple doses is $\approx 10^{-6}$ mol/L. Thus, clinical concentrations of fluvastatin may induce significant inhibition of MMP-1 expression in ECs in vivo and influence the plaque stability and progression of coronary artery disease.

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**References**

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