Simvastatin Promotes Atherosclerotic Plaque Stability in ApoE-Deficient Mice Independently of Lipid Lowering

Florian Bea, Erwin Blessing, Brian Bennett, Michael Levitz, Elizabeth P. Wallace, Michael E. Rosenfeld

Objective—This study sought to determine whether simvastatin, a 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor, has stabilizing effects on vulnerable atherosclerotic plaques that are independent of their lipid-lowering capabilities.

Methods and Results—Simvastatin (50 mg/kg per day) was administered to 30-week-old apolipoprotein E–deficient mice exhibiting advanced unstable atherosclerotic lesions within the innominate/brachiocephalic artery. Simvastatin was administered in the chow to separate groups of mice for 6, 12, 18, or 24 weeks. Simvastatin significantly increased serum cholesterol after 12, 18, and 24 weeks of treatment. The average cross-sectional area of atherosclerotic lesion increased in the innominate artery after 12 and 24 weeks of treatment, concomitant with the increase in serum cholesterol. However, histological analysis of sections of the innominate artery stained with Movat and von Kossa stains demonstrated a 49% reduction in the frequency of intraplaque hemorrhage and a 56% reduction in the frequency of calcification, both markers of advanced and unstable atherosclerotic plaques.

Conclusions—These data suggest that despite an increase in serum cholesterol and lesion size, simvastatin has stabilizing effects on advanced atherosclerotic lesions. (Arterioscler Thromb Vasc Biol. 2002;22:●●●●.●●●.)

Key Words: arteriosclerosis □ statins □ lipids □ plaque stability
Methods

Animals
Male apoE−/− mice on a C57BL/6J background (n = 64) were purchased from Jackson Laboratories (Bar Harbor, Me). Mice were fed a chow diet and water ad libitum throughout the study. At 30 weeks of age, simvastatin (50 mg/kg per day) was added to the chow of half of the mice. The dose of simvastatin used in the present study was based on the doses used in previous studies with hyperlipidemic mice and on the results of a small pilot study. The present study was approved by the University of Washington Institutional Animal Care and Use Committee.

Animal Euthanasia and Preparation of Arterial Tissues
After 6, 12, 18, and 24 weeks of simvastatin treatment, the mice were sedated (Avertin, Aldrich), blood was collected from the inferior vena cava, and the animals were euthanized by exsanguination. The animals were perfused with 10 mL PBS at physiological pressure, followed by perfusion with 10% buffered formalin via the left ventricle for 4 minutes. The entire innominate/brachiocephalic artery from each animal was dissected out, embedded in paraffin, and serially sectioned (5 μm). Every fifth section was stained with a modified Movat pentachrome stain. To identify vascular calcification, adjacent slides were stained with the von Kossa stain. Total serum cholesterol was measured colorimetrically by using a commercially available cholesterol oxidase enzymatic kit (401-25P, Sigma Chemical Co).

Evaluation of Plaque Composition and Plaque Size
Two independent investigators who were blinded to the study protocol evaluated each section for characteristic features of plaque instability. These included the following: thickness of the fibrous cap (thin fibrous cap was defined as ≤3 cell layers), size of the necrotic core (a large necrotic core was defined as occupying ≥50% of the volume of the plaque), intraplaque hemorrhage (defined as the presence of red blood cells independent of microvessels), medial erosion (defined as the replacement of the normal media by plaque components), calcification (defined on the basis of positive staining with the von Kossa stain), and lateral xanthomas (defined as the presence of aggregates of macrophage-derived foam cells situated on the lateral margins of the plaques). These were recorded as binary outcomes, and the frequency for each animal was determined. The cross-sectional area of lesion in each section was determined by using computer-assisted morphometry (Image Pro, Media Cybernetics) and is reported as maximum plaque area per animal.

Statistical Analyses
All data are expressed as mean ± SE. Significant differences between means in serum cholesterol and lesion size were determined by the Student 2-tailed t test. For analysis of the plaque morphology, mean frequencies between groups were compared by the Mann-Whitney U test. Multiple Poisson regression was used to analyze the overall outcome in plaque morphology adjusted for length of exposure to simvastatin, serum cholesterol, and lesion size. Overdispersion of the model was corrected via the bootstrap method.

Results

Effects of Simvastatin on Serum Cholesterol
There were no significant differences in serum cholesterol levels 6 weeks after the start of the treatment. However, by 12 weeks of treatment, serum cholesterol levels were significantly increased in the simvastatin-treated mice relative to control mice. The cholesterol levels progressively decreased at 18 and 24 weeks but remained significantly higher than the levels in the age-matched control mice (Table 1).

Maximum Lesion Area
There were no significant differences in maximum lesion area 6 weeks after the start of treatment. However, concomitant with the increase in serum cholesterol, maximum lesion area increased in treated mice versus control mice by 12 weeks of simvastatin treatment. Lesion area continued to be larger in the treated mice at 18 weeks but did not reach statistical significance. Lesion area was again significantly larger in the treated mice at 24 weeks (Table 1).

Plaque Composition: Comparison of Group Means at Individual Time Points

Thinning of the Fibrous Cap
There was a nonsignificant trend for a reduced frequency of lesions with thin fibrous caps in the simvastatin-treated animals at all time points throughout the study. The differences were most notable after 6 weeks of treatment (for frequency of thin fibrous caps, 19% in simvastatin-treated animals versus 38% in control animals; P = 0.22; Figure 1).

Frequency of Lesions With Hemorrhage
There was an initial reduction in the frequency of intraplaque hemorrhage 6 weeks after the start of treatment with simvastatin (72% frequency of hemorrhage in control animals versus 18% frequency of hemorrhage in simvastatin-treated animals, P < 0.05). However, again, concomitant with the simvastatin-induced increase in plasma cholesterol, there was a nonsignificant increased frequency of hemorrhage after 12 weeks of treatment (49% for treated animals versus 32% for control animals, P = 0.26). However, as the plasma cholesterol levels started to decline, the frequency of hemorrhage was again reduced in the groups treated with simvastatin (39% for treated animals versus 63% for control animals in the 18-week group, P = 0.473; 21% for treated animals versus 44% for control animals in the 24-week group, P = 0.446; Figure 2). Hemorrhage was most frequently observed within the necrotic cores (Figure 3A), in the lateral xanthomas, and

**Table 1. Serum Cholesterol and Lesion Area in Simvastatin-Treated and Control ApoE−/− Mice**

<table>
<thead>
<tr>
<th>Duration of Treatment</th>
<th>n</th>
<th>Total Cholesterol, mg/dL</th>
<th>Lesion Area, μm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 weeks</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Simvastatin</td>
<td>7</td>
<td>497 ± 32</td>
<td>132 375 ± 16 396</td>
</tr>
<tr>
<td>Control</td>
<td>7</td>
<td>443 ± 50</td>
<td>160 453 ± 28 173</td>
</tr>
<tr>
<td>12 weeks</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Simvastatin</td>
<td>8</td>
<td>884 ± 49*</td>
<td>243 570 ± 14 046†</td>
</tr>
<tr>
<td>Control</td>
<td>9</td>
<td>536 ± 22</td>
<td>137 319 ± 25 488</td>
</tr>
<tr>
<td>18 weeks</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Simvastatin</td>
<td>8</td>
<td>783 ± 47*</td>
<td>205 278 ± 30 364</td>
</tr>
<tr>
<td>Control</td>
<td>9</td>
<td>514 ± 38</td>
<td>175 805 ± 14 143</td>
</tr>
<tr>
<td>24 weeks</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Simvastatin</td>
<td>8</td>
<td>636 ± 51†</td>
<td>322 172 ± 50 558†</td>
</tr>
<tr>
<td>Control</td>
<td>8</td>
<td>471 ± 56</td>
<td>184 738 ± 6922</td>
</tr>
</tbody>
</table>
underneath thin fibrous caps in lateral parts of the lesions (Figure 3B).

**Frequency of Lesions With Calcification**
There was a continuous increase in the frequency of calcified lesions in the control animals, whereas the simvastatin-treated animals exhibited little change in the frequency of calcification during the entire time course of the study (28% in treated animals versus 52% in control animals after 12 weeks, \( P=0.136 \); 22% versus 62%, respectively, after 18 weeks, \( P=0.15 \); and 26% versus 78%, respectively, after 24 weeks, \( P<0.01 \); Figure 4). Calcification of the media was often present (Figure 5C), and calcification was associated with chondrocyte-like cells flanking the calcium deposits (Figure 5D).

**Multiple Poisson Regression Analyses: Adjustment for Duration of Treatment, Cholesterol, and Lesion Area**
To determine whether the stabilizing effects of simvastatin were independent of the increased serum cholesterol and
lesion size and whether these effects were consistent over the entire duration of the study, we performed multiple Poisson regression analyses. These included adjusted rate ratios for characteristics such as the number of sections, the level of cholesterol, and the size of the lesion. For analysis of intraplaque hemorrhage, the rate ratio was further adjusted for fibrous cap thickness, and the rate ratio for calcification was adjusted for the presence of chondrocytes.

As shown in Table 2, there was a significant (49%) reduction in the frequency of hemorrhage and a significant (56%) reduction in the frequency of calcification in the simvastatin-treated animals over the entire duration of treatment after adjustment for cholesterol and lesion size. The frequency of a thin fibrous cap achieved only borderline significance after the above-mentioned adjustments. There were no significant differences in the frequency of a large necrotic core or the frequency of lateral xanthomas.

Discussion

The current paradigm for formation of occlusive thrombi and subsequent coronary events and stroke involves the rupture of the fibrous cap, with intraplaque hemorrhage and exposure of the blood to thrombogenic plaque components. Interestingly, most of these events occur in smaller lesions of <50%.

| Table 2: Multiple Poisson Regression Analysis of Treatment Effects |
|--------------------------|----------------|----------|
| Outcome                  | T Value†       | P Value† |
| Intra-plaque hemorrhage   | 5.6            | 0.01     |
| Calcification             | 9.7            | 0.001    |
| Thinning of fibrous cap   | 3.1            | 0.08     |
| Lateral xanthoma          | 0.93           | 0.33     |
| Large necrotic cores      | 0.02           | 0.88     |

Values shown are the outcomes adjusted for length of exposure to simvastatin, serum cholesterol, and lesion size (log-transformed).
stenosis. Statins have been shown to improve the clinical outcome of coronary events by changing plaque composition rather than plaque size, and angiographic data suggest that clinical improvements with statins far exceed recorded changes in lumen diameter.

**Effect of Simvastatin on Lesion Size**

In the present study, the maximum cross-sectional area of atherosclerotic lesions in the innominate arteries of older apoE−/− mice increased after simvastatin treatment for up to 24 weeks. The unexpected increase in serum cholesterol is likely the cause for this increase in lesion size. These findings are consistent with recent observations by Wang et al., who reported that simvastatin also increased serum cholesterol and lesion size in apoE−/− mice. Graded increases in plasma cholesterol have been associated with increases in lesion size in the aortic root of apoE−/− mice administered high-fat diets. However, lesion size is not predictive of plaque stability in humans, and our data suggest that this is also true for simvastatin-treated apoE−/− mice.

**Effect of Simvastatin on Plaque Composition**

**Thickness of the Fibrous Cap and Size of the Necrotic Core**

In human studies, the thickness of the fibrous cap covering a necrotic core frequently dictates whether a plaque is prone to rupture. In the present study, apoE−/− mice treated with simvastatin, compared with control mice, tended to have thicker fibrous caps, although the frequency was only of borderline significant difference when adjusted for duration, cholesterol, and lesion size (P = 0.08, Table 2). There were no differences in the frequency of a large central necrotic core. This is consistent with previous studies of pravastatin-treated rabbits, in which the fibromuscular components of plaques increased, and the lipid content decreased. Our failure to demonstrate significant effects of simvastatin on the frequency of these parameters is likely due to the nature of the mouse lesions. For example, the mouse lesions are often composed of multiple, layered, and convergent plaques, each containing several necrotic zones and often without a well-defined fibrous cap.

**Hemorrhage**

Recent studies indicate that intraplaque hemorrhage is a risk factor and indicator of plaque rupture and is often associated with thrombus formation in humans. We have previously reported a high frequency of intraplaque hemorrhage in lesions in the innominate artery of older apoE−/− mice fed a chow diet without any associated thrombosis. The absence of thrombi suggests that ruptured microvessels in the plaque may be the source of hemorrhage rather than rupture of the plaque itself. It may also be indicative of the formation of small fissures that do not expose sufficient plaque components to the blood to induce thrombosis or indicative of a very efficient thrombolytic system in the mouse. We have recently observed a complete absence of microvessels in the innominate lesions after immunostaining with antibodies to VE-cadherin (data not shown). This strongly suggests that intraplaque hemorrhage is a marker of plaque fissure and/or rupture. Furthermore, recent studies by Williams et al. in apoE−/− mice fed a high-fat diet have documented the presence of occlusive thrombi as well as hemorrhage at sites of plaque rupture in the innominate arteries.

In the present study, treatment with simvastatin significantly reduced intraplaque hemorrhage in the innominate artery by 49% over the entire time course of the study. There was also a statistically significant association between the frequency of lesions with thin fibrous caps and the frequency of intraplaque hemorrhage as assessed by Poisson regression analysis (r = 0.32, P < 0.05), suggesting that intraplaque hemorrhage likely occurs more frequently in lesions with thin fibrous caps in this model. This is the first report of a direct lipid-independent effect of the statins on the frequency of intraplaque hemorrhage and strongly suggests that treatment with simvastatin promotes plaque stability. However, the high rate of hemorrhage and lack of thrombosis in the mice is not consistent with the pathobiology of human coronary artery disease; thus, the effects of simvastatin in the mice may not be entirely representative of what occurs in humans.

The mechanism(s) by which simvastatin reduces intraplaque hemorrhage is not yet known. One likely explanation is the anti-inflammatory properties of the statins. Several previous studies have demonstrated that treatment with statins reduces the macrophage content of atherosclerotic lesions. We have also observed a reduced macrophage content in the innominate arteries of the simvastatin-treated mice after immunocytochemical staining with macrophage-specific antibodies (data not shown). Activated macrophages within unstable plaques express a variety of proteolytic enzymes capable of degrading the extracellular matrix, and statins may have a direct effect on the expression and activity of these enzymes. For example, pravastatin has been shown to increase expression of transforming growth factor-β1, which is known to reduce the proteolytic activity in macrophages.

**Calcification**

Calcification of the arterial wall may increase the risk of plaque rupture and the risk of adverse effects during intra-arterial interventions, such as balloon angioplasty. Treatment with statins has been shown to reduce vascular calcification in humans and apoE*-Leiden mice, but these effects were associated with reduced serum lipid levels. The present study showed a dramatic reduction in the frequency of calcification within the innominate lesions over the entire duration of the study that was completely independent of the lipid-lowering effects of simvastatin. Our multiple regression analyses with adjustment for serum cholesterol clearly demonstrated a statistically significant reduction in vascular calcification by 56% over the entire 24-week course of the study. There was also a statistically significant association between the frequency of chondrocyte-like cells with calcification (r = 0.36, P < 0.01), suggesting that the chondrocyte-like cells may be the cellular source of calcification.

It is very unlikely that the increase in serum lipids in the present study led to a decrease in vascular calcification. Recent studies have demonstrated a positive correlation between serum lipids and the extent of vascular calcification.
in humans and mice.\textsuperscript{40–42} In addition, Williams et al\textsuperscript{33} reported a lipid-independent reduction in coronary artery calcification after statin treatment in monkeys.

We did not expect simvastatin treatment of the apoE\textsuperscript{−/−} mice to elevate serum cholesterol, inasmuch as Sparrow et al\textsuperscript{17} had previously reported that there were no differences in serum cholesterol in apoE\textsuperscript{−/−} mice treated with simvastatin at 100 mg/kg per day for 6 weeks. In the present study, we did not observe significant increases in liver size and weight in the simvastatin-treated animals, nor did we observe any increase in liver enzymes in the serum of the treated animals, indicating that the dose of simvastatin used in the present study (50 mg/kg per day) was not hepatotoxic (data not shown). It is currently unclear why chronic treatment of the apoE\textsuperscript{−/−} mice with simvastatin increased serum cholesterol. However, this observation is consistent with the recent report by Wang et al,\textsuperscript{27} who reported that apoE\textsuperscript{−/−} mice treated with simvastatin at 300 mg/kg per day had increased serum cholesterol that was associated with a decrease in the HDL/LDL ratio. A possible explanation for this discrepancy is the different modes of administration. In the study by Sparrow et al, simvastatin was administered once daily by gavage. In contrast, in the present study and in the study by Wang et al, the simvastatin was administered with the chow. This mode of administration leads to a more continuous availability of the drug because mice eat throughout much of the light and dark cycles. Therefore, it is likely that the higher continuous dose of simvastatin had stimulatory effects on the expression and activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase in the liver.\textsuperscript{38,43} However, there are also other potential mechanisms that may involve the absence of apoE and/or the effects of inhibiting protein preylation that cannot be ruled out at this time.\textsuperscript{27,44}

Conclusions

Despite an unexpected increase in serum lipids and a consequent increase in lesion size, simvastatin reduces the frequency of intraplaque hemorrhage and calcification in the innominate arteries of apoE\textsuperscript{−/−} mice. Inasmuch as intraplaque hemorrhage and calcification are markers of vulnerable plaques, these data provide direct support for the lipid-independent pleiotropic effects of the statins and help explain the plaque-stabilizing capabilities of the statins that have been observed in recent clinical trials.

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References


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