Association Between Enhanced Soluble CD40L and Prothrombotic State in Hypercholesterolemia
Effects of Statin Therapy

Francesco Cipollone, MD; Andrea Mezzetti, MD; Ettore Porreca, MD; Concetta Di Febbo, MD; Michele Nutini, PhD; Maria Fazia, PhD; Angela Falco, MD; Franco Cuccurullo, MD; Giovanni Davì, MD

Background—Hypercholesterolemia is associated with inflammation and the prothrombotic state. CD40-CD40 ligand (CD40L) interactions promote a prothrombotic response in nucleated cells. The aim of this study was to characterize the in vivo expression of soluble CD40L (sCD40L) in hypercholesterolemia, to correlate it with the extent of the prothrombotic state, and to investigate whether it may be modified by statins.

Methods and Results—We studied 80 hypercholesterolemic patients and 80 matched healthy subjects. Hypercholesterolemic subjects had enhanced levels of sCD40L, factor VIIa (FVIIa), and prothrombin fragment 1+2 (F1+2) compared with healthy subjects. sCD40L correlated with total cholesterol and LDL cholesterol. Moreover, sCD40L was positively associated with in vivo platelet activation, as reflected by plasma P-selectin and urinary 11-dehydro-thromboxane B2, and with procoagulant state, as reflected by FVIIa and F1+2. Inhibition of cholesterol biosynthesis by pravastatin or cerivastatin was associated with comparable, significant reductions in sCD40L, FVIIa, and F1+2.

Conclusions—This study suggests that sCD40L may represent the molecular link between hypercholesterolemia and the prothrombotic state and demonstrates that statin therapy may significantly reduce sCD40L and the prothrombotic state.

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Key Words: risk factors ■ atherosclerosis ■ hypercholesterolemia ■ inflammation

A high incidence of thrombotic complications has been associated with hypercholesterolemia. This may be related to enhanced thrombotic risk. In fact, we have previously documented persistent platelet activation and increased thrombin generation in hypercholesterolemic subjects, as well as the ability of simvastatin to reverse these abnormalities.

The CD40 ligand (CD40L) has been found expressed on CD4+ T cells and on activated platelets. Both membrane-bound and soluble CD40L (sCD40L) interact with CD40 expressed on vascular cells, resulting in inflammatory and prothrombotic responses. Thus, CD40L-CD40 interaction may be the molecular mechanism that links inflammation and the prothrombotic state in the setting of hypercholesterolemia. According to this hypothesis, inhibition of CD40 signaling would prevent the initiation and progression of atherosclerosis. Moreover, sCD40L has been implicated in acute coronary syndromes, and elevated sCD40L predicts an increased risk of future cardiovascular events in healthy subjects. Interestingly, Garlichs et al showed that hypercholesterolemia is associated with an ex vivo activation of the CD40 system that can be downregulated by cerivastatin. However, they did not provide any direct evidence suggesting the intervention of this mechanism in vivo or its contribution to the prothrombotic state observed in hypercholesterolemia.

Therefore, in the present study, we investigated whether upregulation of the CD40 system in hypercholesterolemia is associated with in vivo platelet activation and the procoagulant state. Moreover, we investigated whether the sCD40L-associated prothrombotic state may be normalized by statins and whether this effect is a function of cholesterol reduction or is due to pleiotropic effects of statins.

Methods

Subjects
Eighty consecutive hypercholesterolemic patients and 80 matched healthy normocholesterolemic subjects were asked to participate in the study. Patient recruitment was completed when predetermined groups with laboratory evidence of hypercholesterolemia were formed. The local Ethics Committee approved the study, and patients provided written informed consent. The baseline characteristics of patients and controls are shown in Table 1.
TABLE 1. Characteristics of Study Participants

<table>
<thead>
<tr>
<th>Variable</th>
<th>Patients (n=80)</th>
<th>Controls (n=80)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>57±12</td>
<td>51±18</td>
</tr>
<tr>
<td>Sex, M/F</td>
<td>37/43</td>
<td>37/43</td>
</tr>
<tr>
<td>Diabetes</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Hypertension</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Smoking</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Aspirin</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Concomitant therapies</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>TC, mg/dL</td>
<td>258±25*</td>
<td>187±18</td>
</tr>
<tr>
<td>LDL-C, mg/dL</td>
<td>170±29*</td>
<td>126±13</td>
</tr>
<tr>
<td>Triglycerides, mg/dL</td>
<td>152±91</td>
<td>174±61</td>
</tr>
<tr>
<td>HDL cholesterol, mg/dL</td>
<td>53±12</td>
<td>51±8</td>
</tr>
</tbody>
</table>

*P<0.001.

Design of the Studies
First, a cross-sectional comparison of circulating sCD40L, interleukin-2 receptor (sIL2-R), factor VIIa (FVIIa), and prothrombin fragment 1+2 (F1+2) was performed between all patients and controls. Furthermore, urinary 11-dehydro-thromboxane (TX) B2, and plasma P-selectin were evaluated in 27 of the 80 hypercholesterolemic patients compared with 27 of the 80 healthy controls. Both groups were chosen randomly.

Second, to ascertain whether statin therapy could influence sCD40L, we investigated the effects of 2 structurally different statins (cerivastatin and pravastatin) on sCD40L. Thus, in a first study (study A), we randomly assigned 10 of the 80 hypercholesterolemic patients to 8-week treatment with either cerivastatin (0.2 mg/d) or placebo in a double-blind fashion. All patients were given the American Heart Association step I diet.

In a second study (study B), 16 of the 80 hypercholesterolemic patients were randomly assigned to 8-week treatment with either pravastatin (40 mg/d) or placebo in a double-blind fashion. All patients were given the American Heart Association step I diet.

Sample Collection
Serum, plasma, and urine samples were obtained after a 12-hour fast. Samples were frozen and stored at −20°C until analysis.

Biochemical Measurements
Total cholesterol (TC) and triglycerides were determined by an enzymatic method. HDL cholesterol was measured after phosphotungstic acid/MgCl2 precipitation on fresh plasma. LDL cholesterol (LDL-C) was calculated by the Friedewald formula.

Enzyme Immunoassays
Serum sCD40L and plasma P-selectin, sIL2-R, F1+2, and FVIIa were determined by ELISA (sCD40L: Bender Medsystems; P-selectin and sIL2-R: R&D Systems; F1+2: Behringwerke AG; and FVIIa: Diagnostica Stago).

11-Dehydro-TXB2 Assay
Immunoreactive 11-dehydro-TXB2 was extracted from 20-mL urine aliquots and measured by a previously described radioimmunoassay.

Statistical Analysis
Comparisons between groups were made with the Mann-Whitney U test. The differences between baseline and posttreatment values were analyzed with the Wilcoxon test. Correlations were assessed by Spearman test. All values are reported as mean±SD. Statistical significance was indicated by P<0.05. All calculations were made with the Stat View II program (Abacus Concepts).

Results
Patients had significantly higher TC and LDL-C than controls. No differences were observed with respect to HDL cholesterol and triglycerides (Table 1).

Inflammatory and Prothrombotic State
sCD40L was significantly increased in hypercholesterolemic versus normocholesterolemic subjects (8.3±5.2 versus 2.4±1.3 ng/mL, n=80, P<0.0001; Figure 1A). Fifty-eight (72.5%) of 80 patients had sCD40L in excess of 2 SD above the control mean (Figure 1A). Hypercholesterolemic patients also had higher FVIIa and F1+2 with respect to controls (37±13 versus 24.8±4.8 mU/mL, P<0.001, and 1.52±0.95 versus 0.55±0.22 nmol/L, P<0.001, respectively; n=80). Finally, hypercholesterolemic patients had enhanced 11-dehydro-TXB2 (1319±547 versus 350±164 pg/mg creatinine, n=27, P<0.001) and P-selectin (115±55 versus 60±15 ng/mL, n=27, P<0.001) compared with controls.

Associations
In hypercholesterolemic patients and in control subjects, sCD40L showed a correlation with TC (r=0.353, P=0.0018, and r=0.231, P=0.04, respectively) and LDL-C (r=0.379, P=0.0008, and r=0.292, P=0.0094, respectively; Figure
1B). Moreover, sCD40L was correlated with the intensity of platelet activation, as reflected by 11-dehydro-TXB₂ (Figure 2a) and P-selectin (Figure 2b), both in hypercholesterolemic patients (r=0.443, P=0.024, and r=0.455, P=0.0204, respectively) and in normocholesterolemic subjects (r=0.514, P=0.0087, and r=0.402, P=0.0403, respectively). sCD40L was also correlated with the procoagulant state, as reflected by FVIIa (Figure 2c) and F1+2 (Figure 2d) levels, both in hypercholesterolemic patients (r=0.799, P=0.0001, and r=0.789, P<0.0001, respectively) and in controls (r=0.343, P=0.0023, and r=0.273, P=0.0154, respectively). In contrast, sCD40L was not correlated with T-lymphocyte activation, as reflected by sIL2-R (data not shown).

**Effect of Statin Therapy**
Placebo administration was not associated with any change in blood lipids, sCD40L, or procoagulant state in the 2 intervention studies (Table 2). In contrast, both pravastatin and cerivastatin therapy were associated with comparable, significant reductions in TC and LDL-C (Table 2). More interestingly, patients randomized to both statins showed a significant reduction in sCD40L, FVIIa, and F1+2 (Figure 1B; Table 2). When we compared the effect of cerivastatin and pravastatin on sCD40L, we observed that both drugs produced a reduction in sCD40L comparable to the effects on lipid profile (Table 2). Notably, the correlation between sCD40L and TC (r=0.389, P=0.05), LDL-C (r=0.41, P=0.05), FVIIa (r=0.871, P=0.0001), and F1+2 (r=0.733, P=0.0002) remained significant after statin therapy.

**Discussion**
Despite ex vivo evidence of CD40L upregulation on activated platelets that has been reported recently in the setting of

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**Figure 2.** Correlation between sCD40L and 11-dehydro-TXB₂ (a), P-selectin (b), FVIIa (c), and F1+2 (d) in hypercholesterolemic and normocholesterolemic (box) subjects.

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**TABLE 2. Statin Therapy Is Associated With Reduced Inflammation and Prothrombotic State**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Study A</th>
<th></th>
<th>Study B</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Placebo (n=5)</td>
<td>Cerivastatin (n=5)</td>
<td>Placebo (n=8)</td>
<td>Pravastatin (n=8)</td>
</tr>
<tr>
<td>TC, mg/dL</td>
<td>246±26 vs 247±28</td>
<td>248±29 vs 206±43*</td>
<td>256±17 vs 241±19</td>
<td>272±18 vs 224±22†</td>
</tr>
<tr>
<td>LDL-C, mg/dL</td>
<td>147±21 vs 143.2±26.8</td>
<td>144±21 vs 102±20*</td>
<td>171±16 vs 156±19</td>
<td>189±28 vs 139±27†</td>
</tr>
<tr>
<td>Triglycerides, mg/dL</td>
<td>140±42 vs 154±42</td>
<td>180±46 vs 157±68</td>
<td>165±72 vs 147±54</td>
<td>112±26 vs 126±38</td>
</tr>
<tr>
<td>HDL cholesterol, mg/dL</td>
<td>46±13 vs 50±15</td>
<td>47±3 vs 49±7</td>
<td>57±19 vs 56±15</td>
<td>54±13 vs 60±13</td>
</tr>
<tr>
<td>sCD40L, ng/mL</td>
<td>6.4±1.5 vs 4.7±2.9</td>
<td>6.4±2.9 vs 3.3±1.2*</td>
<td>9.6±6 vs 8.3±4.7</td>
<td>10.7±7 vs 7.5±2†</td>
</tr>
<tr>
<td>FVIIa, mU/mL</td>
<td>29.4±7.1 vs 29.2±5.5</td>
<td>34.4±9.4 vs 25±4.4*</td>
<td>43.4±13.9 vs 40.9±9.7</td>
<td>44±17.4 vs 30±10.4†</td>
</tr>
<tr>
<td>F1+2, nmol/L</td>
<td>1.4±0.7 vs 1.2±0.6</td>
<td>1.6±0.9 vs 1.1±0.5*</td>
<td>1.6±0.8 vs 1.5±0.5</td>
<td>2.1±1.2 vs 1.2±0.5†</td>
</tr>
</tbody>
</table>

Values shown are before vs after therapy.
*P<0.05; †P<0.02.
hypercholesterolemia,\textsuperscript{10} no double-bind, randomized studies have yet demonstrated whether statins are able to reduce circulating sCD40L in vivo, thus improving the prothrombotic state associated with hypercholesterolemia.\textsuperscript{11} Furthermore, little is still known regarding the cellular sources of sCD40L in humans. The present study is the first to (1) provide in vivo evidence that enhanced sCD40L is associated with platelet activation in the setting of hypercholesterolemia, (2) relate the increase in sCD40L to the prothrombotic state previously described in hypercholesterolemia, and (3) provide in vivo evidence that statins may significantly reduce sCD40L and the CD40L-associated prothrombotic state.

In the present study, the association between enhanced sCD40L and platelet activation is supported by the relationship between sCD40L and both 11-dehydro-TXB\textsubscript{2} and P-selectin. In contrast, the nonsignificant correlation between sCD40L and sIL2-R suggests that T cells are unlikely to be a major source of sCD40L in this setting.

In addition to the association with platelet activation, increased sCD40L showed a positive correlation with FVIIa and F1+2, which are in vivo indices of coagulative activation. This could suggest that hypercholesterolemia stimulates the release of sCD40L from activated platelets in vivo and that it may subsequently induce a procoagulant response in endothelial and mononuclear cells through interaction with CD40.\textsuperscript{4,5} Interestingly, because CD40L is a stimulus for cyclooxygenase-2 expression and activated platelets may induce cyclooxygenase-2 in mononuclear cells,\textsuperscript{12} we can speculate that sCD40L might also contribute to this transcellular mechanism of aspirin-insensitive TXA\textsubscript{2} biosynthesis.

In the present study, increased sCD40L in hypercholesterolemic patients showed a correlation with TC and LDL-C; in contrast, a nonsignificant ($P=0.06$) trend was observed in the study from Garlichs et al.\textsuperscript{10} It is likely that the significantly different sample size between the 2 studies (80 versus 15 patients) may be responsible for this discrepancy.

In the present study, statins decreased sCD40L and coagulative activation, 2 effects that could contribute to explain the reduction in cardiovascular events observed in clinical trials with statins. In contrast, Garlichs et al\textsuperscript{10} reported that a short-term (21 day) treatment with cerivastatin 0.3 mg/d did not significantly affect sCD40L. The most plausible explanation for this discrepancy might be the different period of treatment. Thus, adequately long treatment periods with statin might be necessary to significantly downregulate sCD40L.

Notably, the comparable reduction in sCD40L obtained with both cerivastatin and pravastatin argues against any molecule-specific pleiotropic effect and suggests that a reduction in sCD40L and the sCD40L-associated prothrombotic state is a class feature of statins.

In conclusion, the present study addresses the missing link between hypercholesterolemia and the prothrombotic state by demonstrating the association between enhanced sCD40L and platelet and coagulative activation in this setting and by providing evidence that sCD40L suppression by statins is associated with the prothrombotic state reduction in vivo. These findings are potentially important from a fundamental standpoint because they demonstrate the decisive role of sCD40L in all steps of thrombus formation. From a practical standpoint, statins might provide a novel therapy for high-risk patients with a thrombophilic pattern.

References

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