Elastin and Calcium Rather Than Collagen or Lipid Content Are Associated With Echogenicity of Human Carotid Plaques

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Background and Purpose—Echolucent carotid plaques have been associated with increased risk for stroke. Histological studies suggested that echolucent plaques are hemorrhage- and lipid-rich, whereas echogenic plaques are characterized by fibrosis and calcification. This is the first study to relate echogenicity to plaque composition analyzed biochemically.

Methods—Echogenicity of human carotid plaques was analyzed by standardized high-definition ultrasound and classified into echolucent, with gray-scale median (GSM) <32 and echogenic with GSM ≥32. The biochemical composition of the plaques was assessed by fast-performance liquid chromatography and high-performance thin-layer chromatography.

Results—As assessed biochemically (milligrams per gram [mg/g]), echolucent plaques contained less hydroxyapatite (43.8 [SD 41.2] mg/g versus 121.6 [SD 106.2] mg/g; P=0.018), more total elastin (1.7 [SD 0.4] mg/g versus 1.2 [SD 0.4] mg/g; P=0.008), and more intermediate-size elastin forms (1.2 [SD 0.3] mg/g versus 0.8 [SD 0.4] mg/g; P=0.018). There was no difference in collagen amount between echogenic and echolucent plaques, neither biochemically (15.3 [SD 3.7] mg/g versus 14.4 [SD 3.4] mg/g) nor histologically (13.4 [SD 4.9] % versus 13.0 [SD 5.6] %). Cholesterol esters, unesterified cholesterol, and triglycerides were increased in plaques associated with symptoms (22.5 [SD 23.3] mg/g versus 13.3 [SD 3.2]; P=0.04), but no differences were detected between echolucent and echogenic plaques (13.5 [SD 4.0] versus 20.2 [SD 21.5] mg/g). Similar results were obtained by Oil Red O staining (symptomatic 7.6 [SD 4.7] % versus asymptomatic 4.2 [SD 3.6] %; P=0.03; echolucent 5.9 [SD 4.1] % versus echogenic 5.0 [SD 4.0] % of area).

Conclusions—Echogenicity of carotid plaques is mainly determined by their elastin and calcium but not collagen or lipid content. In addition, echolucency is associated to higher elastin content. (Stroke. 2004;35:2795-2800.)

Key Words: atherosclerosis ■ calcium ■ carotid artery plaque ■ carotid stenosis ■ elastin ■ ultrasonography

Carotid artery stenosis is an important risk factor for stroke. The degree of stenosis can be measured by high-definition ultrasonography and is a criterion for surgery for symptomatic and asymptomatic patients. Echolucent plaques are associated with increased risk for ischemic cerebrovascular events independent of or together with the degree of stenosis and cardiovascular risk factors. Echogenicity of atherosclerotic plaques can be assessed by B-mode ultrasound and digital image analysis, calculating the gray-scale median (GSM). Calcium deposits attenuate transmitted ultrasound. Studies comparing plaque histology with ultrasonography have suggested that echolucent plaques have more lipid and hemorrhage and echogenic plaques more fibrous tissue. Analysis of plaque constituents can be achieved more accurately by biochemical assays of plaque homogenates. In this study, extracellular matrix and lipid composition of human carotid plaques were related to their echogenicity.

Methods

Patients

Twenty-eight patients (6 females, 22 males) aged 67.8 (SD 8.5) years underwent carotid endarterectomy (CEA) en bloc by the same surgeon. Informed consent was given by each patient. The study was approved by the local ethical committee. Three patients underwent bilateral CEA: in 1 patient, both plaques were symptomatic; in another, none were symptomatic. In the third, 1 plaque was associated with symptoms. Accordingly, a total of 31 plaques were used in this study, 14 associated with symptoms, and 17 not associated with symptoms. Surgical indications were: (1) plaques associated with ipsilateral symptoms (6 with amaurosis fugax/transient ischemic attack and 8 with stroke in the last 6 months) and stenosis >70%; (2) plaques not associated with symptoms (n=17) causing a stenosis >80%. Patients with atrial fibrillation, aortic valve disease, mechanical heart valves, ipsilateral carotid artery occlusion, or restenosis after previous CEA were excluded from this study. Cardiovascular risk factors, such as hypertension (systolic blood pressure >140mm Hg), diabetes, coronary artery disease, smoking

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(in the past or currently), and fasting lipoproteins (total cholesterol, high-density lipoprotein [HDL] cholesterol, low-density lipoprotein [LDL] cholesterol, and triglycerides) were recorded, as well as the use of statins.

**Ultrasonography**

Carotid high-definition ultrasonography (ATL-HDI 3000; 7–10 MHz probe; 60-dB dynamic range and postprocessing linear maps) of 31 plaques was blindly performed preoperatively by 1 observer. Ultrasonographic data of 4 plaques were accidentally lost. Plaques with any size of acoustic shadow were excluded because that could jeopardize the accurate visualization of the whole plaque area. Color-flow Duplex scan was used to assess severity of stenosis using European Carotid Surgery Trial (ECST) criteria, cross-sectional area reduction, and hemodynamic assessment using an angle of 60°.13 A longitudinal image of each plaque representative of its structure was obtained. Plaques in the near or far wall of the vessel were outlined, as can be seen in the examples in Figure 1. Color imaging was used in parallel to the B-mode images to help in the precise delimitation of the plaque region. The digital images were computer standardized (Adobe Photoshop 3.0) according to previously described and validated methodology, and GSM was determined.8,14-17 Plaques were divided in 2 groups: echolucent, with GSM/H11021 <32 (n=10; Figure 1A through 1C); and echogenic, with GSM ≥32 (n=17; Figure 1D and 1E) because this previously provided optimal discrimination between symptomatic and asymptomatic plaques.8,14,18

**Sample Preparation**

Plaques were immediately snap-frozen in liquid nitrogen after surgical removal. Two-millimeter-thick fragments from the stenotic region of frozen plaques were taken for histology. Plaques were weighed and homogenized as described previously.19

**Analysis of Extracellular Matrix**

Elastin, hydroxyapatite, and sulfated glycosaminoglycans (GAG) were determined as described previously.19 Collagen was assessed by measuring hydroxyproline, assuming that 12.5% of collagen is hydroxyproline.20

**Lipid Analysis**

Plaque lipids were extracted with hexane:isopropanol (3:2, vol:vol) and applied (Linomat IV system; Camag) on high-performance thin-layer chromatography (TLC) Silica gel 60 plates (Merck). Lipids were separated with a 2-phase solvent system21 in an automatic developing chamber (Camag). Lipids were detected with CuSO4.22 Band densities were measured with a TLC scanner (Camag) and the amounts of respective lipids quantified by comparison with standards.

**Histology**

Sections from the 2-mm-thick fragment were fixed with Histochoice (Amresco), dipped in 60% isopropanol, and then in 0.4% Oil Red O in 60% isopropanol (for 20 minutes) to stain lipids. Masson’s trichrome using Ponceau-acid fuchsin (Chroma-Gesellschaft; Clinical Characteristics of the Patients (N=28))

<table>
<thead>
<tr>
<th>Clinical Characteristics</th>
<th>Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>67.8 (SD 8.5)</td>
</tr>
<tr>
<td>Sex</td>
<td>6 females, 22 males</td>
</tr>
<tr>
<td>Degree of stenosis (%)</td>
<td>84 (SD 9)</td>
</tr>
<tr>
<td>Hypertension (%)</td>
<td>19 (68)</td>
</tr>
<tr>
<td>Diabetes (%)</td>
<td>7 (25)</td>
</tr>
<tr>
<td>Coronary artery disease (%)</td>
<td>17 (61)</td>
</tr>
<tr>
<td>Smoking (in the past or currently) (%)</td>
<td>14 (50)</td>
</tr>
<tr>
<td>Fasting lipoproteins (mg/dL):</td>
<td></td>
</tr>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>226 (SD 45)</td>
</tr>
<tr>
<td>HDL cholesterol (mg/dL)</td>
<td>49 (SD 14)</td>
</tr>
<tr>
<td>LDL cholesterol (mg/dL)</td>
<td>147 (SD 34)</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>137 (SD 81)</td>
</tr>
<tr>
<td>Statin use (%)</td>
<td>6 (21)</td>
</tr>
</tbody>
</table>

Figure 1. High-definition ultrasonography image of human carotid plaques. A, Color-flow ultrasound image of an echolucent plaque. B, The contour of the echolucent plaque in the anterior and in the posterior wall seen in A is marked with the white dashed line. C, Histogram of the gray-scale of pixels corresponding to the same plaque delineated in B. After computer standardization (Adobe Photoshop 3.0), GSM is automatically determined (arrow); in this case, GSM=12. D, Echogenic plaque, marked with the white dashed line. E, Histogram of the gray scale of pixels corresponding to the plaque delineated in D. GSM is automatically calculated (arrow); in this case, GSM=64. CC, indicates common carotid artery; IC, internal carotid artery, EC, external carotid artery.
Schimd), and aniline blue (BDH) was used to assess plaque collagen content. Stained area of plaque (percent of area) was quantified blindly using an Olympus BX60 microscope and computer-aided morphometry (Image Plus).

Statistics
Results were normalized to plaque wet weights. Values are presented as mean (SD), and when using Student $t$ test, mean difference and its SE are shown. $\chi^2$ test was used to investigate associations with dichotomous variables. Two-group comparisons were performed with unpaired Student $t$ or Mann–Whitney tests according to the distribution of the variables (normal or not). Spearman’s $\rho$ was used for correlation analysis. Linear regression analysis was performed using GSM as the dependent variable and the biochemical components as independent ones. Differences were considered statistically significant at $P<0.05$.

Results
The clinical characteristics of the patients included in this study are presented in the table. Seven symptomatic patients had plaques with GSM $<32$, and 3 asymptomatic patients had plaques with GSM $\geq 32$ ($P=0.04$ using $\chi^2$ test). Symptomatic and asymptomatic plaques had a mean GSM of 35.8 (SD 22.5) and 48.9 (SD 24.9), respectively. There were no statistically significant differences between symptomatic and asymptomatic patients for hypertension, diabetes, coronary artery disease, or tobacco use in the past or currently. There were also no differences between symptomatic and asymptomatic patients in total cholesterol (227 [SD 35] versus 219 [SD 53] mg/dL; $-7.4$ [SE 19.3]), HDL cholesterol (43 [SD 11] versus 52 [SD 15] mg/dL; 8.7 [SE 5.7]), or LDL

Figure 2. Extracellular matrix components of human carotid plaques. Comparison of different constituents in echolucent (GSM $<32$) or echogenic plaques (GSM $\geq 32$). A, Hydroxyapatite. B, GAG. C, Total elastin. D, Intermediate forms of elastin. E, Collagen amount assessed biochemically and histologically (F). Box plots show the median 25th to 75th percentiles, minimum-maximum observed values.
Elastin content was higher in echolucent plaques (1.7 [SD 0.4] versus 1.2 [SD 0.4] mg/g; P = 0.008; Figure 2C). This was also true for intermediate-size elastin forms (1.2 [SD 0.3] versus 0.8 [SD 0.4] mg/g; −0.37 [SE 0.14]; P = 0.018; Figure 2D), which may represent dysfunctional elastin. In univariate analysis, using GSM as a continuous variable, GSM correlated negatively with total elastin content (r = −0.41; P = 0.03), as well as with intermediate elastin forms (r = −0.53; P = 0.007). Using linear regression, significant independent associations were found between GSM and total elastin (P = 0.03; Figure 3A), explaining 14% of the GSM variation, whereas for intermediate elastin, this model explained 25% of the variation (P = 0.007; Figure 3B).

Collagen content was not significantly different between echolucent and echogenic plaques (15.3 [SD 3.7] versus 14.4 [SD 3.4] mg/g; −0.97 [SE 1.41]; Figure 2E). Similar findings were made histologically using image analysis of Masson trichrome–stained plaque sections (13.4 [SD 4.9] versus 13.0 [SD 5.6] percent of area; Figure 2F). These stainings correlated with plaque collagen content as assessed biochemically (r = 0.43; P = 0.02).

No other components besides elastin showed significant Spearman correlation to GSM. Similarly, no statistically significant results were found for the other components when linear regression models with GSM as the dependent variable were evaluated.

### Lipids

Lipid analysis showed no difference in phospholipids between plaques associated with symptoms and asymptomatic plaques (121.0 [SD 26.3] versus 103.9 [SD 30.3] mg/g; −17.0 [SE 10.3]; Figure 4A). However, cholesterol esters were higher in symptomatic plaques than in asymptomatic ones (11.0 [SD 10.3] versus 6.4 [SD 1.9] mg/g; P = 0.02). Total neutral lipids (22.5 [SD 23.3] versus 13.3 [SD 3.2] mg/g; P = 0.04; Figure 4B) and Oil Red O–stained area (7.6 [SD 4.7] versus 4.2 [SD 3.6] percent of area; −3.4 [SE 1.5]; P = 0.03; Figure 4C) were also increased in symptomatic plaques, whereas no differences were found for unesterified cholesterol (8.0 [SD 8.6] versus 5.3 [SD 2.0] mg/g) and triglycerides (3.4 [SD 4.9] versus 1.5 [SD 1.0] mg/g; −1.9 [SE 1.2]).

Comparison of echolucent (GSM <32) and echogenic (GSM ≥32) plaques demonstrated no differences in phospholipid content (105.1 [SD 23.3] versus 114.6 [SD 32.2] mg/g; 9.5 [SE 11.7]; Figure 4D), neutral lipids (13.5 [SD 4.0] versus 20.2 [SD 21.5] mg/g; Figure 4E) or lipid-stained area (5.9 [SD 4.1] versus 5.0 [SD 4.0] percent of area; −0.9 [SE 1.6]; Figure 4F). In respect to the different subgroups of neutral lipids, unesterified cholesterol content was lower in echolucent than in echogenic plaques (4.2 [SD 2.4] versus 8.0 [SD 7.6] mg/g; P = 0.04), whereas no significant differences were found for cholesterol esters (7.4 [SD 2.7] versus 9.5 [SD 9.6] mg/g) or triglycerides (1.9 [SD 1.2] versus 2.8 [SD 4.6] mg/g; 0.9 [SD 1.5]). No statistically significant correlation was found between GSM and any of the plaque lipid fractions analyzed. No significant results were obtained in the linear regression for any of the plaque lipids (independent variables) and GSM (dependent variable).

### Discussion

The present study shows that echogenic (GSM ≥32) plaques contain more hydroxyapatite and less elastin. In contrast, echogenic and echolucent plaques contained similar amounts of collagen, suggesting that calcification rather than collagen content is the major determinant of plaque echogenicity. This observation contradicts previous reports of an association...
between echogenicity and fibrous tissue based only on histology. The lower amount of hydroxyapatite in echolucent plaques as well as the higher amounts of elastin and intermediate elastin forms are in agreement with the pattern observed earlier for symptomatic plaques. Coronary plaques associated with unstable angina have higher intimal elastin content than those in stable angina. Furthermore, elastin peptides are involved in several processes implicated in atherogenesis and increased elastin turnover contributes to increased oxidative stress in lesions. Elastin is also involved in lipid entrapment in vascular wall. The inverse correlation and the results from the regression models between GSM and elastin and its intermediary forms suggest that, apart from detecting calcification, echography can provide further insight into plaque composition. The fact that no statistically significant associations were found between hydroxyapatite and GSM using linear regression analysis is likely attributable to the relatively small number of samples in this study. In accordance with the present observations, previous histological studies have demonstrated associations between tissue contents of calcium and GSM values. This study confirms these results by biochemical analyses.

The higher levels of neutral lipids as well as the larger Oil Red O–stained areas found in symptomatic plaques agree with the notion that vulnerable plaques have large lipid pools. In contrast to previous studies, where more lipid was found histologically in echolucent plaques, we found no differences between echoluent and echogenic plaques, neither biochemically nor histologically. In our data, unesterified cholesterol content was actually lower in echolucent plaques than in the echogenic.

Histological analysis has several advantages, allowing detailed assessment of the spatial distribution of plaque constituents, as well as the identification of specific cell types and proteins by immunohistochemistry. However, biochemical analysis is advantageous in the respect that it allows accurate quantification of individual plaque constituents. The risk factor profile of the patients included in this study shows no apparent difference from what generally is observed in patients with high-grade carotid stenosis. However, the small sample number can limit the representativity of these findings. On the other hand, the specificity of the biochemical analysis allows the identification and quantification of the differences between the groups.

In conclusion, echogenic plaques are characterized by a high degree of calcification, whereas echoluent plaques contain increased intermediate elastin forms. This is the first study in which elastin content is related to echogenicity of human carotid plaques. Differences in collagen or lipid content did not explain differences in echogenicity. The increased understanding of the relationship between echostucture and plaque composition may ultimately improve identification and stratification of patients with high-risk plaques.

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