Statins Reduce Inflammation in Atheroma of Nonhuman Primates Independent of Effects on Serum Cholesterol

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Objective—Some of the statin-induced reduction in cardiac events in patients with atherosclerosis may be derived from mechanisms independent of lipid lowering. This study tested in nonhuman primates whether statins can influence inflammation (indicated by vascular cell adhesion molecule-1, interleukin-1β, tissue factor, and macrophages) and features of plaque stability (indicated by collagen and smooth muscle cells) independent of their effect on plasma cholesterol level.

Methods and Results—Adult male cynomolgus monkeys (n=12 per group) consumed an atherogenic diet for 12 months while receiving (1) no treatment (control), (2) pravastatin (Prava, 40 mg/kg per day), or (3) simvastatin (Simva, 20 mg/kg per day). Dietary cholesterol was adjusted to equalize plasma cholesterol levels among groups. Although the intima/media ratio in the abdominal aorta did not differ among groups, drug treatment reduced inflammation and features of plaque vulnerability. Macrophage content in the lesions of statin-treated animals was lowered (2.4-fold with Prava and 1.3-fold with Simva; both P<0.001 versus control). Furthermore, lesions had ≈2-fold less vascular cell adhesion molecule-1, interleukin-1β, and tissue factor expression in statin-treated versus control animals (P<0.005). Lesional smooth muscle cell and collagen content was 2.1-fold greater in the Prava-treated group (P<0.001) and 1.5-fold greater in the Simva-treated group (P<0.005) than in the control group.

Conclusions—In primates, these results provide further support for the beneficial effect of statins on plaque inflammation and stability in addition to cholesterol lowering. (Arterioscler Thromb Vasc Biol. 2002;22:1452-1458.)

Key Words: atherosclerosis ▪ statins ▪ inflammation ▪ macrophages ▪ serum cholesterol

Traditional angiographic evaluation of atheroma has focused on the degree of luminal stenosis. Recent clinical evidence from several angiographic studies suggests that many lesions that cause clinical events do not produce high degrees of luminal narrowing. Moreover, angiographically monitored lipid-lowering studies in humans have shown marked reduction in acute coronary events in the face of modest increases in luminal diameter.

These findings indicate that qualitative aspects of atheroma (and not solely the degrees of luminal stenosis) critically influence the propensity of atheroma to cause acute coronary syndromes, including unstable angina pectoris and myocardial infarction.

We now recognize that plaque disruption, either frank fracture of the fibrous cap or superficial erosion, underlie the thromboses that cause most of these events.

Sites of fatal plaque disruption in humans have abundant inflammatory cells, especially macrophages. We and others have proposed roles for macrophage-derived proteases that degrade the arterial extracellular matrix in plaque disruption. Previous studies have shown proteolytic activity in the shoulder regions of human atheroma, frequent sites of plaque rupture, and have defined macrophages as a major source of matrix metalloproteinases (MMPs) in vivo and in vitro.

Intimal tissue factor (TF), a potent procoagulant, may accelerate thrombus formation at sites of plaque disruption. We recognize now that macrophages, by expressing both MMPs and TF, contribute importantly to 2 major determinants of fatal coronary events, plaque disruption and thrombus formation.

Several clinical trials have demonstrated that statin therapy reduces cardiovascular events and mortality, probably by “stabilizing” atherosclerotic plaques. Furthermore, animal studies have shown that lipid lowering can improve features of plaque stability, a concept supported by pilot observations in humans.

One recent study indicated that 3 months of pravastatin (Prava) treatment improved the features of plaque stability in patients undergoing carotid endarterectomy. The degree to which mechanisms beyond lipid lowering contribute to improved clinical outcomes remains controversial. Several independent studies by Ridker and colleagues and Albert et al have demonstrated that various statins decrease C-reactive protein, an index of inflammation,
independent of serum cholesterol changes. Importantly, statins consistently reduce cerebrovascular events, although the LDL cholesterol (LDL-C) level poorly predicts the risk of stroke.23,24

Despite these intriguing hints, direct mechanistic assessment of cholesterol-independent mechanisms of cardiovascular event reduction in patients presents considerable obstacles. Therefore, we have used nonhuman primates with atherosclerosis as a way to pose mechanistic questions in this regard.

Our previous experiments have shown that Prava produces cholesterol-independent improvement in endothelial cell (EC) vasodilator function, decreased macrophage content, and increased smooth muscle cell (SMC) content in atheroma of nonhuman primates.25 Although a decrease in macrophage content reflects an anti-inflammatory effect, the previous study did not examine more specific markers of inflammation. The present observations on a different cohort tested the hypothesis that statins reduce inflammation, indicated by vascular cell adhesion molecule (VCAM)-1, interleukin (IL)-1β, or vascular TF expression, under conditions that held serum cholesterol constant in nonhuman primates with advanced fibrofatty lesions resembling, in many respects, human atheroma. The present study also evaluated 2 different members of the statin family to determine whether non–cholesterol-dependent anti-inflammatory action depended on a specific structure of a particular agent.

Methods

Animal Model/Experimental Design

Thirty-nine adult male cynomolgus monkeys (Macaca fascicularis) were initially fed a moderately atherogenic diet containing 0.28 mg cholesterol per calorie of diet, with 16.7% from protein, 45.1% from lipids, and 38.1% from carbohydrates. After consuming the atherogenic diet for 3 months, the monkeys were divided into 3 groups (n=13 each) that were equivalent in their total plasma cholesterol (TPC), LDL-C, and HDL cholesterol (HDL-C) concentrations; these groups consumed the atherogenic diet and received statin (or control) treatment for an additional 15 months. Control monkeys were fed the atherogenic diet with no added statins. Prava-treated monkeys had 40 mg Prava/kg body wt per day added to the atherogenic diet. Simvastatin (Simva)-treated monkeys consumed 20 mg Simva/kg body wt per day. All procedures performed on the monkeys were approved by the Institutional Animal Care and Use Committee.

Because 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors work primarily on endogenously produced cholesterol and because in cholesterol-fed monkeys plasma cholesterol is derived primarily from exogenous sources, monkeys must receive a large dose of the drug. We sought to examine the cholesterol-lowering independent effects of these two 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors on cardiovascular pathophysiology. Therefore, dietary cholesterol was adjusted on a monthly basis to maintain equivalent plasma TPC, HDL-C, and LDL-C concentrations among groups. This required only minor adjustments in the dietary cholesterol. Cholesterol concentrations at the end of the treatment period were 0.28 mg cholesterol per calorie of diet in the control group, 0.31 mg cholesterol per calorie of diet in the Prava-treated group, and 0.30 mg cholesterol per calorie of diet in the Simva-treated group.

Plasma cholesterol concentrations were measured monthly during the treatment period. Cholesterol and triglyceride analyses used enzymatic methods on the COBAS FARA II analyzer, with protocols and reagents supplied by Boehringer-Mannheim. HDL-C concentrations were determined by using the heparin-manganese precipitation procedure (Burstein and Samaille test, 1960) as described in the Manual of Laboratory Operations of the Lipid Research Clinical Program.26

Angiography

Coronary artery angiography was performed as described previously.27,28 Briefly, a custom-designed 3F catheter was advanced into the left main coronary artery through the left femoral artery by using fluoroscopic guidance. This was followed by a series of 2-minute intracoronary infusions: (1) 5% dextrose in water (control), (2) acetylsalicylic (10−4, 10−3, and 10−2 mol/L final concentration in the coronary circulation), (3) control, and (4) nitroglycerin (50 μg/min), with a 5-minute delay between infusions.

Immunocytchemistry

Serial cryostat sections (6 μm) from 3 levels of abdominal aorta (celiac artery, left renal artery, and just proximal to iliac artery bifurcation) were fixed in acetone (−20°C, 5 minutes), air-dried, and stained with the avidin-biotin-peroxidase method. Tissue sections were treated with 0.3% hydrogen peroxide to inhibit endogenous peroxidase activity and incubated with primary antibodies diluted in PBS supplemented with 4% of the species-respective normal serum. Subsequent processing was performed according to the manufacturer’s recommendations (Universal DAKO LSAB Kit, peroxidase; DAKO Co). The reaction was visualized with 3-amino-9-ethylcarbazole as the substrate (Sigma Chemical Co). Sections were counterstained with Gill’s hematoxylin solution (Sigma Chemical Co).

Antibodies used for the present study were raised against human antigens. Cross-reactivity of some antibodies has been shown.29 Please see details in the expanded Methods section (available online at http://www.atvb.ahajournals.org).

Staining of Collagens Type I and III by Picrosirius Red

For staining of collagens type I and III, we used a method published by Junqueira et al.30 Please see details in the expanded Methods section (available online at http://www.atvb.ahajournals.org).

Statistical Analyses

Values shown are mean±SEM. ANOVA was used to detect a treatment effect on morphometric analysis of lesion size, vascular reactivity, and plasma lipids and lipoproteins. For post hoc analysis of the data, the Duncan multiple comparison procedure was used.

For immunohistochemical analysis, a computer-assisted color image quantification (ImagePro Plus) was used. The percentage of the total area with positive color for each section was recorded. Two observers unaware of the origin of the samples performed grading of VCAM-1, MMP, and tissue inhibitor of metalloproteinases (TIMP) immunostaining. Data are presented as mean±SD and were compared between groups by the Student t test. A value of P≤0.05 was considered significant.

Results

Plasma Lipid and Lipoprotein Concentrations

Consumption of the atherogenic diet increased TPC, LDL-C, and triglyceride concentrations and reduced HDL-C concentrations in all groups (P<0.05 versus baseline). Dietary adjustment of cholesterol maintained equivalent plasma cholesterol concentrations among groups (please see online Table 1, available at http://www.atvb.ahajournals.org).

EC Function in Statin-Treated Monkeys

Vascular Reactivity

Acetylsalicylic produced a −15±3% change in the left circumflex coronary arterial diameters of control monkeys.
Monkeys that received either statin showed a diminished constrictor response to $10^{-6}$ mol/L acetylcholine: Prava to $-3.1\%$ (by $80\%$) and Simva to $-5.4\%$ (by $64\%$). $P<0.05$ for each versus control. There was no significant difference between 2 groups treated with different statins. Intracoronary infusion of nitroglycerin produced similar dilation of the left circumflex coronary artery in all groups.

**Expression of VCAM-1**

The expression of VCAM-1 was less pronounced in the Prava and Simva groups compared with the control group. The semiquantitative score for VCAM-1 immunoreactivity of almost 3 ($2.8\pm0.99$, indicating strong staining in 10% to 50% of ECs) in the control arteries decreased to $1.4\pm0.8$ ($P<0.02$) in monkeys treated with Prava and to $1.7\pm0.8$ ($P<0.05$) in monkeys treated with Simva (indicating weak staining in $<50\%$ or strong staining in $<10\%$ of ECs). Grading criteria were as follows: grade 0, no staining; grade 1, weak staining in $<50\%$ of ECs; grade 2, weak staining in $<50\%$ or strong staining in $>10\%$ of ECs; grade 3, strong staining in 10% to 50% of ECs; and grade 4, strong staining in $\approx100\%$ of ECs.

**Morphological Characteristics of Features of Plaque Stability and Vulnerability**

Plaque size, measured as intimal area, medial area, and intima/media ratio, was similar in all groups tested. These data indicate that treatment with statins did not affect the size of aortic atheroma under these conditions of constant cholesterol (data not shown).

More detailed analysis of plaque composition disclosed substantial differences between treated and control groups. We used certain well-established characteristics of vulnerable plaque, such as relative amount of SMCs and macrophages, as well as collagen and lipid content. Compared with the control group, both statin-treated groups had significantly higher intimal SMC content in the abdominal aortas (Figure 1A). Levels of interstitial collagen in Prava- and Simva-treated atherosclerotic plaques ($26.3\pm5.7\%$ and $20.4\pm3.8\%$,
respectively) significantly \((P<0.005)\) exceeded the levels in nontreated animals \((12.7\pm2.4\%)\), corresponding to the content of SMCs, the source of most arterial extracellular matrix. In contrast, intimal macrophage and lipid content were lower in the statin-treated groups. In all groups, macrophages localized mostly in the shoulder region of plaques and in areas surrounding the lipid core. However, the percentage of intimal area positive for macrophages disclosed by staining with a specific monoclonal antibody was significantly less \((P<0.001, n=10\text{ per group})\) only in the Prava-treated group \((13.3\pm6.1\%)\) compared with control group \((30.4\pm8.3\%)\); see Figure 1B. Lipid accumulation also was less pronounced in the Prava-treated group \((17.5\pm3.5\%, P<0.001)\) compared with the untreated control group \((31.6\pm2.6\%)\). The Simvastatin-treated group showed a trend toward reduced macrophage \((21\%)\) and lipid \((22\%)\) content compared with the control nontreated group, defined as 100%: however, these variables did not achieve statistically significant changes \((P<0.1\text{ for macrophages, } 24.0\pm6.7\% \text{ and } 24.6\pm7.6\% \text{ for lipid content})\) by the Student \(t\) test (Figure 1B).

Expression of MMPs and Their Inhibitors in Monkey Atheroma

In sections of abdominal aortas uninvolved in atherosclerosis, the medial SMCs contained immunoreactive MMP-2 and its selective inhibitor TIMP-2 (Figure 2A, left; Table). Our previous biochemical studies have shown that constitutively expressed MMP-2 in SMCs is complexed with TIMP-2 in its inactive zymogen form. These uninvolved aortic segments did not contain immunoreactive MMP-3 (Figure 2A, top right) or MMP-9 (data not shown) but consistently displayed expression of TIMP-1 in SMCs (Figure 2A, bottom right; Table). These findings indicate that the inhibitors of MMPs in

**Figure 2.** A, Inverse distribution of MMPs and TIMPs in the nonatherosclerotic aorta. Medial SMCs express MMP-2 as well as its inhibitor TIMP-2 (left). However, they do not contain immunodetectable MMP-3 but do express its inhibitor TIMP-1 (right). B, Increased MMP expression in a fatty streak type intimal lesion. Intimal macrophages contain MMP-3, whereas medial SMCs express little or no immunodetectable MMP-3. Conversely, only medial SMCs show TIMP-1 staining. C, Increased MMP expression in intima of fibrofatty plaque. SMCs and macrophage-derived foam cells express MMP-3, whereas medial SMCs showed more pronounced staining for TIMP-1 (bottom right).

**Expression of MMPs and TIMPs in Atherosclerotic Lesions in Monkeys**

<table>
<thead>
<tr>
<th>Lesion Type</th>
<th>MMP-2</th>
<th>MMP-3</th>
<th>MMP-9</th>
<th>TIMP-1</th>
<th>TIMP-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal aorta ((n=9))</td>
<td>I</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Fatty streak ((n=6))</td>
<td>I</td>
<td>++</td>
<td>±</td>
<td>−</td>
<td>+++</td>
</tr>
<tr>
<td>Atheroma ((n=5))</td>
<td>I</td>
<td>++</td>
<td>±</td>
<td>++</td>
<td>±</td>
</tr>
</tbody>
</table>

Semiquantitative grading of immunohistochemistry for MMPs and TIMPs: (−) no staining, (±) patchy and weak staining, (+) uniform weak staining, (+) moderate staining, and (++++) uniform, intense staining. I indicates intima; M, media; NA, not available.
the normal uninvolved arteries prevail over active enzymes, as we have shown in human arteries. Fatty streak-like atherosclerotic lesions generally exhibited higher levels of expression of MMPs than did uninvolved arteries, particularly in neointimal foam cells (Figure 2B). Conversely, the expression of the MMP inhibitors was much more prominent in the underlying media than in the expanded intima (Figure 2B, Table), indicating an excess of active protease over inhibitors in these intimal lesions.

This pattern increased in more advanced atheroma, containing clusters of macrophages in the shoulder area and the lipid core. Intimal SMCs and macrophages frequently colocalized with immunoreactive MMPs (Figure 2C, Table) but did not produce TIMP-1. Conversely, medial SMCs contained much less immunoreactive MMP but constitutively exhibited TIMPs (Figure 2C, bottom; Table).

Intimal macrophage-derived foam cells produce certain cytokines (IL-1β) that can augment MMP expression. Interestingly, control group lesions, which contain more macrophages (Figure 1B), have more pronounced staining for IL-1β (8.6±4.9%) than those in the Prava-treated (1.4±1.7%, P<0.01) or Simva-treated (3.4±3.4%, P<0.05) groups (Figure 3, top).

TF Expression in Atheroma of Prava-Treated Monkeys

All cell types in monkey lesions expressed TF protein, but intimal macrophages by far predominated as a source of this procoagulant (Figure 3, bottom left panel). These results resemble observations with rabbit atheroma. Color image analysis showed substantially (~30-fold) less TF staining in the intimal lesions of the Prava-treated animals compared with the control animals (0.22±0.4 versus 6.2±4.9, respectively; P<0.02). The TF content of monkey atheroma was significantly correlated with the macrophage area, consistent with a key role for these inflammatory cells in the regulation of plaque thrombogenicity. Treatment with Simva did not produce a significant difference in TF expression compared with no treatment (control). Lack of staining with an irrelevant mouse IgG type and class matched for the TF antibody established the specificity of the signal (data not shown).

Discussion

Pharmacological lipid lowering reduces clinical events in patients despite relatively modest improvement in luminal stenosis monitored angiographically. Recently, plaque characteristics (other than size alone) that may influence propensity to rupture and cause thrombosis have begun to receive attention. The mechanism by which statin treatment prevents atherosclerotic events in humans remains uncertain. Lipid lowering likely contributes to these clinical benefits. However, the extent to which non–lipid-dependent effects of statins influence plaque biology in vivo has engendered controversy. Studies of human atherosclerotic lesions do not permit systematic analysis of optimally preserved specimens sampled from the same arterial site. Moreover, it is difficult to control for confounding variables in human studies, eg, heterogeneity in concomitant drug treatment, prior surgery, and cardiovascular risk factors. The use of nonhuman primates obviated many of these limitations and also furnished tissue that has many important characteristics of human atherosclerosis.

The present work extends our previous finding that statins improve endothelium-mediated responses in nonhuman primates independent of their effect on serum cholesterol. For this purpose, dietary cholesterol was adjusted on a monthly basis to maintain equivalent plasma total cholesterol, HDL-C, and LDL-C concentration among groups (see details in Methods). The healthy vascular endothelium exhibits vasodilatory, anti-inflammatory, and anticoagulant functions. In contrast, hypercholesterolemia and atherosclerosis lead to endothelial dysfunction. The beneficial cholesterol-independent effect of statins may include improvement of...
endothelium-dependent vasodilatation, decreased adhesion molecule expression and leukocyte recruitment, and plaque stabilization.\textsuperscript{30,33,34} Simva has an anti-inflammatory effect on carrageenan-induced foot pad edema.\textsuperscript{35} In the present study, we tested the possibility that decreased expression of adhesion molecules that promote leukocyte recruitment accompany improved endothelial function. Indeed, VCAM-1 expression and the macrophage population in the intima (percent positive area) were lower in the Prava-treated group. Interestingly, Prava-treated and, to a lesser extent, Simvatreated monkeys showed a lower level of the inflammatory cytokine IL-1\(\beta\) that can induce VCAM-1 expression by ECs\textsuperscript{40–42} in intimal lesions. Reduction of this proinflammatory cytokine, a well-known prototypical inducer of MMP expression, may also have importance in the context of proteolytic degradation of extracellular matrix and plaque destabilization.

The present demonstration of MMPs in association with macrophages found in monkey atherosclerotic lesions agrees with findings in human atheroma. Abundant macrophages characterize plaques that cause fatal disruption in humans.\textsuperscript{2–4} Accumulation of monocytes/macrophages in the intima may contribute importantly to 2 crucial aspects of atheroma complication, plaque rupture and thrombosis. We and others have shown that macrophages within atheroma overexpress inflammatory mediators, various extracellular matrix–degrading proteases,\textsuperscript{3,41,43–46} and highly thrombogenic TF.\textsuperscript{1,4,32} We have colocalized macrophage-derived collagenases MMP-1, MMP-13,\textsuperscript{6} and MMP-8\textsuperscript{47} with sites of cleaved collagen in human atheroma, and we have demonstrated in vivo and in vitro that macrophages furnish most of the MMPs in human plaques. In this manner, the cholesterol-independent reduction of macrophages and the macrophage-derived MMPs after statin treatment may contribute to lesion stabilization and, hence, to the reduction of clinical events. The lipid-lowering independent effects of statin treatment documented in the present study in vivo agree with in vitro experiments showing that statin treatment directly reduces proteolytic activity of MMP-9 and TF expressed by cultured human macrophages.\textsuperscript{31a,48,49}

Inflammation with an accumulation of activated mononuclear cells may also contribute to thrombosis after plaque disruption by producing TF in the lesion. Our in vivo results showing lipid-independent reduction of TF expression in lesions of Prava-treated monkeys bolster the relevance of in vitro experiments on isolated human monocytes/macrophages that show lipid-independent reduction in TF activity due to statin treatment.\textsuperscript{31a,50}

In the present study, our observations on atheroma in nonhuman primates provide important support for a component of lipid-independent mechanisms of benefit in statin treatment. These in vivo experimental results provide a new link between in vitro studies of statin pleiotropic effects on LDL lowering by statins on the other. These findings furnish additional evidence of the importance of inflammation in the biology and treatment of atherosclerosis.

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