Changes Related to Age and Cerebrovascular Symptoms in the Extracellular Matrix of Human Carotid Plaques

Isabel Gonçalves, MD; Jonatan Moses, PhD; Nuno Dias, MD; Luís M. Pedro, MD; José Fernandes e Fernandes, MD, PhD; Jan Nilsson, MD, PhD; Mikko P.S. Ares, PhD

Background and Purpose—Many processes involved in the pathogenesis of atherosclerosis result in modifications of the extracellular matrix. These changes not only determine the mechanical stability of atherosclerotic lesions but can directly or indirectly influence further development of the lesions. The purpose of the present study was to compare the matrix composition of human carotid plaques from symptomatic patients with those obtained from patients without symptoms. Furthermore, matrix changes related to age were studied.

Methods—Thirty atherosclerotic carotid plaques were removed by endarterectomy from 27 patients and divided into 2 groups on the basis of the presence of ipsilateral symptoms. The plaques were homogenized, and the total levels of the major components of the extracellular matrix were determined.

Results—Plaques associated with symptoms were characterized by increased levels of elastin (1.58±0.46 versus 1.24±0.40 mg/g wet wt; P=0.03) and decreased levels of hydroxyapatite (45.1±46.3 versus 131.4±111.7 mg/g wet wt; P=0.02) compared with asymptomatic plaques. The increase in elastin in plaques from symptomatic patients was due to elevated levels of an intermediate-size fraction, as determined by liquid chromatography. Collagen and sulfated glycosaminoglycans were present in equal amounts in both groups. Elastin content in carotid plaques decreased with age.

Conclusions—Carotid plaques from symptomatic patients have lower levels of hydroxyapatite than those from asymptomatic patients. The present study also raises the possibility that non–cross-linked forms of elastin, increased in plaques associated with symptoms, could be a marker of plaque vulnerability and/or directly induce harmful cellular activities or increase lipoprotein retention in the vascular wall. (Stroke. 2003;34:616-622.)

Key Words: aging ■ atherosclerosis ■ carotid stenosis ■ elastin

Carotid atherosclerotic plaques can be related to ipsilateral symptoms of cerebral ischemia by reduction of blood flow due to high-grade stenosis or by distal embolization. An important determinant of plaque vulnerability is the mechanical strength and stability of the fibrous cap. Ruptured caps are usually thinner and contain less collagen, fewer smooth muscle cells, and more macrophages. These characteristics, as well as the presence of a large lipid core close to the lumen, have been associated with cerebrovascular symptoms. However, the relation of main components of the extracellular matrix of human carotid plaques to sympotms is not clear.

Collagens, the major proteins of the extracellular matrix, form fibrils and networks that resist tensile stress. Elastin is a major component of the extracellular matrix of large blood vessels, which need to cope with large variations of blood pressure. Most of the elastin biosynthetic activity in aortic lesions of hyperlipidemic rabbits is detected in intimal regions, and 25% to 60% of the dry weight of intima and media in mechanically induced rabbit lesions consists of elastin. Elastin is synthesized as a monomer termed tropoelastin. The elastic properties of cross-linked elastin are due to a variety of random coil conformations of monomers, which allow repeated stretching and recoiling. Elastic fibers in tissues such as the aorta consist of elastin and microfibrils. The fibrous proteins of the extracellular matrix are embedded in proteoglycans, which resist compressive forces.

Morphological studies suggest that coronary plaques associated with unstable angina have increased intimal elastin content compared with stable plaques. Atheromatous tissues also exhibit elevated elastase activity. Elastin-lipid interactions in the arterial wall can contribute to lipid deposition in atherosclerosis. Elastin from plaque intima contained more cholesterol and less triglycerides and phospholipid than elastin from intima without plaque. Elastin also binds LDL, IDL, and VLDL, most probably contributing to their retention in the vascular wall.
Although calcification impairs the mechanical function of the vascular wall, lesions responsible for unstable angina or myocardial infarction are less calcified, suggesting that calcium could stabilize plaques, at least if the most vulnerable regions of the plaque are encased by fibrocalcified tissue.

In the present study we quantified the main components of the extracellular matrix in human carotid plaques and related them to age and presence of ipsilateral symptoms. Plaques associated with symptoms had increased levels of intermediate (non-cross-linked) forms of elastin. This may be functionally important because degradation products of elastin have been shown to stimulate cells in several different ways. In contrast, aging was associated with decreased plaque content of elastin.

Subjects and Methods

Patients

Thirty atherosclerotic plaques obtained by carotid endarterectomy (CEA) for 8 months were snap-frozen for subsequent matrix analysis. The same and only surgeon performed CEA as the standard technique. After the longitudinal arteriotomy, plaques were dissected through a subadventitial plan and removed en bloc without fragmentation. Plaques were removed from 27 patients (21 men and 6 women) aged 67±8.5 (mean±SD) years. Fourteen plaques were removed from patients referred for CEA with internal carotid artery stenosis >70% (European Carotid Surgery Trial [ECST]) and ipsilateral symptoms (6 with amaurosis fugax/transient ischemic attack and 8 with stroke in the last 6 months; mean time from event, 3 months). Sixteen plaques were removed from asymptomatic patients who had an internal carotid artery stenosis >80% (ECST). Three patients underwent bilateral CEA. In one, both plaques were associated with symptoms; in another, both plaques were associated with no symptoms. In the last patient, one plaque was associated with symptoms, while the other one was not. Patients with atrial fibrillation, aortic valve disease, mechanical heart valves, ipsilateral carotid artery occlusion, or restenosis after previous CEA were excluded. All the patients underwent a complete neurological and cardiological evaluation. The use of lipid-lowering drugs (statins) and blood pressure–lowering drugs (diuretics and angiotensin-converting enzyme inhibitors) was noted for each patient. Patients were not receiving other types of medication considered to potentially influence atherosclerosis. The severity of carotid stenosis was assessed by duplex Doppler imaging by the same observer, using internationally established criteria (ECST and measurement of cross-sectional area reduction). Cardiovascular risk factors such as hypertension (systolic blood pressure >140 mm Hg), diabetes, coronary artery disease, and tobacco use (in the past or current) were recorded. Routine laboratory analyses to investigate associations with dichotomous variables.

Sample Preparation

Plaques were removed by endarterectomy and immediately snap-frozen in liquid nitrogen. Two-millimeter-thick fragments from the stenotic region of the frozen plaques were removed for histology. Plaques were weighed, cut into pieces while still frozen, and homogenized with a motorized blender (1600 rpm) attached to a loose-fitting Teflon pestle. Each plaque was homogenized in 5 mL of a homogenization buffer consisting of 50 mmol/L Tris-HCl (pH 7.5), 0.25 mol/L sucrose, 2 mmol/L tris[2-carboxyethyl]phosphine HCl, 50 mmol/L NaF, 1 mmol/L Na-orthovanadate, 10 mmol/L Na-glycerophosphate, 5 mmol/L Na-pyrophosphate, protease inhibitor cocktail (Roche Complete, EDTA-free), 1 mmol/L benzamidine, and 10 mmol/L phenylmethylsulfonyl fluoride.

Analysis of Elastin and Collagen

An aliquot of plaque homogenate was centrifuged at 200g for 2 minutes to remove heavy calcium/apatite salts. Guanidine hydrochloride (4 mol/L) was added to aliquots of plaque, and thereafter the total of elastin or collagen was dye-precipitated and quantified according to the manufacturer’s instructions for the Sircol collagen assay (Biocolor) or the Fastin elastin assay (Biocolor). Alternatively, dye-precipitated samples were subjected to liquid chromatography for the analysis of elastin or collagen fractions of different molecular sizes. The samples were analyzed by gel filtration with the use of an HR17 column coupled to a fast performance liquid chromatography (FPLC) triple-wavelength system (BioRad). Samples were identified by online spectrophotometry for peptide bonds and tryptophan residues with a linear flow of 4 mol/L guanidine hydrochloride (pH 5.8) and 0.1% Triton X-100. Dye pellets were washed with the buffer supplied by the manufacturer, and the specificity of the dye precipitation was controlled by parallel immunoprecipitation. The elution position identities were determined by the use of standard forms of collagen (Sigma) and elastin (Sigma) among other pure matrix components (versican, decorin, and biglycan, antibody purified) used as position equilibrators. Dyel separation from the sample was determined with the use of wavelengths specific for each dye. Column stability was constantly monitored online. Prefilters as well as postfilters were analyzed for impurity after each column wash to verify that no material had avoided separation in the included volume of the column. Spectrophotometric were set to zero against buffer between runs. The medium recovery rate, including test samples, was >90% (154 column runs measured in total). The exclusion volume of the column was determined by blue dextran (Amersham Biosciences), and total column volume was determined by radioactive sulfate.

Glycosaminoglycan Measurement

To release serine glycosaminoglycans, proteoglycans were proteolytically cleaved by adding 1 µg Pronase supplemented with Ca²⁺ (Sigma) to 10 µL of plaque homogenate, followed by incubation at 65°C for 90 minutes. Afterward Blyscan proteoglycan and glycosaminoglycan assay (Biocolor) was used for the measurement of total sulfated glycosaminoglycans. Separation into galactosaminoglycans and glucosaminoglycans is not included in this study, nor is assessment of hyaluronic acid, which is not detected by the assay because of lack of sulfate groups.

Hydroxyapatite Measurement

In atherosclerotic lesions, the predominant type of calcium salt is hydroxyapatite. The plaque homogenate (1 mL) was centrifuged at 200g for 2 minutes, and the pellet was dried at 55°C. After the pellet was weighed, 0.5 mol/L EDTA (pH 7.5) was added, and the mixture was incubated at 37°C for 24 hours. The samples were centrifuged again, at 10 000g, for 5 minutes. After the supernatant was removed, the samples were dried at 95°C and weighed again. The difference of the 2 weights measured corresponded to the amount of hydroxyapatite in the sample.

Histological Procedure

Two-millimeter-thick fragments from the stenotic regions of the frozen plaques were embedded in O.C.T. compound (Tissue-Tek, Sakura), cryo-sectioned in serial 8-µm sections, and mounted on coated slides. The sections were fixed with 4% formaldehyde in phosphate buffer (0.1 mol/L, pH 7) and stained with elastin stain (Sigma Diagnostics Accustain). Plaques were observed blindly to assess location and pattern of distribution of elastin with the use of an Olympus BX60 microscope and the program Image Plus. Images were taken with a digital camera (Sony, DRC-5000).

Statistical Analysis

Results were normalized to the wet weight of the plaques. Values are presented as mean±SD. We used χ² analyses or Fisher’s exact test analyses to investigate associations with dichotomous variables. Two-group comparisons were performed with the use of the unpaired
Student’s *t* test. Fisher’s *r* to *z* test was used for the correlation analyses. Values of *P*≤0.05 were considered to indicate statistically significant findings. Statistical analysis was performed with the use of StatView for Windows, version 5.0.1 (SAS Institute Inc).

**Results**

Most studies focusing on the composition of atherosclerotic plaques have been based on histology. Analysis of total levels of plaque components is, however, more appropriately done by biochemical assays of whole plaque homogenates. In the present study carotid plaques were removed by endarterectomy and immediately snap-frozen in liquid nitrogen. A 2-mm fragment of the stenotic area was removed for histology, and the rest was weighed (mean±SD wet weight, 0.922±0.245 g), cut into pieces while still frozen, and homogenized at low temperature. Components of the extracellular matrix were subsequently analyzed in the homogenates.

Cardiovascular risk factors were assessed in the patients. 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 for total cholesterol (226.5±35.3 versus 219.1±53.1 mg/dL), HDL (42.9±10.7 versus 51.6±15.4 mg/dL), or LDL (150.2±33.1 in versus 144.4±35.8 mg/dL), respectively. Only plasma triglycerides were higher in symptomatic patients than in asymptomatic patients (171.2±101.2 versus 102.9±46.3 mg/dL; *P* =0.03).

Seventeen patients were treated with blood pressure-lowering drugs, and 6 were taking lipid-lowering drugs. Of the 14 plaques associated with symptoms, only 2 were from patients receiving lipid-lowering drugs. Four asymptomatic patients were taking lipid-lowering drugs. Among the plaques associated with symptoms, 7 were from patients treated with blood pressure-lowering drugs. Eleven of the patients from whom asymptomatic plaques were obtained were taking blood pressure-lowering drugs. No statistically significant differences were found between the groups (*P* >0.05, χ² or Fisher exact test).

Carotid plaques associated with ipsilateral hemispheric symptoms had 30% higher amounts of elastin (Figure 1A) and 70% lower amounts of hydroxyapatite (Figure 1B) compared with plaques not associated with symptoms. In contrast, there was no statistically significant difference in total sulfated glycosaminoglycan content between these 2 groups of patients (Table).

Elastin content was also analyzed by FPLC, which enables separation of degraded, intermediate, and cross-linked forms of elastin (Figure 2; Table). Plaques associated with symptomatic patients contained more elastin than plaques from asymptomatic patients (Figure 2C). Only plasma triglycerides were higher in symptomatic patients than in asymptomatic patients (171.2±101.2 versus 102.9±46.3 mg/dL; *P* =0.03).

### Collagen, Elastin, Glycosaminoglycan, and Hydroxyapatite (mg/g wet weight, mean±SD) in Carotid Plaques From Symptomatic and Asymptomatic Patients

<table>
<thead>
<tr>
<th></th>
<th>Symptomatic</th>
<th>Asymptomatic</th>
<th><em>P</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen (total)</td>
<td>6.26±2.73</td>
<td>6.50±2.05</td>
<td>NS</td>
</tr>
<tr>
<td>Collagen fractions</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crossed-linked</td>
<td>4.35±2.34</td>
<td>4.52±1.74</td>
<td>NS</td>
</tr>
<tr>
<td>Intermediate</td>
<td>1.04±0.69</td>
<td>1.19±0.63</td>
<td>NS</td>
</tr>
<tr>
<td>Degraded</td>
<td>0.87±0.52</td>
<td>0.79±0.30</td>
<td>NS</td>
</tr>
<tr>
<td>Elastin (total)</td>
<td>1.58±0.46</td>
<td>1.24±0.40</td>
<td>0.03</td>
</tr>
<tr>
<td>Elastin fractions</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crossed-linked</td>
<td>0.29±0.20</td>
<td>0.24±0.20</td>
<td>NS</td>
</tr>
<tr>
<td>Intermediate</td>
<td>1.16±0.38</td>
<td>0.86±0.42</td>
<td>0.04</td>
</tr>
<tr>
<td>Degraded</td>
<td>0.13±0.06</td>
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<tr>
<td>Glycosaminoglycan</td>
<td>0.63±0.33</td>
<td>0.63±0.27</td>
<td>NS</td>
</tr>
<tr>
<td>Hydroxyapatite</td>
<td>45.1±46.3</td>
<td>131.4±111.7</td>
<td>0.02</td>
</tr>
</tbody>
</table>

NS indicates not significant.
Toms were characterized by increased intermediate elastin, which was also the dominant form (72% of the total on average) in all plaques. The contents of cross-linked and degraded elastin were similar in the 2 patient groups.

Histologically, elastin was found to be present in the plaques mostly in the core (Figure 3A), in the transition between the core and the fibrous cap (Figure 3A and 3B), and in the shoulders (Figure 3A, 3B, 3C, and 3D). Additionally, in plaques associated with symptoms, elastin was more abundant in the subendothelial space (Figure 3E) and in the vicinity of rupture sites (Figure 3F). Although all the shoulder regions observed stained for elastin, strong staining was more frequently observed in those plaques associated with symptoms (6 of 9 plaques) than in the asymptomatic ones (1 of 9). Calcific deposits were scattered in most regions of the plaques. The deposited minerals were lost during the histological procedure and hence observed as holes in the tissue sections. Histological quantification of that substance is difficult, but calcific deposits were more frequent in the asymptomatic plaques, in accordance with our more precise biochemical results.

In contrast to elastin, collagen fractions analyzed by FPLC were similar in plaques from symptomatic and asymptomatic patients (Table). In all plaques, predominant collagen forms were the cross-linked ones (70% of the total on average), in accordance with earlier studies. A correlation was found between cross-linked collagen and cross-linked elastin in the plaques ($r=0.452; P=0.011$) (Figure 4). Total glycosaminoglycans correlated with total elastin ($r=0.45; P=0.01$) and intermediate elastin ($r=0.533; P=0.002$). However, glycosaminoglycans were not increased in plaques associated with symptoms, suggesting that total glycosaminoglycan content is less likely to be a marker of plaque vulnerability than the non–cross-linked forms of elastin, found to be increased in plaques associated with symptoms.

Total elastin content correlated negatively with the age of the patients ($r=-0.439; P=0.01$) (Figure 5A). A similar correlation was found between age and intermediate elastin ($r=-0.39; P=0.03$) (Figure 5B). In contrast, there were no statistically significant correlations between the age of the patients and hydroxyapatite, collagen, or glycosaminoglycan. A decreased elastin content results in an increased collagen/elastin ratio, which is known to be associated with aging and the increasing stiffness of blood vessels.
Discussion

The present study identified disturbed elastin homeostasis in carotid plaques as a possible marker of increased risk of ipsilateral symptoms. Plaques associated with symptoms had decreased levels of calcium salts, supporting the notion that calcification generally makes plaques more stable. In contrast, collagen levels were similar in the patient groups with or without ipsilateral symptoms, suggesting that there is no general defect in collagen synthesis in unstable plaques. It is clear that collagen is an important determinant of the stability of vulnerable areas of atherosclerotic plaques such as cap and shoulder regions, but the present study assessed the levels of matrix constituents in the entire plaque.

Elastin is the major component of elastic fibers present in the arterial wall. Vessel wall ability to adapt to new circulatory conditions often translates into an increase of elastin biosynthesis. Coronary plaques associated with unstable angina are characterized by increased intimal elastin content compared with stable plaques. On the other hand, increased elastolytic activity is associated with severity of atherosclerosis in the aorta. We found elastin in the subendothelial space and in the ruptured areas in the plaques associated with symptoms. Shoulders of these plaques had stronger elastin staining, possibly related to the turbulence and strong hemodynamic forces that these regions have to attenuate. The shoulder regions are also known to have high biochemical activity.

The increase in elastin levels in plaques associated with symptoms was due to increased levels of intermediate elastin forms. The intermediate elastin fraction can represent molecules on the way either to become cross-linked or to be degraded by proteases. It can be argued that at least in the rupture-prone regions infiltrated by macrophages, degradation of elastin should dominate. However, plaques associated with symptoms did not have increased levels of degraded elastin. This may be explained by the fact that degraded elastin forms are cleared from the plaques more rapidly than intermediate forms. In any case, our data suggest that elastin synthesis is increased in plaques associated with symptoms, but the newly synthesized elastin is not effectively incorporated into elastic fibers (since the levels of cross-linked elastin did not change). The intermediate forms can, however, be assumed to be more susceptible to proteolysis than the fully cross-linked elastin, suggesting that the turnover of incompletely cross-linked elastin may be increased in plaques associated with symptoms.

Age has been shown to be a major determinant of carotid artery cross-sectional area, and arteries are known to get...
stiffer with increasing age. In the present study plaques from older patients were found to have decreased total elastin and decreased intermediate elastin forms. The collagen/elastin ratio and the stiffness of the plaques are therefore expected to increase with age, a result in accordance with earlier studies.

Thus, 2 kinds of changes in elastin content were discovered in the present study. First, total elastin content in carotid plaques decreased with age. Second, the increase in total elastin content in plaques associated with symptoms was due to increased levels of intermediate elastin rather than cross-linked elastin. The possible harmful effects of intermediate elastin may be due not only to altered elasticity of the affected atherosclerotic plaques. Tropoelastin, the monomeric form of elastin, has been shown to be chemotactic, and degradation products of elastin can stimulate a number of processes, summarized below.

The increase in intermediate forms of elastin in plaques associated with symptoms suggests that the turnover of elastin is increased in these plaques and/or that the assembly of stable elastin fibers is impaired. The intermediate elastin forms could contribute to the pathogenesis of atherosclerosis in several different ways. Physiological degradation of elastic fibers, enhanced in vascular pathologies, leads to the presence of circulating elastin peptides, which have been demonstrated to induce cell migration and proliferation. Low concentrations (1 μmol/L) of α-elastin (elastin peptides) have been shown to increase total protein and fibronecctin biosynthesis, while higher concentrations (1.35 μmol/L) induce cell death. These effects are thought to be mediated by the elastin-laminin receptor (ELR), which is expressed in vascular smooth muscle cells, endothelial cells, monocytes, and fibroblasts. Activated human lymphocytes were also shown to express the ELR in plaques, and these cells secrete elastase and cathepsin G on exposure to elastin peptides. In a human fibrosarcoma cell line, elastin peptides induced matrix metalloproteinase-2 (MMP-2) production. The ELR has also been associated with increased turnover of glycosaminoglycans.

On the other hand, the ELR has been shown to trigger NO-dependent vasodilation and downregulation of cholesterol synthesis. However, these functions decrease or disappear with increasing age, while the upregulation of elastase release is increased. During aging, the ELR is uncoupled from its transmission pathway (G-protein, phospholipase C, protein kinase C) but continues to stimulate production of superoxide. Thus, increased elastin turnover could contribute to cell proliferation, migration, and oxidative stress in atherosclerotic lesions.

Moreover, the alterations in the extracellular matrix observed in the present study may increase trapping of lipids and lipoproteins in the vascular wall. Elastin, collagen, and proteoglycans are all known to bind LDL. Elastin appears to bind cholesterol so strongly that the bound cholesterol cannot be removed by HDL. Apart from direct interactions between matrix constituents and lipoproteins, the permeability of the elastic lamina can affect the rate of lipoprotein entrapment. Furthermore, lipid deposits have been found to be localized predominantly in the musculoelastic layer of the vascular wall, inside vacuolated elastin fibers. Our histological data showed the presence of elastin in the core, in the transition between the core and cap, and in the shoulders. Finally, serum elastase activity, circulating elastin peptides, and serum elastase inhibitor titers correlate with several risk factors for atherosclerosis.

The present study demonstrates an association of increased levels of presumably dysfunctional, intermediate forms of carotid plaque elastin with ipsilateral symptoms. Taken together, our results and previous studies implicate increased elastin turnover in a vicious cycle of cell activation and protease secretion, leading to gradual weakening of atherosclerotic plaques. The pathophysiological significance of these mechanisms remains to be established, but it may be of interest to initiate further studies of markers of elastin catabolism in relation to atherosclerosis.

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References


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