Associations Between Soluble CD40 Ligand, Atherosclerosis Risk Factors, and Subclinical Atherosclerosis
Results from the Dallas Heart Study

James A. de Lemos, Andreas Zirlik, Uwe Schönbeck, Nerea Varo, Sabina A. Murphy, Amit Khera, Darren K. McGuire, Greg Stanek, Hao S. Lo, Rebecca Nuzzo, David A. Morrow, Ronald Peshock, Peter Libby

Objectives—The purpose of this study was to evaluate the associations between plasma levels of soluble CD40 ligand (sCD40L), atherosclerosis risk factors, and evidence of subclinical atherosclerosis.

Methods and Results—Plasma levels of sCD40L were measured in 2811 subjects from the Dallas Heart Study, a multiethnic population-based cross-sectional study. Electron Beam Computed Tomography measurements of coronary artery calcium (CAC) and MRI measurements of aortic plaque were performed in 2198 and 1965 subjects, respectively. No association was observed between quartiles of sCD40L and age, sex, race, body mass index, diabetes, smoking, creatinine clearance, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, or C-reactive protein. In contrast, weak but statistically significant associations were observed between sCD40L and total cholesterol and triglycerides. The prevalence of detectable CAC (CAC score ≥ 10) and aortic plaque did not differ across sCD40L quartiles, and individuals with CAC scores < 10, 10 to 100, > 100 to 400, and > 400 had similar sCD40L levels.

Conclusions—In a large and representative multiethnic population-based sample, sCD40L was not associated with most atherosclerotic risk factors or with subclinical atherosclerosis. These findings suggest that sCD40L will not be useful as a tool to screen for the presence of subclinical atherosclerosis in the population. Further evaluation of this biomarker should focus on settings in which platelet activation is common, such as following acute coronary syndromes or coronary revascularization procedures. (Arterioscler Thromb Vasc Biol. 2005;25:2192-2196.)

Key Words: sCD40 ligand ■ CD40 ■ atherosclerosis ■ diabetes ■ cholesterol

Multiple cell types in the developing atherosclerotic lesion express the proinflammatory and immunoregulatory molecule CD40 ligand (CD40L; CD154) and its receptor, CD40.1 CD40/CD40L interactions promote processes that likely contribute to the initiation, progression, and complications of atherosclerosis, including endothelial cell activation,2 release of inflammatory cytokines3 and matrix degrading enzymes,4 and tissue factor production.5,6 Recently, interest has focused on the measurement of a circulating soluble form of CD40L (sCD40L) for risk stratification of patients with or at risk of developing coronary artery disease. Pilot studies have found that individuals with hypercholesterolemia7 and diabetes8 have elevated sCD40L levels and that very high levels of sCD40L may identify apparently healthy women at increased risk of having a first adverse cardiovascular event.9 In contrast with membrane-bound CD40L, which is expressed on multiple cell types, platelets appear to contribute most circulating sCD40L.10 In patients with acute coronary syndromes, levels of sCD40L correlate with measures of platelet activation and serve to identify patients at risk of having recurrent ischemic events.11,12 Few data on sCD40L are available from population-based studies. Therefore, we measured plasma sCD40L in a large multiethnic population and correlated plasma levels of sCD40L with coronary artery disease risk factors and with measures of subclinical atherosclerosis.

Methods
The Dallas Heart Study is a multiethnic population-based study of 6101 randomly selected adult subjects from Dallas County between the ages of 30 and 65. The initial cohort underwent a detailed in-home health survey visit, and 3557 subjects returned for blood and urine collection. Subsequently, 2971 subjects returned for a detailed clinic visit that included cardiac and aortic MRI and electron beam computed tomography (EBCT). Two EBCT measurements were performed and the average coronary artery calcium (CAC) score was reported using standard calcium scoring methods.13 Abdominal MRI was performed using a 1.5 Tesla whole-body MRI system (Intera, Original received April 26, 2005; final version accepted August 2, 2005.
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Arterioscler Thromb Vasc Biol. is available at http://www.atvbaha.org DOI: 10.1161/01.ATV.0000182904.08513.60

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Association Between Plasma Levels of Soluble CD40 Ligand and Selected Clinical Variables

<table>
<thead>
<tr>
<th>Quartile 1 (n=703)</th>
<th>Quartile 2 (n=703)</th>
<th>Quartile 3 (n=703)</th>
<th>Quartile 4 (n=702)</th>
<th>P Value for Trend</th>
<th>≤95% (n=2668)</th>
<th>&gt;95% (n=143)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>sCD40L, ng/mL</td>
<td>0.82</td>
<td>1.40</td>
<td>2.48</td>
<td>5.42</td>
<td>5.43–11.97</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age, years</td>
<td>44 [36, 52]</td>
<td>43 [36, 51]</td>
<td>44 [27, 52]</td>
<td>44 [36, 53]</td>
<td>0.54</td>
<td>43 [36, 52]</td>
<td>44 [37, 54]</td>
</tr>
<tr>
<td>Male sex</td>
<td>262 (37%)</td>
<td>265 (38%)</td>
<td>235 (33%)</td>
<td>285 (41%)</td>
<td>0.48</td>
<td>980 (37%)</td>
<td>67 (47%)</td>
</tr>
<tr>
<td>White race</td>
<td>182 (26%)</td>
<td>198 (28%)</td>
<td>213 (30%)</td>
<td>178 (25%)</td>
<td>0.94</td>
<td>731 (27%)</td>
<td>30 (28%)</td>
</tr>
<tr>
<td>Diabetes</td>
<td>80 (11%)</td>
<td>70 (10%)</td>
<td>97 (14%)</td>
<td>80 (11%)</td>
<td>0.47</td>
<td>314 (12%)</td>
<td>13 (9%)</td>
</tr>
<tr>
<td>Hypertension</td>
<td>219 (32%)</td>
<td>236 (34%)</td>
<td>254 (37%)</td>
<td>255 (37%)</td>
<td>0.02</td>
<td>907 (35%)</td>
<td>57 (40%)</td>
</tr>
<tr>
<td>Current smoker</td>
<td>194 (28%)</td>
<td>185 (26%)</td>
<td>186 (27%)</td>
<td>186 (27%)</td>
<td>0.54</td>
<td>732 (27%)</td>
<td>29 (20%)</td>
</tr>
<tr>
<td>Family history of CAD</td>
<td>228 (32%)</td>
<td>233 (33%)</td>
<td>243 (35%)</td>
<td>227 (32%)</td>
<td>0.89</td>
<td>878 (33%)</td>
<td>53 (37%)</td>
</tr>
<tr>
<td>HDL, mg/dL</td>
<td>47 [41, 56]</td>
<td>48 [39, 58]</td>
<td>49 [40, 59]</td>
<td>48 [40, 58]</td>
<td>0.15</td>
<td>48 [40, 58]</td>
<td>49 [41, 60]</td>
</tr>
<tr>
<td>Triglycerides, mg/dL</td>
<td>90 [82, 136]</td>
<td>97 [68, 147]</td>
<td>99 [69, 142]</td>
<td>96 [69, 143]</td>
<td>0.01</td>
<td>96 [67, 144]</td>
<td>96 [69, 140]</td>
</tr>
<tr>
<td>hs-CRP, mg/L</td>
<td>2.9 [1.1, 7.1]</td>
<td>3.1 [1.2, 7.9]</td>
<td>3.2 [1.3, 8.0]</td>
<td>3.3 [1.3, 7.6]</td>
<td>0.28</td>
<td>3.1 [1.2, 7.7]</td>
<td>2.6 [1, 6.3]</td>
</tr>
</tbody>
</table>

Values are medians and interquartile ranges for continuous variables, and percentages for categorical variables.

BMI indicates body mass index; CAD, coronary artery disease; hs-CRP, high sensitivity C-reactive protein.

Philips Medical Systems). Six transverse slices of the infrarenal abdominal aorta were obtained using a free-breathing, ECG-gated, T2-weighted turbo spin-echo (black-blood) sequence. Images were analyzed by trained observers using the Magnetic Resonance Analytical Software Systems (MASS) cardiac analysis software package (Version 4.2 beta, Medis Medical Imaging Systems, Inc). The criteria for aortic wall lesions have been previously defined.14 Adventitial and luminal borders were drawn for each slice using a free-hand manual contour drawing tool. Areas of increased signal intensity, luminal protrusion, and focal wall thickening were identified as atherosclerotic plaque.

Details of the study design,15 variable definitions,16 and EBCT imaging techniques17 have been reported previously. Medical history, demographic data, body mass index (BMI), and blood pressure were similar between subjects completing the home survey, blood collection visit, and the detailed clinic visit, and laboratory parameters were similar between subjects completing the blood collection and clinic visits.15 All patients who had plasma volume sufficient for measurement of sCD40L (n=2811) were included in the present substudy. Of this group, 2198 underwent EBCT measurement of coronary calcification and 1965 underwent MRI measurement of aortic atherosclerosis. Subject characteristics were similar among the overall cohort with blood drawn, and among the subsets with sCD40L measured, with EBCT performed, and with aortic MRI performed (data not shown).

Measurement of sCD40L and Other Analytes

Venous blood was collected into ethylenediaminetetraacetic acid (EDTA) tubes and centrifuged at 1430g for 15 minutes at 4°C. The platelet-poor plasma component was stored at −80°C. Samples were thawed and assays were performed for high sensitivity C-reactive protein (Roche Diagnostics) and monococyte chemoattractant protein (MCP-1) (Biosite, Inc) as previously described.16 Because whole blood samples were not available for measurement of glycosylated hemoglobin, serum fructosamine was measured to estimate level of glycemic control using a standardized Roche/Hitachi fructosamine assay (Roche Diagnostics). Samples were refrozen and shipped to the core laboratory at the Brigham and Women’s Hospital, where assays for sCD40L (Bender MedSystems) were performed in 96-well plates by a single investigator (A.Z.), blinded to subject information, as described previously.8,9,12,18 The intra- and interassay variation coefficients were <10% and <15%, respectively, at concentrations relevant to this study.

Statistical Analysis

Subjects were divided into quartiles based on the level of sCD40L. Categorical variables were compared across quartiles using the χ² trend test. Trends for continuous variables were assessed using the χ² trend test across ordered groups. A threshold value of sCD40L at 95% was also evaluated, and comparisons between subjects > versus ≤95% were performed using the χ² test for categorical variables and the Wilcoxon rank-sum test for continuous variables. The correlation between sCD40L and continuous variables was explored using Spearman correlation coefficients. Summary statistics are reported as medians (interquartile ranges) for continuous variables and percentages for categorical variables. All analyses were performed using STATA version 7-intercooled (STATA Corp). Probability values of <0.05 (2-tailed) were considered to indicate statistical significance.

Results

No association was observed between quartiles of sCD40L and age, sex, race, BMI, diabetes, smoking, or creatinine clearance (Table). Distribution of these variables was also similar between subjects with sCD40L >95% (5.42 ng/mL) versus those with sCD40L ≤95% (Table). Weak but statistically significant associations were observed between sCD40L quartiles and hypertension, total cholesterol, and triglycerides. In contrast, sCD40L did not associate with low-density lipoprotein (LDL) cholesterol, high-density (HDL) cholesterol, C-reactive protein, or fructosamine (Table). When the analyses were restricted to subjects with diabetes, no difference in fructosamine concentration was observed across sCD40L quartiles (P=0.17). The strongest association was observed between sCD40L and MCP-1 (Spearman’s ρ 0.15; P<0.0001). The associations between sCD40L and demographic and clinical variables were similar.
when these analyses were restricted to subjects with available EBCT or aortic MRI data.

The prevalence of detectable CAC (CAC score ≥10) and aortic plaque did not differ across sCD40L quartiles (Figure 1). Similarly, no difference in the prevalence of CAC ≥10 or aortic plaque was observed for subjects with sCD40L >95% versus those with levels ≤95% (Figure 2). Individuals with CAC scores <10, ≥10 to 100, >100 to 400, and >400 had similar sCD40L levels (Figure 3). The associations above did not change when subjects taking statin medications (n = 216) were excluded.

**Discussion**

In a large, multiethnic, population-based sample, we observed minimal association between sCD40L and coronary disease risk factors and no association between sCD40L and evidence of subclinical atherosclerosis. These findings suggest that sCD40L will not be useful as a tool to screen for the presence of subclinical atherosclerosis in low-risk subjects.

**Validity of the Present Null Findings**

Several issues require consideration in regards to these null findings, including type II error, assay methodology, and sample processing and storage issues. A type II statistical error is unlikely in the present study, as it had 99% power to detect a 25% difference in sCD40L levels between patients with and without CAC ≥10 and 93% power to detect a 20% difference. In addition, power was 99% to detect a 50% increase in the prevalence of CAC ≥10 between different sCD40L quartiles. We used a standard well-validated ELISA kit (Bender Medsystems) that has been used in most previous studies of sCD40L performed to date.7–10,12–18 Plasma levels of sCD40L seen in our study are similar to those in other population-based studies.9,11,12,25

We used platelet-poor EDTA plasma, the optimal sample type for determination of in vivo circulating sCD40L, for measurement of sCD40L in this study. Sample processing methods can profoundly affect measured levels of sCD40L, with serum yielding levels 3- to 5-fold higher than plasma.10,19 Differences between serum and plasma sCD40L levels are partially attenuated when serum is frozen immediately after collection,10 demonstrating that ex vivo release from platelets contributes to higher serum versus plasma levels.10,19,26 Moreover, platelet count influences serum levels of sCD40L, a confounding factor avoided by use of platelet-poor plasma in the present study.10 Finally, EDTA inhibits the release of CD40L from the platelet surface when administered before platelet activation,21,27,28 thus preventing ex vivo release of sCD40L.

The cross-sectional design of the present study does not permit delineation of cellular source of sCD40L, and the plasma levels measured likely reflect an aggregate measurement from all cell sources, including platelets (resulting from in vivo activation), endothelial cells, smooth muscle cells, monocytes, and T cells.1

**Association Between sCD40L, Risk Factors, and Atherosclerosis**

Several small studies have reported elevated levels of sCD40L among patients with diabetes,8,20–24 but other studies have not observed a similar association.9,11,12,25 Our population-based study, much larger than any of the previously published studies, found similar levels of sCD40L in subjects with and without diabetes. We also found no corre-
lation between levels of fructosamine, an intermediate-term measure of glucose control, and sCD40L, a finding supported by other studies that reported no correlation between Hemoglobin A1C and sCD40L.\textsuperscript{20,29,31}

Previous small studies of highly selected patients have reported elevated levels of sCD40L among patients with hypercholesterolemia and a reduction in sCD40L after treatment with statin agents.\textsuperscript{7,18,22,23} However, these findings have not consistently been observed,\textsuperscript{31} and a recent large study found no effect of high dose atorvastatin on levels of sCD40L after an acute coronary syndrome event.\textsuperscript{33} We observed only a weak association between total cholesterol and sCD40L in the present population-based study of unselected individuals and no significant associations between sCD40L and LDL or HDL cholesterol levels. The discordance in findings may be attributable to differences in the patient populations studied.

In the general population, which consists predominantly of low-risk individuals, hyperlipidemia appears to contribute only modestly to variability in sCD40L. In contrast to the weak associations observed with lipid parameters, a more robust correlation was observed between sCD40L and MCP-1, a finding that supports the role of CD40/CD40L signaling in stimulating monocyte activation.\textsuperscript{34,35}

Most importantly, we found no association between sCD40L and 2 measures of prevalent atherosclerosis, CAC and aortic plaque. In several of the pilot studies described above, sCD40L was associated with diabetes but not with the presence of coronary artery disease.\textsuperscript{22,30,31} Thus, although cellular interactions between CD40 and CD40L play an important role in the development of atherosclerosis, plasma levels of sCD40L do not appear useful as a marker of atherosclerosis burden.

Clinical and Research Implications

Although the present study does not support the utility of sCD40L as a screening tool for subclinical atherosclerosis, long-term follow-up studies will be needed to determine whether sCD40L can serve as a marker of future cardiac events in healthy populations. Indeed, it is possible that sCD40L will perform better as a marker of plaque vulnerability than of plaque burden: pilot studies have suggested that sCD40L is more closely associated with plaque composition than plaque volume.\textsuperscript{36} The present findings cannot be extrapolated to situations in which platelet activation is more common, and more clinically relevant, such as acute coronary syndromes, percutaneous coronary intervention, and coronary artery bypass surgery. In these settings, where platelet activation has proven to be an important therapeutic target, higher levels of sCD40L have been correlated with other markers of platelet activation, an increased risk for recurrent ischemic events, and the benefit of antiplatelet therapies.\textsuperscript{11,12,24,33,37}

Conclusions

In a large and representative multiethnic population-based sample, sCD40L was not associated with most atherosclerotic risk factors or with subclinical atherosclerosis. Evaluation of the utility of this biomarker should focus on settings in which platelet activation is common, such as in patients with acute coronary syndromes or those undergoing revascularization procedures.

Acknowledgments

This work was supported by grants from the Donald W. Reynolds Foundation to UT Southwestern Medical Center and the Brigham and Women’s Hospital and were partially supported by United States Public Health Service General Clinical Research Center (USPHS GCRC) grant #8M01RR0633 from National Institutes of Health/National Center for Research Resources-General Clinical Research Center (NCCRR-CTSC). Dr de Lemos was supported by a career development award from GlaxoSmithKline. Dr Zirlik was supported by a grant from the Deutsche Forschungsgemeinschaft (ZIT43/1-1), and Hao Lo was supported by a Doris Duke Charitable Foundation Clinical Research Fellowship for Medical Students.

References


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*Arterioscler Thromb Vasc Biol. 2005;25:2192-2196; originally published online August 18, 2005;
doi: 10.1161/01.ATV.0000182904.08513.60

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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