Comparative Effects of Diet and Statin on NO Bioactivity and Matrix Metalloproteinases in Hypercholesterolemic Patients With Coronary Artery Disease

Kwang Kon Koh, Ji Won Son, Jeong Yeul Ahn, Dong Kyu Jin, Hyung Sik Kim, Yu Mi Choi, Dae Sung Kim, Euy-Myoung Jeong, Gi Soo Park, In Suck Choi, Eak Kyun Shin

Objective—We investigated the effects of statin compared with the American Heart Association (AHA) Step I Diet on lipoproteins, vasomotor function, tumor necrosis factor (TNF)-α, and serological markers of plaque stability. Furthermore, we investigated the mechanism of regulation suggested by experimental studies.

Methods and Results—For 14 weeks, we administered AHA diet + placebo and AHA diet + simvastatin (20 mg daily) to 31 and 32 randomly selected patients with coronary artery disease, respectively. Compared with diet alone, simvastatin significantly improved the percent flow-mediated dilator response to hyperemia from 3.37±2.28% to 5.89±2.35% (P<0.001) and lowered plasma levels of C-reactive protein from 0.48 to 0.10 mg/dL (P<0.001), TNF-α from 3.38 to 2.79 pg/mL (P<0.001), total matrix metalloproteinase (MMP)-9 from 36 to 28 ng/mL (P=0.006), and tissue inhibitor of matrix metalloproteinase-1 from 80±30 to 74±23 ng/mL (P=0.041), and simvastatin lowered to a greater extent MMP-9 activity (from 71 to 52 ng/mL, P=0.006) and MMP-9 activity/tissue inhibitor of matrix metalloproteinase-1 ratios (P=0.018), although this difference did not reach statistical significance. There were significant correlations between the degree of changes in TNF-α and the degree of changes in MMP-9 activity (r=0.424, P=0.016). However, no significant correlations between lipoprotein levels and flow-mediated dilation percentages and levels of plaque stability markers were determined (r=−0.208, P=0.243).

Conclusions—Simvastatin reduced serological markers of inflammation and plaque stability, independent of lipoprotein changes. (Arterioscler Thromb Vase Biol. 2002;22:___.-___.)

Key Words: 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors ■ endothelial function ■ nitric oxide ■ plaque stability ■ atherosclerosis

In soft lipid-rich plaque obtained from patients who died of acute coronary syndrome (ACS), inflammatory cells, including activated macrophages and mast cells, are shown to accumulate at a high concentration.1 There is an increased rate of formation of matrix metalloproteinase (MMP) enzymes in the ruptured atherosclerotic plaque.2 Active rupture of the vulnerable plaque by these proteinases is one of the triggers causing subsequent thrombus formation and ACS, and serial changes in the peripheral blood levels of MMP-2 and MMP-9 have been observed in patients with ACS, which implicates the role of these enzymes in the molecular mechanism of plaque instability in ACS.3 Macrophages and smooth muscle cells of human atherosclerotic plaques have been shown to synthesize MMP-3 and MMP-9.4 Lipoproteins,5 NO,6 tumor necrosis factor (TNF)-α, or mevalonate7 regulate MMP-9 and tissue inhibitor of matrix metalloproteinase (TIMP) expression in experimental studies.

Recent studies have suggested that the beneficial effects of statins on clinical events may involve nonlipid mechanisms that affect endothelial function: inflammatory responses, thrombus formation, and plaque stability.8–10 Statins stimulate endothelial NO synthase and release NO11; furthermore, they improve NO bioactivity in humans.12,13 Furthermore, cholesterol level lowering by diet and by statin therapy in experimental rabbits increased the content of interstitial collagen and suppressed the growth of macrophages expressing MMP, which may contribute to atherosclerotic plaque stability.14 Accordingly, atherosclerotic plaque stability with statin may explain the reduction of cardiovascular risk.

The purpose of the present study was to determine the following: (1) whether statin, compared with the American Heart Association (AHA) Step I Diet, improves NO bioactivity and reduces serological markers of inflammation and plaque stability and (2) whether statin-induced reduction in...
markers of plaque stability is mediated by lipoprotein changes, improvement in NO bioactivity, or TNF-α changes, as suggested by experimental studies.4–7

Methods

Study Population and Design

Sixty-three patients with angiographically documented coronary artery disease were enrolled in the present study. All patients were categorized as Canadian Cardiovascular Society class I or II. All patients were familiarized with and placed on the AHA Step I-Diet through the study period. We administered the AHA diet-placebo and AHA diet+simvastatin (20 mg daily) for 14 weeks to 31 and 32 randomly selected patients with coronary artery disease, respectively; a single-blind prospective randomized design was used. The clinical characteristics of these patients are summarized in Table 1.

Laboratory Assays

Blood samples for laboratory assays were obtained at ~8:00 AM after overnight fasting at pretreatment and after simvastatin treatment for 14 weeks and were immediately coded so that the investigators performing the laboratory assays would be blinded to subject identity and study sequence. Assays for lipids, TNF-α, plasma MMP-3, total MMP-9 (active MMP-9 plus pro-MMP-9, Quantikine MMP-9 kit), MMP-9 activity (Fluorokine E Active MMP-9 kit), and TIMP-1 were performed in duplicate by ELISA (R & D Systems), as previously described.10,13,15 For all patients, serum was collected for the measurement of C-reactive protein (CRP) levels. CRP levels were determined with an immunonephelometry system according to methods described by the manufacturer (rate nephelometry, IMMAGE, Beckman Coulter). The measurement range was 0.1 to 98 mg/dL. All samples from the same patient (batch samples) were measured in blinded pairs on the same ELISA kit to minimize run-to-run variability. The interassay and intra-assay coefficients of variation were <6%.

Vascular Studies

Imaging studies of the right brachial artery were performed by using an ATL HDI 3000 ultrasound machine equipped with a 10-MHz linear-array transducer according to a previously published technique.13,15 Measurements were performed by 2 independent investigators (D.K.J. and H.S.K.) who were blinded to each subject’s identity and medication status.

Statistical Analysis

Data are expressed as mean±SD or median (range 25% to 75%). After testing the data for normality, we used the Student paired t test or the Wilcoxon signed rank test to compare values between baseline and treatment for 14 weeks and the Student unpaired t test or Mann-Whitney rank sum test to compare baseline values and percent changes between diet+placebo and diet+simvastatin for 14 weeks, as reported in Table 2. Pearson or Spearman correlation coefficient analysis was used to assess associations between measured parameters. We calculated that 30 subjects would provide 80% power for detecting the difference of absolute increase, ≥2.1% flow-mediated dilation of the brachial artery between baseline and simvastatin, with α=0.05 according to our previous studies.13,15 A value of P<0.05 was considered to be statistically significant.

Results

There were no significant differences in baseline characteristics and baseline values (lipids, vascular function [diameter and flow], CRP, TNF-α, and markers of plaque stability) between diet+placebo and diet+simvastatin groups.

Effects of Therapies on Lipids and Vasomotor Function

The effects of therapies on lipids are shown in Table 2. Both diet alone and simvastatin treatment significantly improved the percent flow-mediated dilator response to hyperemia relative to pretreatment measurements (both P<0.001, Figure 1 and Table 2); however, compared with diet alone, simvastatin treatment significantly improved the response (P<0.001). The brachial artery dilator responses to nitroglycerin between each therapy were not significantly different (P=0.987, Table 2).

Effects of Therapies on CRP, TNF-α, and Markers of Plaque Stability

Simvastatin significantly lowered serum levels of CRP by 46±44% (P<0.001), and the reduction was greater than with diet alone (P<0.001). Furthermore, we observed that patients with the highest baseline CRP levels showed the greatest extent of reduction on simvastatin (r=−0.582, P<0.001).

TABLE 1. Baseline Characteristics of the Study Population

<table>
<thead>
<tr>
<th></th>
<th>Diet+Placebo (n=31)</th>
<th>Diet+Simvastatin (n=32)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>62±8</td>
<td>62±7</td>
</tr>
<tr>
<td>Sex, M:F</td>
<td>13:18</td>
<td>13:19</td>
</tr>
<tr>
<td>Risk factors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypertension</td>
<td>21 (68)</td>
<td>23 (72)</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>7 (23)</td>
<td>8 (25)</td>
</tr>
<tr>
<td>Current smoking</td>
<td>11 (35)</td>
<td>10 (31)</td>
</tr>
<tr>
<td>Medications</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-adrenergic blockers</td>
<td>25 (81)</td>
<td>26 (81)</td>
</tr>
<tr>
<td>Calcium channel blockers</td>
<td>15 (48)</td>
<td>15 (47)</td>
</tr>
<tr>
<td>ACE inhibitors</td>
<td>7 (23)</td>
<td>9 (28)</td>
</tr>
<tr>
<td>Long-acting nitrates</td>
<td>25 (81)</td>
<td>27 (84)</td>
</tr>
<tr>
<td>Aspirin</td>
<td>26 (84)</td>
<td>28 (88)</td>
</tr>
</tbody>
</table>

Values are expressed as means±SD or n (%).

Marketable

8th Annual Meeting of the American Heart Association
2 Arterioscler Thromb Vasc Biol.
September 2002
Simvastatin significantly lowered plasma levels of TNF-\(\alpha\) by 14±22% from the respective baseline levels (\(P<0.001\)), and reduction was greater than with diet alone (\(P<0.001\)). The effects of therapies on markers of plaque stability are shown in Table 2. Neither diet alone nor simvastatin treatment significantly changed plasma levels of MMP-3 compared with respective baseline levels. However, simvastatin significantly lowered the plasma levels of total MMP-9, MMP-9 activity, TIMP-1, and the ratio of MMP-9 activity over TIMP-1 (MMP-9 activity/TIMP-1) by 24±6, 18±37, 5±15, and 13±38%, respectively (\(P<0.001\), \(P=0.006\), \(P=0.045\), and \(P=0.018\), respectively; Figures 2 and 3).

Compared with diet alone, simvastatin significantly lowered plasma levels of total MMP-9 and TIMP-1 (\(P=0.006\) and \(P=0.041\), respectively) and reduced MMP-9 activity and MMP-9 activity/TIMP-1 ratios, although this difference did not reach statistical significance. Furthermore, there were significant inverse correlations between pretreatment total MMP-9, MMP-9 activity, TIMP-1, or MMP-9 activity/TIMP-1 levels and the degree of change in those levels after simvastatin treatment (\(r=-0.793\) [\(P<0.001\)], \(r=-0.442\) [\(P=0.011\)], \(r=-0.437\) [\(P=0.012\)], and \(r=-0.356\) [\(P=0.045\)], respectively).

To identify a mechanism for the effects of simvastatin on lipoproteins, vasomotor function, TNF-\(\alpha\), and plaque stabil-

### Table 2. Effects of Diet and Simvastatin in Hypercholesterolemic Patients with Coronary Artery Disease

<table>
<thead>
<tr>
<th></th>
<th>Diet + Placebo</th>
<th>After Diet</th>
<th>Diet + Simvastatin</th>
<th>After Therapy</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lipids, mg/dL</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>240±41</td>
<td>221±31*</td>
<td>226±33</td>
<td>163±37†</td>
</tr>
<tr>
<td>LDL cholesterol</td>
<td>153±27</td>
<td>134±36*</td>
<td>141±33</td>
<td>82±32†</td>
</tr>
<tr>
<td>Apolipoprotein B</td>
<td>118±26</td>
<td>110±24</td>
<td>110±18</td>
<td>81±17†</td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>50±11</td>
<td>46±10*</td>
<td>46±10</td>
<td>48±11†</td>
</tr>
<tr>
<td>Apolipoprotein A-I</td>
<td>128±24</td>
<td>119±24*</td>
<td>116±23</td>
<td>123±20†</td>
</tr>
<tr>
<td><strong>Vasomotor function, %</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flow-mediated dilation</td>
<td>4.13±1.41</td>
<td>4.92±1.55*</td>
<td>3.37±2.28</td>
<td>5.89±2.35†</td>
</tr>
<tr>
<td>Nitroglycerin dilation</td>
<td>12.11±3.73</td>
<td>12.68±3.49</td>
<td>12.33±3.19</td>
<td>12.59±3.36</td>
</tr>
<tr>
<td><strong>Cytokines</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CRP, mg/dL</td>
<td>0.19 (0.12–0.48)</td>
<td>0.20 (0.11–0.51)</td>
<td>0.48 (0.15–1.23)</td>
<td>0.10 (0.10–0.26)*†</td>
</tr>
<tr>
<td>TNF-(\alpha), pg/mL</td>
<td>2.59 (2.09–3.38)</td>
<td>2.81 (2.30–3.97)*</td>
<td>3.38 (1.24–4.45)</td>
<td>2.79 (1.20–3.87)*†</td>
</tr>
<tr>
<td><strong>Plaque stability</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP-3, ng/mL</td>
<td>22±19</td>
<td>18±17</td>
<td>15±12</td>
<td>16±14</td>
</tr>
<tr>
<td>Total MMP-9, ng/mL</td>
<td>25 (18–49)</td>
<td>26 (17–41)</td>
<td>36 (22–68)</td>
<td>28 (19–34)*†</td>
</tr>
<tr>
<td>MMP-9 activity, ng/mL</td>
<td>95 (63–132)</td>
<td>76 (59–117)</td>
<td>71 (49–118)</td>
<td>52 (40–85)*</td>
</tr>
<tr>
<td>TIMP-1, ng/mL</td>
<td>84±25</td>
<td>86±26</td>
<td>80±30</td>
<td>74±23†</td>
</tr>
<tr>
<td>MMP-9 activity/TIMP-1</td>
<td>1.05 (0.74–1.47)</td>
<td>0.98 (0.67–1.44)</td>
<td>1.00 (0.59–1.81)</td>
<td>0.79 (0.46–1.24)*†</td>
</tr>
</tbody>
</table>

Data are expressed as means±SD or median (25%–75%).

*\(P<0.05\) for comparison with the baseline value.

†\(P<0.05\) for comparison with the value after therapy with diet.
ity, we assessed correlations between lipoprotein levels, flow-mediated dilation percentage, or TNF-α and levels of plaque stability markers on simvastatin. Of interest, there were significant inverse correlations between LDL cholesterol or the ratio of LDL to HDL cholesterol levels and flow-mediated dilation percentage ($r = -0.342 \ [P = 0.009]$ and $r = -0.356 \ [P = 0.006]$, respectively). There were significant correlations between the degree of changes in TNF-α and the degree of changes in MMP-9 activity ($r = 0.424$, $P = 0.016$). Of interest, no significant correlations between lipoprotein levels or flow-mediated dilation percentage and levels of plaque stability markers were determined ($-0.208 \leq r \leq 0.243$).

**Discussion**

Lowering blood LDL cholesterol levels may facilitate plaque stability either through a reduction in size or by an alteration of the physiochemical properties of lipid cores. However, changes in plaque size by lipid lowering tend to occur over an extended period of time and are quite minimal, as assessed by angiography. Rather, the clinical benefits from lipid lowering are probably due to decreases in macrophage accumulation in atherosclerotic lesions and inhibition of MMP production by activated macrophages. Indeed, statins inhibit the expression of MMPs, with the cholesterol-independent or direct macrophage effects occurring within a much earlier time frame. Therefore, the plaque-stabilizing properties of statins are mediated through a combined reduction in lipids, macrophages, and MMPs. However, we did not observe significant correlations between lipoprotein levels and total MMP-9, MMP-9 activity, or TIMP-1 levels despite significant changes of total MMP-9, MMP-9 activity, and TIMP-1 levels on simvastatin treatment. Although diet alone tended to reduce MMP-9 activity and MMP-9/TIMP-1 ratios from the respective baseline levels, diet alone did not change CRP, TNF-α, total MMP-9, and TIMP-1 levels despite significant changes in lipoproteins. Our present observations support nonlipid mechanisms of statins.

Statins have been shown to inhibit MMP-9 production by macrophages in culture, an inhibition reversed by the addition of mevalonate, providing further insight regarding their direct antiatherosclerotic potentials. Meanwhile, endothelial NO

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**Figure 2.** Changes in plasma levels of total MMP-9 associated with diet + placebo and diet + simvastatin. Compared with diet alone, simvastatin significantly lowered plasma levels of total MMP-9. Median values are identified by open circles.

**Figure 3.** Changes in plasma levels of MMP-9 activity associated with diet + placebo and diet + simvastatin. Simvastatin significantly lowered plasma levels of MMP-9 activity, and the diet alone tended to reduce the respective baseline levels. Compared with diet alone, simvastatin reduced the levels to a greater extent, although this difference did not reach statistical significance. Median values are identified by open circles.
synthase gene transfer significantly decreased MMP-2 and MMP-9 activities simultaneously, with an increase of TIMP-2 levels in the conditioned medium. Furthermore, TNF-α, a proinflammatory cytokine, stimulated the synthesis and secretion of MMP-9. In the present study, we observed significant correlation between the degree of changes in TNF-α and the degree of changes in MMP-9 activity. Indeed, Lee et al recently demonstrated TNF-α-induced expression of MMP-1, MMP-9, and MMP-13. However, contrary to our hypothesis based on experimental studies, we observed no significant correlations between lipoprotein levels or flow-mediated dilation percentage and levels of plaque stability markers. One study observed that pravastatin decreased serum MMP-9 levels independently of changes in lipid levels. Recently, Williams et al demonstrated that compared with arteries of monkeys not receiving pravastatin, the arteries of pravastatin-treated monkeys had better dilator function and plaque characteristics more consistent with plaque stability. Of interest, these beneficial arterial effects of pravastatin occurred independently of plasma lipoprotein concentrations, which were consistent with our data.

References

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Arterioscler Thromb Vasc Biol. published online July 25, 2002;
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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