HMG-CoA Reductase Inhibitors Reduce MMP-9 Secretion by Macrophages


Abstract—Macrophages secrete matrix metalloproteinases (MMPs) that may weaken the fibrous cap of atherosclerotic plaque, predisposing its fissuration. The 92-kDa gelatinase B (MMP-9) has been identified in abdominal aortic aneurysms and in atherosclerotic tissues. Fluvastatin, through the inhibition of the isoprenoid pathway, inhibits major processes of atherogenesis in experimental models (smooth muscle cell migration and proliferation and cholesterol accumulation in macrophages). We studied the effect of fluvastatin on the activity of MMP-9 in mouse and human macrophages in culture. Conditioned media of cells treated for 24 hours with fluvastatin were analyzed by gelatin zymography. In mouse macrophages, fluvastatin (5 to 100 μmol/L) significantly inhibited in a dose-dependent manner MMP-9 activity from 20% to 40% versus control. The drug, at a concentration as low as 5 μmol/L, inhibited MMP-9 activity (~30%) in human monocyte-derived macrophages as well. Phorbol esters (TPA, 50 ng/mL) stimulated MMP-9 activity by 50%, and fluvastatin inhibited this enhanced activity up to 50% in both mouse and human macrophages. The above results on the secretion of MMP-9 were confirmed by Western blotting and ELISA. The inhibitory effect of fluvastatin was overcome by the simultaneous addition of exogenous mevalonate (100 μmol/L), a precursor of isoprenoids. Fluvastatin’s effect was fully reversible, and the drug did not cause any cellular toxicity. The statin did not block directly the in vitro activation of the secreted protease. Similar data were obtained with simvastatin. Altogether, our data indicate an inhibition of MMP-9 secretion by the drug. This effect is mediated by the inhibition of synthesis of mevalonate, a precursor of numerous derivatives essential for several cellular functions. (Arterioscler Thromb Vasc Biol. 1998;18:1671-1678.)

Key Words: statins ■ macrophages ■ plaque stability ■ metalloproteinases

Advanced human atherosclerotic plaques are characterized by a lipid core covered by a fibrous cap composed of smooth muscle cells (SMCs) and extracellular matrix. These lesions also have chronic inflammatory infiltrates of macrophages and T lymphocytes. Composition and stability of the plaque, rather than its volume (ie, severity of stenosis), are the most important determinants of atherosclerosis complications. Plaque disruption with superimposed thrombosis is the main cause for the acute coronary syndrome of unstable angina, myocardial infarction, and sudden death. Plaque instability, manifesting as ulceration of the fibrous cap, plaque rupture, or intraplaque hemorrhage, is characteristic of plaques with a high lipid content and excess macrophages in the cap.

Disrupted aortic caps contain fewer SMCs (collagen-synthesizing cells) and less collagen than intact caps. Collagen is the main component of fibrous caps responsible for their tensile strength. In addition to the plaque rupture, the matrix composition participates in several key events in the development of the atherosclerotic lesion: cell migration and proliferation, lipoprotein retention, cell adhesion, calcification, thrombosis, coagulation, and apoptosis. A wide range of proteases may be produced at focal sites in plaques. Macrophages are capable of degrading extracellular matrix by phagocytosis or by secreting proteolytic enzymes, in particular a family of metalloproteinases (MMPs) that may weaken the fibrous cap, predisposing its rupture. The 92-kDa gelatinase B, or MMP-9, is the most prevalent form, expressed by virtually all activated macrophages, and has been shown to be more common in atherectomy materials from unstable angina and abdominal aortic aneurysm. In addition to macrophages, SMCs may release MMPs, an event relevant not only to atherogenesis but especially to the process of restenosis after angioplasty. MMPs play a major role in restenosis by liberating the SMC from its pericellular matrix cage as a prerequisite to proliferation and migration. MMPs are a family of Zn²⁺- and Ca²⁺-dependent enzymes, which are important in the resorption of extracellular matrices in both physiological and pathological processes. The MMPs are secreted as the proenzyme and, once activated, can completely degrade all extracellular matrix components. The regulation of these enzymes is very important and occurs at 3
levels: transcription, activation of latent proenzymes, and inhibition of proteolytic activity. Interleukin-1, platelet-derived growth factor, and tumor necrosis factor-α stimulate the synthesis of MMPs; transforming growth factor-β (TGF-β), heparin, and corticosteroids have an inhibitory effect. Plasmin is a potent proenzyme activator of most MMPs, but plasmin-independent pathways also exist. In vitro experiments have shown that phorbol esters (12-O-tetradecanoylphorbol 13-acetate (TPA)) and lipopolysaccharide can stimulate MMP secretion. MMPs are inhibited by a family of naturally occurring specific inhibitors (tissue inhibitors of metalloproteinases (TIMPs)), which are secreted multifunctional proteins essential in the regulation of connective tissue metabolism.

In addition to lipid-lowering therapy, which most probably affects the size of the atheromatous core, plaque stabilization could be achieved by a direct inhibition of MMPs in the arterial wall. Along with TGF-β, corticosteroids, and heparin, several synthetic inhibitors have been investigated: namely, tetracyclines, antracyclines, and synthetic peptides. The efficacy of these molecules in therapy, however, is questionable. The inhibition of MMP activity also has been investigated in tumor therapy.

In our laboratory, we demonstrated that fluvastatin, a hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase inhibitor (statin), may directly interfere with the major processes of atherogenesis occurring in the arterial wall. SMC migration and proliferation are inhibited by the drug, and cholesterol accumulation is prevented in macrophages by reducing modified-LDL endocytosis. Interestingly, the inhibitory activity observed in macrophages was more pronounced in cholesterol-loaded cells than in normal cells, suggesting a potential selectivity of the drug for the foam cells and hence for the atherosclerotic lesion. All cellular effects mentioned above are mediated by inhibition of the isoprenoid pathway. Because the isoprenoid pathway is involved in several cellular processes and macrophage-derived foam cells constitutively produce MMPs, we studied the effect of fluvastatin on secreted MMP-9 activity in macrophages in vitro.

Methods

Cell Culture

Mouse peritoneal macrophages (MPMs) were collected by peritoneal lavage with PBS from mice given a 0.3 mL intraperitoneal injection of 4% thioglycollate in water. The MPMs were pelleted, washed twice with serum-free Dulbecco’s modified Eagle’s medium (DMEM; GibCO BRL), plated at a density of 3×10^6 cells/35-mm dish, and allowed to adhere to dishes for 2 hours in DMEM containing 10% FBS. Then, plates were washed 3 times with DMEM to remove nonadherent cells and incubated in DMEM containing 10% FBS until the day of the experiment.

Circulating human monocyte-derived macrophages (HMs) were isolated from the blood of healthy donors. In brief, blood was centrifuged, and the buffy coats were underlayered with Ficoll-Paque (Pharmacia) and centrifuged at 320g for 35 minutes at 25°C. The monocytes in a broad band below the interface were collected using a siliconized Pasteur pipette and washed 3 times with cold PBS. The final pellet was resuspended in serum-free DMEM, and the cells were plated at a density of 3×10^6 cells in a 35-mm dish. After 2 hours, cell monolayers were washed twice, and the adherent cells were incubated for 10 to 14 days with DMEM containing 10% human AB serum and insulin (8 µg/mL).

To generate the conditioned media, cells were incubated for 24 hours at 37°C with DMEM supplemented with 0.2% BSA (Sigma) and increasing concentrations of fluvastatin in the absence or presence of phorbol esters (TPA, 50 ng/mL; Sigma). At the end of the incubation, the conditioned media were collected, and the activity of secreted MMP-9 was analyzed by zymography. Cellular protein content was measured according to Lowry et al. Cellular toxicity caused by the drug was assessed using the dimethylthiazol-diphenyltetrazolium bromide assay (MTT), which relied on the ability of viable cells to actively metabolize a tetrazolium dye.

SDS–Polyacrylamide Gel Electrophoresis Zymography

Electrophoresis was performed on samples (40 µL, for MPMs and 5 µL for HMs of conditioned medium per lane) at 4°C on 7.5% polyacrylamide gels containing 10% SDS and gelatin (1 mg/mL) under nonreducing conditions and without boiling. After electrophoresis, SDS was removed from gels in 2 washes with 2.5% Triton X-100 (Sigma) at room temperature. After washes, the gels were incubated overnight at 37°C with gentle shaking in Tris (50 mmol/L; pH 7.5) containing NaCl (150 mmol/L), CaCl_2 (110 mmol/L), and ZnCl_2 (1 µmol/L) to activate the MMP’s ability to digest the substrate. For inhibition studies and to confirm the identity of MMPs, identical gels were incubated in the above buffer containing either EDTA (20 mmol/L), an inhibitor of MMPs, or PMSF (1 mmol/L), an inhibitor of serine proteinases. The addition of PMSF did not alter MMP-9 activity, whereas the treatment with EDTA completely abolished it (data not shown). At the end of the incubation, the gels were stained with a solution of 0.1% Coomassie brilliant blue R-250 (Sigma) in 25% methanol and 7% acetic acid. Clear zones against the blue background indicated the presence of proteinolytic activity. It is important to note that in this SDS-containing gel, the latent form of MMP-9, pro–MMP-9, and the activated gelatinase develop gelatinolytic activity. Therefore, we used the word “activity” to indicate the total gelatinolytic capacity measured in the conditioned media, which, in our experimental conditions, is due entirely to the 92-kDa pro–MMP-9, as assessed by zymography and Western blot analysis (see experimental data), and after activation by incubation for 2 hours at 37°C with 2 mmol/L APMA (4-aminophenylmercuric acetate), the propeptide is cleaved to the active form (<90-kDa; data not shown).

Western Blot Analysis

Aliquots of the conditioned media (40 µL per lane) were run on 10% polyacrylamide gel containing SDS, under nonreducing conditions. The proteins were blotted to nitrocellulose membranes (Bio-Rad) and incubated with a 3% solution of defatted dried milk in PBS containing 0.1% Tween 20 (PBS-T) to block nonspecific binding. Then a mouse monoclonal antibody anti–human MMP-9 (clone 6-6B; Calbiochem) diluted in PBS-T was added, and the incubation continued for 1 hour. This antibody recognizes both the latent (92 kDa) and active (83 kDa) forms of human MMPs-9 under nonreducing conditions but only the latent form under reducing conditions. The bound primary antibody was detected using an anti-mouse secondary antibody conjugated to horseradish peroxidase (Sigma) and the enhanced chemiluminescence kit (Amersham) according to the manufacturer’s instructions.

Northern Blot Analysis

RNA was extracted (RNAzol B; Tel-Test Inc.) from 3×10^3 to 4×10^4 primary HMs cultured for 12 days and treated with and without 50 µmol/L fluvastatin for 24 hours before harvest. Equal amounts of total RNA (10 µg) were denatured in 2.2 mol/L formaldehyde, 50% deionized formamide, and 50 mmol/L MOPS (pH 7.0) at 55°C and electrophoresed on 1% agarose gels containing 3.4% formaldehyde. RNA was blot-transferred to GeneScreen Plus membranes using 10× SSC and fixed with UV irradiation. Blots were prehybridized at 68°C for 1 hour. An MMP-9 probe was generated from THP-1 cells.
treated with TPA by reverse transcription–polymerase chain reaction (PCR) of total RNA using the following primers: sense, 5′ TGGGCAGATTCCAAACCTTTGAGGGC 3′ and antisense, 5′ CCATTCACGTCGTCCTTATGCAAGGG 3′. The 1003-bp product was subcloned into the pCRII vector, and its sequence was verified as MMP-9. Purified MMP-9 probe (QIAquick, Quiagen, 25 ng) generated by PCR from the vector or a G3PDH probe (Clontech) were labeled with [32P]dCTP by random priming and incubated with the blots in ExpressHyb (Clontech) for 1 hour at 68°C. Blots were washed (2× SSC; 0.05% SDS) exhaustively at room temperature and given a final wash at 50°C (0.1× SSC; 0.1% SDS) for 10 minutes. Blots were exposed for autoradiography (X-OMAT AR, Kodak) and then placed on a Bio-Rad BI screen for quantitative analysis in the Bio-Rad GS 363 Molecular Imager. After autoradiography to detect MMP-9 mRNA, the blot was stripped by incubation in 0.01% SDS, 0.01× SSC for 15 minutes at 100°C. The blot then was reprobed for the G3PDH housekeeping gene.

ELISA
The amount of secreted MMP-9 protein was quantified using the highly specific Biotrak Matrix MMP-9 ELISA systems (Amersham). The MMP-9 assay uses 2 antibodies directed against different epitopes of MMP-9 and does not show detectable cross-reactivity with MMP-1, -2, and -3 and TIMP-1 and -2 (Amersham). Aliquots of conditioned media were analyzed as suggested by the manufacturer.

Statistical Analysis
For quantitation of Western blots and zymograms, densitometric scanning was performed using a system incorporating a video camera and a computer analysis package (NIH Image 1.52 image analysis software). Each experiment was performed at least 3 times with different preparations of cells. Background was set for each gel, and each lane was analyzed sequentially. Results were normalized by cellular protein content and expressed as optical density units. To validate the method, a linear response of optical density units versus dilution was obtained for different serial dilutions on 2 separate samples (data not shown). To standardize conditions between gels, an aliquot of a standard sample was loaded on each gel, and values for each band then were normalized to the value of the band of the reference sample run on the same gel. Data are presented as mean±SD and analyzed using the Student t test. P values <0.05 were considered statistically significant.

Results
To study the effect of fluvastatin on MMP-9 activity, we incubated MPMs for 24 hours with increasing concentrations of the drug. The conditioned media then were collected and analyzed by gelatin zymography. This technique allows the visualization of both the active and proenzyme forms of gelatinases. In our experimental conditions, we observed only the pro–MMP-9 form (Figure 1). The analysis of the data obtained in 3 different experiments showed that the addition of fluvastatin (5 to 100 μmol/L) significantly inhibited in a dose-dependent manner MMP-9 activity from 20% to 40% versus control (nontreated cells) (Figure 2A). As expected, dexamethasone, a corticosteroid known to inhibit MMP gene transcription,7 suppressed almost completely the enzyme activity (Figure 2A). In the next series of experiments, we stimulated MMP activity by incubating the cells with phorbol ester (TPA, 50 ng/mL), a known enhancer of MMP expression.7 Treatment with TPA increased MMP-9 activity >50%.
and fluvastatin was still effective in inhibiting the enhanced protease activity by almost 50% (Figure 2B).

Our previous data have shown that fluvastatin may directly interfere with the major processes of atherogenesis occurring in the arterial wall through the inhibition of the isoprenoid pathway. Because mevalonate is the precursor of isoprenic compounds, we postulated that the inhibitory effect of fluvastatin might be due to a deprivation of mevalonate caused by the drug. To test this hypothesis, we incubated the cells with fluvastatin in the presence of exogenous mevalonate (100 μmol/L). As shown in Figure 3, the addition of mevalonate fully overcame the inhibitory effect of the statin, in both the absence and presence of TPA.

To determine whether the inhibitory effect of fluvastatin was due to an interference with the activation process after MMP-9 had been secreted, we incubated MPMs with medium alone. After 24 hours, the conditioned media were collected, due to an interaction of the drug with the protease already secreted into the media, as shown by gelatin zymography. Fluvastatin still reduced the amount of MMP-9 protein released into the incubation media, as shown by Western blot analysis (Figure 6B).

To exclude a possible drug toxicity as the reason of its inhibitory effect, MPMs were incubated for 24 hours with fluvastatin (5 μmol/L) or fluvastatin + mevalonate (100 μmol/L) in the absence or presence of TPA (50 ng/mL). Conditioned media were collected and MMP-9 activity measured by gelatin zymography. Data are mean ± SD of 3 experiments performed in duplicate. Student t test: a indicates P < 0.05 vs control; b, P < 0.05 vs fluvastatin alone; c, P < 0.05 vs TPA alone; and d, P < 0.05 vs TPA + fluvastatin.

Figure 3. Mevalonate prevents the inhibitory effect of fluvastatin on MMP-9 activity in MPMs. Mouse macrophages were incubated for 24 hours with fluvastatin (5 μmol/L) or fluvastatin + mevalonate (100 μmol/L) in the absence or presence of TPA (50 ng/mL). Conditioned media were collected and MMP-9 activity measured by gelatin zymography. Data are mean ± SD of 3 experiments performed in duplicate. Student t test: a indicates P < 0.05 vs control; b, P < 0.05 vs fluvastatin alone; c, P < 0.05 vs TPA alone; and d, P < 0.05 vs TPA + fluvastatin.

Table 1. Reversibility of the Inhibitory Effect of Fluvastatin on MMP-9 Activity

<table>
<thead>
<tr>
<th>Additions, μmol/L</th>
<th>Treatment</th>
<th>Washout</th>
</tr>
</thead>
<tbody>
<tr>
<td>No addition</td>
<td>1153 ± 108</td>
<td>1139 ± 66</td>
</tr>
<tr>
<td>Fluvastatin, 5</td>
<td>899 ± 38</td>
<td>1261 ± 15</td>
</tr>
</tbody>
</table>

Cells were incubated for 24 hours with medium alone or containing fluvastatin (5 μmol/L). Media then were collected, and the cells were incubated for 24 hours in the absence of the drug. MMP-9 activity was measured by zymography at both time points. Student t test: *P < 0.05 vs control (nontreated cells).

Data further confirm that fluvastatin is not toxic and that its inhibitory effect is reversible.
incubation of mouse macrophages with increasing concentrations of the latter statin caused an inhibition in MMP-9 activity similar to that observed with fluvastatin.

Finally, we evaluated by Northern blot analysis whether the reduced levels of secreted MMP-9 in fluvastatin-treated HMs could reflect a reduction in MMP-9 gene expression. As shown in Figure 7, Northern blots of HMs treated for 24 hours with 50 μmol/L fluvastatin demonstrated no reduction in the level of MMP-9 gene expression. Instead, an almost 2-fold increase in mRNA levels for MMP-9 was consistently observed. In contrast, essentially no change was observed in the levels of the housekeeping gene G3PDH.

Discussion

In this study, we have demonstrated that the in vitro incubation of mouse macrophages and HMs with fluvastatin or simvastatin, 2 hypolipidemic and antiatherosclerotic agents, reduced the amount of MMP-9 secreted, suggesting that the effect on MMP-9 activity belongs to the statins as a class of drugs. Cells were treated for 24 hours with increasing concentrations of fluvastatin, and the data clearly show that the inhibition of the HMG-CoA reductase enzyme reduces the amount of protease released into the incubation media. This effect is reversible and appears to be mediated through a starvation of mevalonate and its derivatives, because the coincubation of the cells with fluvastatin and mevalonate completely overcame the inhibitory effect of the drug. The experiments show that fluvastatin reduced MMP-9 activity in gelatin zymography and that the effect of the drug was also maintained when MMP-9 production was induced by the coincubation with TPA, a known inducer of MMP gene transcription. TPA induced a 1.5- to 2.5-fold increase in

Figure 4. Fluvastatin inhibits MMP-9 activity in HMs. HMs were incubated for 24 hours with increasing concentrations of fluvastatin in the absence (A) or presence (B) of TPA (50 ng/mL). Conditioned media were collected and MMP-9 activity measured by gelatin zymography. Data are mean±SD of 3 experiments performed in duplicate. Student t test. A, a indicates P<0.05; b, P<0.005 vs control (non-treated cells). B, a indicates P<0.01 vs control; b, P<0.01 vs TPA alone.

Figure 5. Representative Western blot analysis of MMP-9 secretion by HMs and quantitation by densitometry. HMs were incubated for 24 hours with fluvastatin (5 or 50 μmol/L) or fluvastatin (5 μmol/L) plus mevalonate (100 μmol/L). Conditioned media were collected and MMP-9 secretion assessed by Western blot analysis.
MMP-9 activity in mouse macrophages and HMs, respectively. In these conditions, fluvastatin inhibited MMP-9 activity up to 60% compared with the value obtained in cells incubated with TPA alone.

The inhibitory effect of fluvastatin on MMP-9 activity detected by zymography could be the consequence of direct interference of the drug with the activation process of the MMPs after this has been secreted. Our data exclude this hypothesis, because the drug did not have any effect on the activity of MMP-9 already secreted into the growth media. In addition, as evaluated by Western blot analysis and ELISA, fluvastatin reduced the amount of MMP-9 protein released by the cells, suggesting a direct effect on the secretion process.

The reduced levels of MMP-9 detected in the media by ELISA, Western blot analysis, and zymography could also be the result of a fluvastatin-mediated decrease in MMP-9 gene expression. Our data clearly indicate that this is not the case, because mRNA levels actually rose in treated cells. This would suggest a complex mechanism of action of the drug affecting posttranscriptional processes for MMP-9. A more detailed examination of these processes, beyond the scope of this article, will be required to fully understand the mechanism(s) involved.

In addition to TGF-β, corticosteroids, and heparin, several synthetic inhibitors of MMPs have been investigated: viz, tetracyclines, antracyclines, and synthetic peptides. The synthetic inhibitors of MMPs have been investigated: viz, HMs.
several protein-protein interactions and in signal transduction. 36 Prenylated proteins of the Rab subgroup, small Ras-like GTPases in mammalian cells, have an important role in regulating membrane traffic and exocytic and endocytic transport processes. 37 An inhibition of Rab prenylation causes a block in protein secretion, 38 and the Rab3A is deeply involved in regulated secretion in neuronal cells. 36 Therefore, it is conceivable that the inhibition of isoprenoids formation by statins could lead to a reduced MMP-9 secretion by macrophages, by inhibiting factor(s) essential for the secretion process.

Extrapolation of our in vitro results to the in vivo condition is difficult, of course. The final net level of proteinase activity depends on several factors, such as the relative concentrations of active enzymes and specific inhibitors (ie, TIMPs), and further studies are required to assess the in vivo relevance of our observation. Nevertheless, several clinical studies demonstrated the ability of statins to reduce the incidence of coronary heart disease, 19,40 most probably by increasing the stability of the atherosclerotic plaque rather than by reducing the stenotic occlusion. 3 This effect may involve, in addition to cholesterol reduction, a direct effect of these drugs on the arterial wall. We previously suggested that statins may reduce modified LDL endocytosis and cholesterol accumulation in macrophages. 34 The inhibitory effect of statins on MMP-9 secretion observed in the present study suggests an additional potential mechanism for the stabilizing effect of these drugs on atherosclerotic plaque. 41

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References


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