Soluble CD40 Ligand Levels Indicate Lipid Accumulation in Carotid Atheroma

An In Vivo Study With High-Resolution MRI

Gavin J. Blake, Robert J. Ostfeld, E. Kent Yucel, Nerea Varo, Uwe Schönbeck, Michael A. Blake, Marie Gerhard, Paul M. Ridker, Peter Libby, Richard T. Lee

Objective—The CD40/CD40 ligand pathway mediates inflammatory processes important in atherogenesis and the formation of the intraplaque lipid pool. We tested the hypothesis that plasma levels of soluble CD40 ligand are elevated in patients with evidence of a lipid pool on high-resolution magnetic resonance imaging (MRI) of carotid stenoses.

Methods and Results—We recruited 49 patients with evidence of carotid atherosclerosis on ultrasonography; 3 patients could not undergo carotid MRI because of claustrophobia. The remaining 46 patients underwent high-resolution MRI of the carotid arteries. Two radiologists blinded to all other data determined the presence or absence of an intraplaque lipid pool based on the loss of signal between the 20-ms echo time (TE20) and the fat-suppressed TE55 fast spin-echo images. Plasma levels of soluble CD40 ligand were determined by ELISA. Baseline levels of soluble CD40 ligand were higher among patients with evidence of intraplaque lipid (n = 14) than among those without it (n = 32; median, 2.54 ng/mL; interquartile range [IQR], 1.85 to 3.52) vs median, 1.58 ng/mL; IQR, 1.21 to 2.39; P = 0.02). In contrast, soluble CD40 ligand levels were not correlated with percent diameter stenosis (r = −0.19; P = 0.21). The relative risk for intraplaque lipid associated with soluble CD40 ligand levels above the median was 6.0 (95% confidence interval, 1.15 to 31.23; P = 0.03). The magnitude of this predictive effect did not substantially change after adjustment for traditional cardiovascular risk factors (relative risk, 5.12; 95% confidence interval, 0.78 to 33.73; P = 0.09).

Conclusions—Plasma levels of soluble CD40 ligand may predict patients with features of high-risk atherosclerotic lesions. These data provide novel insight into the mechanism through which elevated levels of soluble CD40 ligand may reflect cardiovascular risk in humans and illustrate the potential value of interfacing high-resolution MRI with studies of vascular inflammation. (Arterioscler Thromb Vasc Biol. 2003;23:1111–1119.)

Key Words: CD40 ligand ▪ atherosclerosis ▪ lipid pool ▪ MRI

The structure and dynamic biology of the atheroma, rather than the severity of stenosis, largely determine cardiovascular events. Large lipid pools and thin fibrous caps characterize vulnerable plaques, and inflammatory mechanisms play a pivotal role in determining plaque stability.1,2 Much of our knowledge of the unstable atheroma derives from postmortem examination, and less is known about the relationships of inflammatory mechanisms and lesion structure in vivo.

The binding of CD40 ligand with its receptor CD40 mediates many inflammatory responses important in atherosclerosis. A wide variety of inflammatory cells express CD40 ligand, and stimulation by other proinflammatory cytokines increases endothelial cell expression of CD40 ligand.3 Ligation of CD40 induces the expression of leukocyte adhesion molecules3 and triggers the release of chemoattractants overexpressed in human atheroma.4,5 Furthermore, CD40 ligation potently induces the expression of tissue factor,7,8 an important prothrombotic component of the intraplaque lipid pool. Thus, the spectrum of functions of CD40 ligand appears to span a wide range from early atherogenesis to late thrombotic complications.

Evidence from animal studies supports the importance of CD40 ligand because inhibition of CD40 signaling in atherosclerosis-prone mice reduced the size and lipid content of aortic lesions and yielded a relative increase in smooth muscle content and fibrillar collagen.9 Moreover, recent data show that elevated plasma levels of soluble CD40 ligand at baseline prospectively predict cardiovascular events among apparently healthy women.10

Recent advances in magnetic resonance imaging (MRI) have permitted noninvasive assessment of carotid plaque composition.11–13 Specifically, use of a custom-made, phased-array carotid coil has demonstrated high levels of agreement...
between carotid MRI findings and results of histological examination among 22 patients undergoing carotid endarterectomy (89% agreement; kappa = 0.83; 95% confidence interval, 0.67 to 1.0). Furthermore, high-resolution carotid MRI accurately detects intraplaque lipid-rich cores.2,13,15,16

This study tested the hypothesis that elevated plasma levels of soluble CD40 ligand are correlated with features suggestive of lipid-rich cores on high-resolution carotid MRI. For comparison, we also measured levels of soluble intercellular adhesion molecule-1 (sICAM-1), a marker of endothelial inflammation.

Methods

Study Participants

From January 2001 to January 2002, we invited men and women with stenoses >30% in either the internal or common carotid artery as assessed by carotid ultrasonography to participate in the study. Any patient with a pacemaker or implantable cardioverter defibrillator was excluded, as well as patients who had received surgical clips or coronary stents in the previous 2 months. Patients with a systemic inflammatory condition or those requiring systemic corticosteroids were also excluded. The study population comprises the 49 consecutive patients who gave informed, written consent to participate in the study. The study was approved by the Human Research Committee of Brigham and Women’s Hospital.

A detailed medical history, including prior cardiovascular history, risk factors, and medication use, was recorded by a study physician for each participant. A blood sample was drawn by nontraumatic venipuncture and centrifuged, and the plasma was stored in EDTA at −80°C.

MRI Protocol

The patients underwent high-resolution MRI of the carotid arteries with a dedicated phased-array carotid coil (IGC, Inc) on a 1.5-T Signa CV/i MRI scanner (GE Medical Systems). Three-dimensional time-of-flight images, proton density–weighted images, and fat-suppressed T2-weighted images were obtained. For the 3D time-of-flight sequences, parameters were as follows: echo time (TE) 3.5 ms, repetition time (TR) 33 to 40 ms, slice thickness (mm), 32 slices, acquired matrix 512×512, reconstructed matrix 256×256, R-R intervals, echo train length 16, bandwidth 62.5 kHz, field of view 12 to 14 cm, slice thickness 2 mm, interpolated to 1 mm, 32 slices, acquired matrix 256×256, reconstructed matrix 512×512, and 1 excitation. For the proton density–weighted sequence, a TE of 21 to 22 ms was used, and for the T2-weighted acquisition, a TE of 53 to 58 ms was used with chemical selective fat suppression. Parameters for these scans were as follows: TR of 2 R-R intervals, echo train length 16, bandwidth 62.5 kHz, field of view 14 cm, slice thickness 3 mm, acquired matrix 256×256, reconstructed matrix 512×512, and 1 excitation. Slice levels were centered at the carotid bifurcation in each patient. Two board-certified radiologists blinded to all other information determined the presence or absence of intraplaque lipid, based on the loss of signal between the proton density–weighted images and the fat-suppressed, T2-weighted fast spin-echo images with isointense signal on 3D time-of-flight imaging.13 The percent diameter stenosis was calculated as the difference between reference and stenotic diameters on 3D time-of-flight axial images, divided by the reference diameter and multiplied by 100. The images were read independently off-line. Interobserver variability was <10%.

Laboratory Studies

Baseline plasma soluble CD40 ligand concentrations were measured by ELISA as previously described.10 In brief, 1:5-diluted plasma samples were applied in triplicate to 96-well plates precoated with mouse anti-human CD40 ligand antibody and mixed (1:2) with a horseradish peroxidase–labeled secondary mouse anti-human CD40 ligand antibody for 2 hours. Plates were then washed, and antibody binding was determined by colorimetry with 3,3′,5,5′-tetramethylbenzidine substrate. Absorbance was read at 650 nm, and plasma concentrations of soluble CD40 ligand were determined by comparison with serial dilutions of recombinant human CD40 ligand. Intra-assay variation among the triplicates for all samples was <10%, and interassay variability was 7.4%. Plasma levels of sICAM-1 were determined by ELISA (R&D Systems).

Statistical Analysis

We divided the study participants into 2 groups, those with evidence of intraplaque lipid on carotid MRI and those without evidence of intraplaque lipid. Median levels of soluble CD40 ligand were computed and compared between the 2 groups with Wilcoxon’s ranked sum test. Relative risks of having intraplaque lipid associated with elevated soluble CD40 ligand levels were computed by use of logistic regression models that divided the study sample according to the median level of soluble CD40 ligand among those without evidence of intraplaque lipid.

Results

Of the 49 patients enrolled, carotid MR images could not be obtained for 3 patients because of claustrophobia. We report the results for the remaining 46 patients. Table 1 shows the baseline clinical characteristics of the patients. There was a high prevalence of a history of hypertension, diabetes, and hypercholesterolemia in the overall study cohort. Thirteen of the 46 patients (28.3%) had a prior history of transient ischemic attack, (n = 8) or stroke (n = 6); 1 patient had suffered both a transient ischemic attack and a stroke. The remaining 33 patients (71.7%) were asymptomatic.

Figure 1 demonstrates the typical appearance of intraplaque lipid on proton density–weighted and fat-suppressed T2-weighted images. Fourteen patients had evidence of intraplaque lipid and 32 did not. Patients with evidence of intraplaque lipid more likely had diabetes (P = 0.02) than those patients without evidence of intraplaque lipid. There was also a trend toward an increased proportion of women (P = 0.1), patients with a history of hypertension (P = 0.16), and current smokers (P = 0.13) in the group with intraplaque lipid. Mean percent carotid diameter stenosis (58 ± 20% vs 56 ± 24%) did not differ between those with and without evidence of intraplaque lipid.

Subjects with intraplaque lipid had higher baseline levels of soluble CD40 ligand than those without lipid accumulations (median, 2.54 ng/mL; interquartile range [IQR], 1.85 to 3.52 vs median, 1.58 ng/mL; IQR, 1.21 to 2.39; P = 0.02). In contrast, soluble CD40 ligand levels were not correlated with the percent diameter stenosis (r = −0.19; P = 0.21). Plasma levels of sICAM-1 did not differ between those with intraplaque lipid and those without (Table 1).

Plasma CD40 levels tended to be higher among diabetic patients (n = 13) compared with nondiabetics (n = 33; 2.21 ng/mL; IQR, 1.66 to 2.77 vs 1.57 ng/mL; IQR, 1.16 to 2.48; P = 0.14) and among women (n = 15) compared with men (n = 31) (2.31 ng/mL; IQR, 1.16 to 3.84 vs 1.72 ng/mL; IQR, 1.37 to 2.48; P = 0.40), although these differences did not reach statistical significance. The relative risk for intraplaque lipid associated with soluble CD40 ligand levels above the median was 6.0 (95% confidence interval, 1.15 to 31.23; P = 0.03). The magnitude of this predictive effect did not substantially change when analyzed by a multivariable model that controlled for the effects of sex, diabetes, hypertension, current smoking, percent stenosis, and ratio of total choles-
terol to HDL cholesterol (relative risk, 5.12; 95% confidence interval, 0.78 to 33.73; \(P = 0.09\)).

### Discussion

Previous work has demonstrated that baseline plasma levels of soluble CD40 ligand prospectively predict cardiovascular events among apparently healthy women.\(^{10}\) The current data provide novel insight into the mechanism through which elevated levels of soluble CD40 ligand may reflect future cardiovascular risk in humans. We found an association between elevated plasma levels of soluble CD40 ligand and carotid plaques with features of high risk without relation to the severity of stenosis. These data agree with evidence from studies showing that interruption of CD40 signaling reduced the size and lipid content of aortic lesions in atherosclerosis-prone mice.\(^{9}\) In contrast, plasma levels of sICAM-1, a marker of endothelial inflammation, showed no relationship with the presence of carotid intraplaque lipid.

Our study has limitations. The cohort comprises a group of elderly patients with carotid atherosclerosis and a high prevalence of traditional risk factors. Thus, these data may not apply generally to other populations. We do not have histological confirmation of an intraplaque lipid pool. Nonetheless, previous work has found that high-resolution carotid MRI, using a similar phased-array carotid coil, can accurately predict histological findings of a lipid pool after carotid endarterectomy\(^{15}\); our MRI protocol relied more on T2-weighted protocols, which other studies have shown can accurately distinguish the lipid pool.\(^{12,13,16}\) It is possible that the potential confounding effect of diabetes or female sex, both of which tended to be associated with higher CD40 ligand levels in our study, or other unknown confounders may have affected the results of this cross-sectional study. However, we believe that this is unlikely, given that the magnitude of the predictive effect of soluble CD40 ligand persisted after adjustment for traditional cardiovascular risk factors, although the confidence intervals did widen in the adjusted analysis, as might be expected given that CD40 ligation appears to represent a common causal pathway in lipid pool formation. Finally, although imaging parameters were kept constant during this study, intrapatient variability in MR images could not be assessed with our study design.

In conclusion, we believe that this study establishes a link between plasma levels of CD40 ligand and intraplaque lipid, which represents 1 potentially important marker of plaque vulnerability. Further advances in plaque imaging, including reproducible measurement of fibrous cap thickness, may further enhance our understanding of the relationship between inflammation and plaque vulnerability.

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**Baseline Clinical Characteristics of the Study Population**

| Age, y (mean±SD) | 70.5±8.1 | 71.0±7.9 | 69.4±8.8 | 0.5 |
| Male gender, n (%) | 31/46 (67.4) | 24/32 (75) | 7/14 (50) | 0.10 |
| History of diabetes, n (%) | 15/46 (32.6) | 7/32 (21.9) | 8/14 (57.1) | 0.02 |
| History of hypertension, n (%) | 37/46 (80.4) | 24/32 (75) | 13/14 (92.9) | 0.16 |
| Current smoker, n (%) | 5/46 (10.9) | 2/32 (6.3) | 3/14 (21.4) | 0.13 |
| History of high cholesterol, n (%) | 34/46 (73.9) | 23/32 (71.9) | 11/14 (78.6) | 0.6 |
| Previous TIA or stroke, n (%) | 13/46 (28.3) | 8/32 (25) | 5/14 (35.7) | 0.46 |
| Statin use, n (%) | 31/46 (67.4) | 20/32 (62.5) | 11/14 (78.6) | 0.28 |
| Percent diameter stenosis | 57±23 | 56±24 | 58±20 | 0.6 |
| Plaque thickness, mm | 3.6±1.5 | 3.5±1.6 | 3.9±1.3 | 0.4 |
| Total cholesterol/HDL cholesterol ratio | 4.97±1.98 | 4.72±1.56 | 5.54±2.66 | 0.20 |
| sICAM-1, ng/mL (median [interquartile range]) | 273.4 [239.4–325.5] | 282.8 [238.8–342.1] | 267.9 [240.4–285.7] | 0.5 |
| Soluble CD40 ligand, ng/mL (median [interquartile range]) | 1.89 [1.35–2.64] | 1.58 [1.21–2.39] | 2.54 [1.85–3.52] | 0.02 |

TIA indicates transient ischemic attack.

Proton density–weighted (A) and fat-suppressed, T2-weighted (B) images showing a large lipid pool (arrow) in an internal carotid artery plaque.
studies of inflammation with high-resolution MRI may provide novel insights into the pathogenesis of atherosclerosis.

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