Increased Expression of Interleukin-1 in Coronary Artery Disease With Downregulatory Effects of HMG-CoA Reductase Inhibitors

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Background—Inflammation is important in atherogenesis. Interleukin (IL)-1 is the prototypic inflammatory cytokine. We hypothesized a dysbalance between inflammatory and anti-inflammatory mediators in the IL-1 family in coronary artery disease (CAD) and a possible modulation of these mediators by HMG-CoA inhibitors (statins).

Methods and Results—In a microarray screening experiment examining peripheral blood mononuclear cells (PBMCs) from 6 CAD patients and 4 healthy control subjects, IL-1β was identified as 1 of 25 genes whose expression were upregulated in CAD and downregulated by statins. In the following, we studied the role of IL-1β and related mediators in CAD. Our major findings were as follows. (1) Although mRNA levels of IL-1α and IL-1β were markedly reduced in PBMCs from CAD patients after 6 months of simvastatin (20 mg/d, n=15) and atorvastatin (80 mg/d, n=15) therapy, the reduction in IL-1 receptor antagonist (IL-1Ra) was more modest. Statins also reduced the spontaneous release of IL-1β and IL-1Ra from PBMCs in CAD patients. (2) mRNA levels of IL-1α, IL-1β, and IL-1Ra were increased in PBMCs from patients with stable (n=20) and unstable (n=20) angina compared with healthy control subjects (n=15). Although the unstable patients had particularly high levels of IL-1β and IL-1α, IL-1Ra was not correspondingly increased. (3) IL-1β induced release of proatherogenic cytokines from PBMCs, whereas atorvastatin partly abolished this effect.

Conclusions—Our findings suggest that cytokines in the IL-1 family may represent therapeutic targets in CAD. The ability of statins to modulate these cytokines in an anti-inflammatory direction underscores their immunomodulatory potential. (Circulation. 2004;109:1966-1972.)

Key Words: interleukins ▪ coronary disease ▪ inflammation ▪ statins

Atherosclerosis has elements of both lipid deposition and inflammation. Thus, although hypercholesterolemia is an established risk factor for atherosclerosis and coronary artery disease (CAD), these patients also have raised plasma levels of inflammatory cytokines, enhanced activation of circulating leukocytes, and extensive infiltration of blood-derived leukocytes into atherosclerotic plaques, indicating an important pathogenic role of inflammation in atherogenesis.1

Interleukin (IL)-1 is the prototypically inflammatory cytokine produced by several cell types in response to various stimuli, being a critical early mediator of inflammation.2 The naturally occurring antagonist IL-1 receptor antagonist (IL-1Ra) is also released during inflammation and may limit the potentially deleterious effects of IL-1. Thus, the IL-1/IL-1Ra balance may determine the severity of both acute and chronic inflammation, and the relative absence of IL-1Ra is suggested to play a role in the pathogenesis of some inflammatory disorders.2 With relevance to CAD, studies in hypercholesterolemic mice suggest that lack of IL-1β or overexpression of IL-1Ra can partly protect against atherosclerosis.3,4 However, the role of the IL-1 in human atherosclerotic disease is far from clear.

Clinical trials with HMG-CoA reductase inhibitors (statins) have demonstrated an improved prognosis for CAD.5 This effect has been attributed to their lipid-lowering properties, but recent studies suggest that immunomodulatory effects of statins also may be beneficial in CAD.5 However, our knowledge of the effects of statins on the cytokine levels in humans is thus far limited.

During recent years, the role of inflammatory cytokines in CAD and acute coronary syndromes (ACS) has been illuminated through numerous studies. Several mediators are iden-
tified, but their relative importance in atherosclerosis is still unknown. In the present study, we used DNA microarrays to identify genes in peripheral blood mononuclear cells (PBMCs) that were (1) upregulated in angina patients and (2) downregulated by statin therapy. These screening experiments identified IL-1 as a potentially important mediator of atherosclerosis, the expression of which could be modulated by statins.

**Methods**

**Patients and Control Subjects**

The design of the statin study was described previously. Thirty patients with previous myocardial infarction and without statin medication were included and randomized to simvastatin 20 mg/d (n=15) or atorvastatin 80 mg/d (n=15) in an open study (Table 1). Venous blood was collected at baseline and after 6 months of therapy. As expected, statins reduced the plasma levels of total cholesterol, LDL cholesterol, and triglycerides after 6 months of therapy, with a tendency toward a greater lipid-lowering effect in the atorvastatin group (Table 1). In the cross-sectional study, 20 patients with previous myocardial infarction and without statin medication were included and randomized to simvastatin 20 mg/d and atorvastatin group (Table 1). In the cross-sectional study, 20 patients with previous myocardial infarction and without statin medication were included and randomized to simvastatin 20 mg/d and atorvastatin group (Table 1).

**Isolation of Cells**

PBMCs were obtained from heparinized blood by Isopaque-Ficoll (Lymphoprep, Nycomed) gradient centrifugation. Cells were immediately cryopreserved or stored in liquid nitrogen as pellets. Freshly isolated PBMCs from healthy blood donors were used in some of the in vitro experiments.

**Cell Culture Experiments**

Thawed cryopreserved or freshly isolated PBMCs were cultured in RPMI 1640 (Gibco) supplemented with 5% human AB serum (Bio-Whittaker) in 96-well or 24-well trays (Costar; 10^6 cells/mL [cryopreserved cells] or 2×10^6 cells/mL [freshly isolated cells]). Cell pellets and cell-free supernatants were harvested after 6 and 20 hours, respectively. Comparisons of data were made only between experiments performed with the same cell concentrations and in the same plate format. In some experiments, cells were stimulated with IL-1β (R&D Systems), with or without preincubation with atorvastatin or its metabolite ortho-hydroxy atorvastatin (gifts from Pfizer) and/or mevalonate (Sigma) for 1 hour before addition of IL-1β. The endotoxin level in the atorvastatin and ortho-hydroxy atorvastatin was <10 pg/mL (Limulus Amoeocyte Lysate Assay; Bio-Whittaker).
TABLE 2. Characteristics of the Angina Patients in the Cross-Sectional Study

<table>
<thead>
<tr>
<th></th>
<th>Unstable Angina (n=20)</th>
<th>Stable Angina (n=20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>66.3±9.4</td>
<td>63.3±9.7</td>
</tr>
<tr>
<td>Sex, F/M</td>
<td>4/16</td>
<td>5/15</td>
</tr>
<tr>
<td>Smokers, +/−</td>
<td>9/11</td>
<td>3/17</td>
</tr>
<tr>
<td>Diabetes, +/−</td>
<td>4/16</td>
<td>1/19</td>
</tr>
<tr>
<td>Previous myocardial infarction, +/−</td>
<td>4/16</td>
<td>6/14</td>
</tr>
<tr>
<td>Medication, +/−</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-Blockers</td>
<td>16/4</td>
<td>17/3</td>
</tr>
<tr>
<td>Aspirin</td>
<td>18/2</td>
<td>17/3</td>
</tr>
<tr>
<td>Statins</td>
<td>15/5</td>
<td>16/4</td>
</tr>
<tr>
<td>Calcium antagonists</td>
<td>9/11</td>
<td>10/10</td>
</tr>
<tr>
<td>ACE inhibitors</td>
<td>3/17</td>
<td>5/15</td>
</tr>
<tr>
<td>Warfarin</td>
<td>1/19</td>
<td>3/17</td>
</tr>
<tr>
<td>LMW heparin</td>
<td>18/2*</td>
<td>20/0</td>
</tr>
<tr>
<td>No. of affected arteries</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td>2</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>Total cholesterol, mmol/L</td>
<td>5.0±0.9</td>
<td>4.5±1.0</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/L</td>
<td>1.1±0.3*</td>
<td>1.4±0.4</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
<td>1.6±1.3</td>
<td>1.4±0.3</td>
</tr>
</tbody>
</table>

Data are mean±SD unless otherwise indicated. LMW indicates low molecular weight.

*P<0.05 vs stable angina.
†Last dosage >12 hours before blood sampling.

Real-Time Quantitative RT-PCR
Quantitative Taqman or Sybrgreen real-time reverse transcription–polymerase chain reaction (RT-PCR) was performed by use of the ABI Prism 7700 (Applied Biosystems). The primers and probes were designed with the Primer Express Software, version 1.5 (Applied Biosystems); tumor necrosis factor (TNF)-α, TRAIL (TNF-related apoptosis inducing ligand), FasL, CD40L, and LIGHT were as previously described. IL-1–converting enzyme (ICE) (forward primer [fp], 5′-AAATTTCCGGCAAGGTTCGA-3′; reverse primer [rp], 5′-AACACATGCTAAAGTCACCTTTCA-3′), and IL-1β (fp, 5′-GGCCTCTTATTTGAAGATATGACTGATT-3′, rp, 5′-GGCCTCTTATTTGAAGATATGACTGATT-3′) were amplified in Figure 1A, the CAD patients had markedly increased gene expression of IL-1β compared with healthy controls and downregulated by at least 50% after atorvastatin therapy. IL-1β was identified as one of these 25 genes, being markedly upregulated in 5 of 6 CAD patients (4- to 19-fold increase), reaching levels comparable to healthy controls after therapy.

Enzyme Immunoassays
Protein levels of IL-1β, IL-1Ra, soluble IL-1 receptor type I (IL-1sRI) and type II (IL-1sRII), ICE, TNF-α, and LIGHT were determined by enzyme immunoassays (R&D Systems).

Statistical Analyses
For comparisons of 2 groups of individuals, the Mann-Whitney U-test was used. When comparing 3 groups of individuals, the nonparametric Kruskal-Wallis test was used. If a significant difference was found, Mann-Whitney U-test was used to calculate the difference between each pair of groups. For comparisons within the same individuals, Wilcoxon’s matched-pair test was used. In the in vitro studies, Student’s t test was used. Probability values of P<0.05 (2-sided) were considered statistically significant.

Results
Microarray Experiments
To screen for inflammatory related genes in PBMCs that were upregulated in CAD patients and downregulated by statins, we used oligonucleotide microarrays encoding 8500 human genes to analyze the gene expression profiles in PBMCs isolated from 4 healthy controls and 6 randomly selected patients from the atorvastatin group of the statin study before and after 6 months of therapy. Except for slightly lower HDL levels, the baseline lipid levels and the lipid-lowering response to atorvastatin in these patients were comparable to those in the total study population. The clustering analysis identified 25 genes to be upregulated at least 2-fold in the CAD patients at baseline compared with healthy controls and downregulated by at least 50% after atorvastatin therapy. IL-1β was identified as one of these 25 genes, being markedly upregulated in 5 of 6 CAD patients (4- to 19-fold increase), reaching levels comparable to healthy controls after therapy.

Effects of Statins on the IL-1 System in PBMCs From CAD Patients
To examine the role of IL-1 in CAD further, we examined the gene expression of IL-1α, IL-1β, and their endogenous inhibitor IL-1Ra by RPA, and that of IL-18, another proatherogenic member of the IL-1 family, by quantitative RT-PCR, in all 30 patients in the statin study. Several significant findings were revealed (Figure 1). First, as exemplified in Figure 1A, the CAD patients had markedly increased gene expression of IL-1α and IL-1β (P<0.001) and slightly elevated expression of IL-1Ra (P=0.1) compared with healthy controls. Second, the mRNA levels of IL-1α and IL-1β decreased significantly after 6 months of both atorvastatin and simvastatin therapy, with a particularly marked effect of atorvastatin on IL-1β. Third, the gene expression of IL-1Ra was also downregulated after statin therapy, although less pronounced than IL-1, shifting the IL-1/IL-1Ra balance in an anti-inflammatory direction. Finally, no effect of statins was found on the gene expression of IL-18 (not shown).

To examine whether the modulatory effects of statin therapy also were present at the protein level, we analyzed the spontaneous release of IL-1β and IL-1Ra from cryopreserved PBMCs isolated before and after 6 months of atorvastatin therapy. Although the secretion of IL-1β was markedly reduced, the decrease in IL-1Ra levels was more modest, resulting in a marked reduction in the IL-1β/IL-1Ra ratio after statin therapy (Figure 2).
patients before and after statin therapy. Although statin therapy significantly reduced IL-1, ICE tended to increase at both the mRNA ($P<0.1$) and protein ($P<0.3$) levels, suggesting that the downregulatory effects of statins on IL-1 do not involve suppressive effects on ICE. Finally, the soluble IL-1 receptors IL-1sRI and IL-1sRII may bind IL-1 and alter IL-1 effects in an inflammatory and an anti-inflammatory direction, respectively. When analyzing the release of these receptors from PBMCs before and after atorvastatin therapy, only minor changes were seen in IL-1sRII levels (669 ± 64 pg/mL before versus 640 ± 50 pg/mL after therapy, $P=0.08$). Secretion of IL-1sRI was not detected either before or after therapy.

During statin therapy, there were no significant correlations between the reduction in total or LDL cholesterol and the changes in mRNA levels of IL-1$\beta$ (array and RPA), IL-1$\alpha$, and IL-1Ra (RPA), or protein secretion of IL-1$\beta$, whereas there was a weak correlation between the reduction in protein secretion of IL-1Ra and the decrease in total (P=0.08) and LDL (P=0.05) cholesterol.

**Gene Expression of IL-1 and IL-1Ra in Stable and Unstable Angina**

We next examined the gene expression of IL-1$\alpha$, IL-1$\beta$, and IL-1Ra in PBMCs by RPA in another population with 20 stable angina patients, 20 unstable angina patients, and 15 healthy controls (cross-sectional study, see Methods). These experiments confirmed the expression pattern from the statin study showing elevated mRNA levels of IL-1$\alpha$, IL-1$\beta$, and IL-1Ra in PBMCs from patients with stable CAD (Figure 3). Furthermore, although IL-1Ra was equally expressed in stable and unstable angina, higher expression of IL-1$\alpha$ and IL-1$\beta$ was seen in those with unstable disease, suggesting a net inflammatory dominance in these patients.

**Effects of IL-1$\beta$ and Atorvastatin on Proatherogenic Members of the TNF Superfamily in PBMCs**

TNF-α and related cytokines have been implicated in atherogenesis, and in addition to IL-1$\beta$, TNF-α was also identified in the microarray experiment as a gene that was upregulated in CAD and downregulated by statins. To further elucidate possible consequences of increased IL-1$\beta$ levels in angina patients, and eventually modulating properties of atorvastatin on these IL-1$\beta$-mediated effects, we next examined the ability of IL-1$\beta$ and atorvastatin to modulate the expression of suggested proatherogenic members of the TNF super family (ie, TNF-α, FasL, TRAIL, LIGHT, and CD40L) in PBMCs. First, to screen for the effects of IL-1$\beta$ on these cytokines, we examined their gene expression by real-time RT-PCR in freshly isolated PBMCs before and after atorvastatin therapy, only minor changes were seen in IL-1sRII levels (669 ± 64 pg/mL before versus 640 ± 50 pg/mL after therapy, $P=0.08$). Secretion of IL-1sRI was not detected either before or after therapy.

During statin therapy, there were no significant correlations between the reduction in total or LDL cholesterol and the changes in mRNA levels of IL-1$\beta$ (array and RPA), IL-1$\alpha$, and IL-1Ra (RPA), or protein secretion of IL-1$\beta$, whereas there was a weak correlation between the reduction in protein secretion of IL-1Ra and the decrease in total (P=0.08) and LDL (P=0.05) cholesterol.
IL-1 is the prototypic inflammatory cytokine, and in the present study, we report markedly raised levels of both IL-1 isotypes in CAD patients with particularly high levels in unstable disease. Importantly, in unstable angina, these high IL-1 levels were not accompanied by a corresponding increase in IL-1Ra, suggesting an inflammatory dominance. Although we have no data on IL-1 bioactivity, these findings suggest that IL-1 may represent a potential target for therapy in CAD and acute coronary syndromes.

The balance between proinflammatory and anti-inflammatory cytokines is thought to be important for the development of several inflammatory disorders, including atherosclerosis and ACS. Thus, it has been shown that the marked rise in inflammatory cytokines during ACS is not accompanied by elevation of the anti-inflammatory cytokine IL-10. In the present study, we show a similar pattern in the balance between IL-1 and the naturally occurring antagonist IL-1Ra, with markedly increased mRNA levels of IL-1α and IL-1β in circulating mononuclear leukocytes in both stable and particularly in unstable angina, accompanied by only modestly increased IL-1Ra levels in the unstable patients. Hence, although IL-1 is highly inflammatory and potentially plaque-destabilizing, for example by inducing adhesion molecules, clotting factors, chemokines, and matrix metalloproteinases, IL-1Ra may protect against those deleterious IL-1 effects.

The findings in this study that IL-1β can stimulate the expression not only of TNF-α but also of LIGHT, a more recently described member of the TNF superfamily with potential proatherogenic properties, further suggest a role of IL-1 in atherogenesis and plaque destabilization. Agents that reduce the production and/or activity of IL-1 have a proposed clinical impact in various disorders, and our results suggest that such a therapeutic approach may also be applied in CAD and ACS. Indeed, recent studies have shown that IL-1 deficiency decreases the severity of atherosclerosis in apolipoprotein E–deficient mice and that overexpression of IL-1Ra may protect against ischemia-perfusion injury in rat cardiomyocytes.

A major finding in this study was the marked reduction in IL-1 in PBMCs after statin therapy, as shown at both the mRNA and the protein level, reaching levels comparable to those in healthy controls. The lack of a placebo group may limit the possible interpretations of these results. However, the vigorous downregulation of IL-1, which was seen in all but 3 patients, suggests that the decrease in IL-1 does not merely reflect variations by chance. Numerous previous studies have demonstrated anti-inflammatory effects of statins, and our study extends these findings in several ways. First, although statin therapy suppressed IL-1 levels, the effect on IL-1Ra was more modest. Plasma levels of IL-1Ra have been reported to be elevated and correlated with impaired clinical outcome in patients with unstable angina, possibly suggesting inflammatory rather than anti-inflammatory effects of IL-1Ra. However, although plasma concentrations of IL-1Ra may be a reliable marker of IL-1 activity, IL-1Ra at the cellular level, as reported in...
the present study, is thought to reflect its anti-
inflammatory capacity. Thus, we believe that statins
seem to have a more pronounced suppressive effect on
IL-1 than on IL-1Ra in PBMCs, this will result in an
overall anti-inflammatory effect. Moreover, the particular
suppressive effect on IL-1 in the atorvastatin group sug-

Figure 4. A and B, Effect of IL-β (10 ng/mL), atorvastatin (Atorva; 1 or 10
µmol/L), or a combination thereof on secretion of LIGHT (A) and TNF-α (B)
from cryopreserved PBMCs isolated from 4 statin-naïve CAD patients and 4 healthy
controls (CTR) and cultured for 20 hours. Data are mean±SEM. *P<0.05 vs
unstimulated levels; #P<0.05 vs IL-1β–stimulated levels. C and D, Effect of IL-1β
and/or atorvastatin and/or mevalonate (100 µmol/L) on secretion of LIGHT (C)
and TNF-α (D) from freshly isolated PBMCs from 5 healthy controls after 20
hours of culturing. Data are mean±SEM. *P<0.05 vs unstimulated levels.

Whereas our statin study suggests an anti-inflammatory
role of statins, the in vitro experiments revealed more
diverse actions of these drugs. Thus, although atorvastatin
and its biologically important metabolite ortho-hydroxy
atorvastatin suppressed the IL-1–mediated induction of
TNF-α and LIGHT in PBMCs, high dosages (10 µmol/L)
of these drugs increased the expression of TNF-α when
given alone. This observation corresponds with a study by
Kiener et al21 demonstrating increased expression of in-
flammatory cytokines in monocytes after incubation with
dosages of lipophilic statins >2.5 to 5 µmol/L. The
concentrations of statins in tissues during therapy is
unknown but is probably <10 µmol/L.22 However, an in
vivo–relevant enhanced cytokine induction by atorvastatin
and other statins cannot be ruled out, at least at high
dosages.

The findings in the present study suggest that IL-1 and
related cytokines may represent targets for therapy in both
stable and unstable CAD. The ability of statins to modulate
this system in an anti-inflammatory direction further un-
erscores their immunomodulatory potential.

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interleukin-1 soluble receptor and hindered by type I interleukin-1


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