Bone Formation in Carotid Plaques
A Clinicopathological Study

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Background and Purpose—Bone formation and dystrophic calcification are present in carotid endarterectomy plaques. The clinical significance of these findings is unknown. The purpose of this study was to determine whether bone formation and extensive dystrophic calcification are associated with stable plaques and protective against ischemic vascular events.

Methods—Carotid endarterectomy plaques were collected from 142 patients (94 men) with carotid stenosis. The specimens were evaluated for lamellar bone formation, dystrophic calcifications, inflammatory infiltrates, neovascularization, and histological type or grade of plaque according to a standard AHA grading system. Immunohistochemical staining was performed to identify vascular endothelial cells in neovascularization (factor VIII) and lymphocytes. Clinical data, including history of cerebrovascular and cardiovascular events, were recorded at the time of surgery.

Results—Patients with calcification of carotid plaques had fewer symptoms of stroke and transient ischemic attack ($P=0.042$) than those without calcification. Stroke and transient ischemic attack occurred less frequently in patients with plaques with large calcific granules ($P=0.021$). Of the patients, 13% had lamellar bone formation, which directly correlated with the presence of sheetlike calcifications ($P=0.0001$) and inversely correlated with ulcerated lesions ($P=0.048$). The presence of bone also correlated with diabetes ($P<0.01$) and coronary artery disease ($P<0.01$). Of the 20 patients with bone, 6 had a history of stroke and transient ischemic attack ($P=0.5$).

Conclusions—The results indicate that bone formation tends to occur in heavily calcified carotid lesions devoid of ulceration and hemorrhage. Patients with extensive calcification of the carotid plaques are less likely to have symptomatic disease. (Stroke. 2002;33:1214-1219.)

Key Words: calcification ■ inflammation ■ stroke

Stroke is the third leading cause of death in the Western world and a major cause of disability in adults. It is well known that carotid artery atherosclerosis predisposes to embolic events, and the histology of atherosclerosis is well defined. However, few published reports provide correlation between the amount of carotid plaque calcification and patient outcome. The pathological changes that are protective from cerebrovascular embolic events and those that stimulate them are unclear.

For the past 1½ centuries, dystrophic calcification (calcification of normal tissue) has been recognized as a common component of the atherosclerotic lesion. Dystrophic calcification can develop as a diffuse pattern or as sheets. Early reports of dystrophic calcification described a link with tissue necrosis. An apoptotic mechanism of cell death is also thought to contribute to dystrophic calcification.

Lamellar bone (ossification) can develop in both arteries and cardiac valves. Although the origin of this pathological change has been attributed to metaplastic osteogenesis along an endochondral pathway, it is unclear whether bone and extensive sheets of calcification are protective from embolic events or are associated with a higher frequency of stroke and transient ischemic attacks (TIAs).

The purpose of this study was to evaluate histological and clinical data from patients who underwent carotid endarterectomy and correlate the presence of bone and sheetlike calcifications with clinical symptoms of ischemia.

Materials and Methods
This study was approved by the University of Pennsylvania School of Medicine Institutional Review Board. Patient information was collected prospectively from a detailed history at the time of presentation and from chart reviews. The presence of symptomatic cardiovascular disease was documented (Table 1), including claudication, coronary artery disease (CAD; angina or myocardial infarction), prior cerebral vascular accident, and prior TIAs. Cardiovascu-
TABLE 1. Clinical Characteristics of the Study Population

<table>
<thead>
<tr>
<th>Symptom</th>
<th>Patients Without Symptoms, n (%)</th>
<th>Patients With Symptoms, n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Claudication</td>
<td>83 (60)</td>
<td>56 (40)</td>
</tr>
<tr>
<td>Coronary artery disease</td>
<td>45 (32)</td>
<td>97 (68)</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>93 (65)</td>
<td>49 (35)</td>
</tr>
<tr>
<td>Prior stroke</td>
<td>120 (84)</td>
<td>23 (16)</td>
</tr>
<tr>
<td>Prior TIA</td>
<td>113 (79)</td>
<td>30 (21)</td>
</tr>
<tr>
<td>Elevated cholesterol</td>
<td>32 (23)</td>
<td>110 (77)</td>
</tr>
<tr>
<td>Statin use</td>
<td>58 (41)</td>
<td>84 (59)</td>
</tr>
<tr>
<td>Tobacco use (former and current)</td>
<td>37 (26)</td>
<td>106 (74)</td>
</tr>
<tr>
<td>Family history of CAD*</td>
<td>45 (56)</td>
<td>35 (44)</td>
</tr>
</tbody>
</table>

*There were patients who did not know their family history of CAD or had no data recorded in their charts.

lar risk factors such as diabetes mellitus, hypercholesterolemia (total cholesterol level >200 mg/dL or use of a cholesterol-reducing drug), hypertension (systolic blood pressure >140 mm Hg), history of tobacco use, and family history of CAD were recorded.

Carotid endarterectomy was performed on a consecutive series of patients by 4 surgeons (R.F., M.M., J.C., M.G.) using conventional surgical techniques. Briefly, the carotid artery was incised and the plaque was removed from within the lumen as a single specimen. The plaques were immediately fixed in 10% formaldehyde. Each plaque was serially sectioned at 2-mm intervals for tissue processing. Selected specimens were subjected to x-ray analysis as a positive control. To allow for tissue sectioning, all specimens with calcifications were decalcified briefly in 10% hydrochloric acid for 2 to 3 hours, a demineralization time that does not affect the histological appearance of calcification, bone, or cellular elements such as osteoblasts or osteoclasts.

After routine paraffin embedding of the specimens, 5-µm sections were prepared with hematoxylin and eosin staining. Selected cases were photographed for review (Figure 1). A detailed microscopic analysis of each plaque profile was performed at low, moderate, and high power (×4, ×10, and ×40, respectively) by a researcher (J.H.) who was blinded to clinical data. The atheromatous plaques were characterized according to well-defined histological features (Table 2).11

Cholesterol deposits were characterized by location (intracellular or extracellular). Intracellular collections of lipid material were defined as macrophages with cytoplasm engorged with foamy material. Extracellular cholesterol was defined as collections of needle-shaped spaces within the extracellular matrix, which contained foamy material.

Plaque ulceration was noted when the inner endothelialized luminal plaque wall was breached by a cleft-shaped tear or by widespread disruption and fragmentation (Figure 2). These features were distinguished from artifactual disorganization by the presence of fibrin, hemorrhage, and inflammatory cell responses, typifying premortem endovascular damage. Intraplaque rupture was similarly defined as the presence of a collection of fibrin, hemorrhage, and thrombus in a cystically dilated, well-circumscribed space within the wall of the plaque. Neovascularization was noted when small-diameter (<1 mm) vascular spaces were present in groups and individually within the normally avascular intimal wall of the plaque (Figure 3).

Dystrophic calcifications were identified on the basis of the typical morphological appearance of aggregates of basophilic crystalline material. These calcifications were classified by size and staining characteristics. Small and large calcifications (<1 and 1 to 9 µm, respectively) were noted when the histological appearance was granular and heterogeneous (Figure 4). Sheetlike calcifications were identified as large plates of lamellated, homogenous calcium deposition (>10 µm in diameter; Figures 4 and 5). Inflammatory cells identified within the plaques included macrophages with cholesterol (see above) and with hemosiderin deposition (brown refractile pigment accumulation). Also noted were lymphocytes, giant cells, and polymorphonuclear cells.

The presence of bone within the atherosclerotic plaque was scored positive when any focus of bone matrix and bone cells (osteoblasts, osteocytes, and osteoclasts) were both present (Figure 6). The

Figure 1. Photomicrograph of gross specimen. This is a cross section of a carotid endarterectomy specimen under a dissecting microscope (approximately ×3 magnification). This plaque has several lumina resulting from recanalization. There is significant ulceration and intraplaque rupture (→), indicating that this is an AHA grade VI lesion. There is also calcification in the wall, surrounded by hemosiderin deposition (◆).

Figure 2. Ulceration and hemorrhage. This plaque demonstrates plaque ulceration. There is a cleft-shaped defect in the endothelialized wall of the plaque (◆). Adherent to the damaged wall is a collection of fibrin, blood, and macrophages (→) (hematoxylin and eosin, magnification ×10).
lamellations of the osseous matrix was verified with polarized light microscopy (Figure 7).

The histological grade of the atherosclerotic plaque (types I through VI) was determined for each specimen according to the AHA classification. In this classification scheme, the higher-grade lesions contain ulceration and plaque hemorrhage, whereas the low-grade lesions demonstrate macrophage accumulation and smooth muscle proliferation. The range is from the initial lesions (grade I) to advanced or complicated lesions (grade VI).

Immunohistochemical stains were performed for inflammatory cells (Leukocyte Common Antigen, 1:150; Dako) and for vascular endothelial cells (factor VIII, 1:1500, Dako).

Statistical analyses were performed to investigate the association between clinical symptomology and medical history and the histological features present in the plaques. We performed $\chi^2$ analyses or Fisher’s exact test analyses when appropriate to investigate associations with dichotomous predictors. Values of potential continuous predictors were expressed as means, and comparisons were made by use of Student’s $t$ test. Statistical significance was established at $P<0.05$. Statistical analysis was performed with Stata 6.0 (Stata Corp).

Results

Patient Demographics

One hundred forty-two patients, 94 men (66%) and 48 women (34%), were enrolled in the study. The patients ranged from 42 to 85 years of age (mean, 69.5 years). The clinical characteristics of the study population are listed in Table 1. Most patients did not have a prior stroke or TIA. Most patients (86%) had at least 1 cardiovascular symptom (CAD, stroke, TIA, or claudication).

Histological Findings

The results of the histologic analysis are presented in Table 2. An advanced (AHA grade VI) carotid endarterectomy plaque is shown in Figure 1. Plaque ulceration and hemorrhage are shown in Figure 2. Calcium sheets were a relatively common pattern of calcification. Inflammatory cells and bone were
Thus, bone, when present, tended to occur in association with foam cells and large sheets of calcium.

**Clinical Symptoms and Histological Findings**

The clinical characteristics of patients and plaque morphology were compared by use of χ² analysis. Of the 52 patients who suffered a TIA or stroke, 35% (n=18) had a calcified plaque (both small and large calcifications), and 65% (n=34) did not have calcification (P=0.042). The presence of large granules of calcification also correlated with a decreased incidence of stroke and TIA, because 17% (n=9) with large granules of calcium and 83% (n=43) without large granules of calcium had a cerebrovascular event (P=0.021). There were 6 patients with bone in the plaque who had a cerebrovascular event and 14 patients who did not have a cerebrovascular event (P=NS).

CAD and diabetes mellitus correlated with bone formation, with 95% of those with bone formation having a clinical history of CAD (P<0.008) and 67% having a history of diabetes (P<0.01). Bone formation in the plaque did not correlate with a clinical history of elevated cholesterol, hypertension, statin use, or sex. Patients with large sheets of calcium in the plaque were also more likely to have CAD (P=0.033). When all clinical symptoms were analyzed together (stroke, TIA, CAD, peripheral arterial disease), there was a direct correlation with smoking (P=0.037) and high cholesterol (P=0.002).

**Discussion**

The results from the present study indicate that bone formation is occurring in patients with histologically low-grade (II, III, and IV), nonulcerated, stable plaques. The atherosclerotic lesions that contain bone have little hemorrhage or ulceration and have large sheets of calcification in their walls. The pathophysiological relationship between bone and sheetlike calcifications is unknown. Most of the higher-stage atherosclerotic lesions do not contain bone, indicating that the patient population with bone formation may be different from the population whose lesions progress to higher-stage, unstable lesions. Further immunohistochemical studies of the microenvironment present in the atherosclerotic plaques containing bone may provide important insight into what cellular elements are producing the growth factors necessary for initiation of ossification.

Ischemic stroke can be subdivided into at least 4 mechanistic categories: large-artery disease, small-artery disease, cardioembolic disease, and cryptogenic.1 Atherosclerosis and thrombosis are important components of large-artery disease in vessels such as the carotid and vertebral arteries.12 Embolic events from atherosclerosis of the carotid artery are well documented as a major contributing factor in the development of stroke,13 and medical treatment of stroke is evolving.14,15 Descriptive pathological studies detailing carotid atherosclerosis with calcification were published,16 but few evaluated for ossification or compared pathological findings with clinical symptoms. Therefore, little is known about the effect of dystrophic calcification and bone formation on cerebrovascular events.

In the present study, patients with calcification of carotid plaques had fewer symptoms of stroke and TIA. Mature lamellar bone was present in 13% of the specimens and correlated with sheets of calcifications. Bone was significantly more common in stable plaques (AHA grades II

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**TABLE 3. Plaques in Each AHA Grade**

<table>
<thead>
<tr>
<th>AHA Grade</th>
<th>Specimen, n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>0</td>
</tr>
<tr>
<td>II</td>
<td>2 (1)</td>
</tr>
<tr>
<td>III</td>
<td>54 (36)</td>
</tr>
<tr>
<td>IV</td>
<td>15 (10)</td>
</tr>
<tr>
<td>V</td>
<td>26 (18)</td>
</tr>
<tr>
<td>VI</td>
<td>53 (35)</td>
</tr>
</tbody>
</table>
through IV) than in unstable plaques (AHA grades V and VI). Patients with large calcific granules in their plaques had significantly fewer stroke and TIA. Although statistical significance was not achieved, fewer patients with bone had stroke and TIA than those without bone.

Histological characteristics associated with clinical plaque instability reported in other studies include an increase in cholesterol content and infiltration of inflammatory cells,17–19 as well as hemorrhage and ulceration.20–22 However, focal accumulation of macrophages can be found in all developmental stages of atherosclerotic carotid arteries.23 Other factors such as proteases24–26 and their inhibitors,25 proinflammatory cytokines,26 and prothrombotic molecules27 are also thought to contribute to plaque instability. Although there is a histological grading scheme for atherosclerotic carotid plaques,11 the current grading scheme does not address the presence of bone or various geometric calcific formations but relies mainly on the presence of hemorrhage and ulceration as a predictor of an advanced lesion.

Recent reports indicate that lamellar bone can form in atherosclerotic plaques in the carotid arteries2 and cardiac valves.10 Because de novo bone formation seems to depend on angiogenesis and inflammatory cells,28,29 it is not surprising that the results from our study and others demonstrated neovascularization, granulation-type tissue, mast cells, macrophages, and osteoclast-like giant cells in the areas of bone.23,30,31 Despite these interesting pathological findings, the relationship of bone formation and dense calcifications with stroke or TIA was previously unknown; there are no published reports evaluating the presence of bone, the AHA grade of atherosclerotic lesions, and cerebrovascular events. Consequently, the current grading scheme does not address the significance of calcium content in the coronary arteries and cardiac valves.

Although there are no other published studies correlating the presence of bone and vascular events, there are published studies evaluating the relationship between calcification and clinical symptoms of vascular disease. Johnson and colleagues32 reported that soft plaques have a greater tendency toward subintimal hemorrhage, ulceration, or primary embolization than better-organized plaques.Investigators from the Tromso Study in Norway found that on high-resolution ultrasound B-mode imaging, echolucent plaques (with higher content of lipid and hemorrhage) were associated with a higher risk of neurological events than echorich plaques that strongly reflect the echo signal.33 Echorich plaques typically contain more calcification and fibrous tissue than echoluent plaques.34 In another Scandinavian study, Gronholt et al35 found that echoluent plaques causing ≥50% diameter stenosis by Doppler ultrasound are associated with a risk of future stroke in symptomatic but not asymptomatic individuals. Cohen et al36 evaluated 334 patients with transesophageal echocardiography at the time of stroke and followed up this cohort for occurrence of vascular events. The highest relative risk for vascular events was found among patients with noncalcified plaques.36

The clinical significance of calcium content in the coronary arteries has also been studied with ultrasound. Mintz et al37 examined the coronary artery atherosclerotic lesions using intravascular ultrasound in 1442 patients. They noted that coronary calcification correlated with plaque burden but not the degree of lumen compromise.37 Also, coronary calcium increased with age and was less common in unstable subsets.37 Beckman et al38 also noted that the extent of coronary artery calcification on intravascular ultrasound was directly related to coronary stability. They speculated that arterial calcification may be a stabilizing force in atherosclerosis and may be more common in stable coronary syndromes.38 Further studies of central and peripheral artery atherosclerotic plaques are needed to confirm the putative stability of calcium.

Noninvasive detection of coronary artery calcification with electron-beam computed tomography (EBCT) is useful in identifying individuals with increased risk of cardiovascular events.39 Although EBCT accurately determines plaque burden, it is not suitable for determining the components of the plaque and therefore which plaques are unstable. Our results do not indicate that EBCT is inaccurate but highlight the importance of the relative amount of calcium and/or bone in atherosclerotic plaques as a determinant of plaque stability.

Although the role of cholesterol as a risk factor for stroke and the effectiveness of cholesterol lowering in reducing stroke have been controversial, recently published studies indicate HMG-CoA reductase inhibitors (statin) therapy is effective in those patients at risk for cerebrovascular events.40 A retrospective study was conducted on the impact of statin treatment on coronary calcium measured on EBCT.41 A calcium volume scoring system was used, and the extent to which the volume of atherosclerotic plaque decreased, stabilized, or increased was directly related to treatment with statins and the resulting low-density lipoprotein cholesterol level. The scoring method used does not take into account the amount of lipid in the plaque, and the reduction in atherosclerotic plaque volume may have been due to a relative reduction in lipid versus calcium content. The mechanism(s) of statin drugs on plaque composition are not yet fully elucidated.15

The salutary role of statins in preventing stroke is likely a result of their beneficial effect on plaque stability throughout the entire vascular tree, both cerebral and precerbral as a result of pleiotropic effects in addition to cholesterol reduction.42,43 Mundy et al44 found that statin drugs stimulate the bone morphogenetic protein promoter and promote bone formation. In addition, statins are thought to enhance angiogenesis, a process necessary for bone formation.45 Thus, perhaps one of the many pleiotropic effects of statin drugs may be enhancement of bone formation and therefore more plaque stability. The present study is limited in that only patients undergoing endarterectomy were included; thus, extrapolation to other populations at earlier stages of disease would be speculative.

The results of the present study support the hypothesis that dystrophic calcification and bone formation in atherosclerotic plaque do not increase the risk of stroke but may even be a stabilizing influence and protective.

Acknowledgments

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References


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