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Original Article

The Relationship between Carotid Plaque Calcification and Stability

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Abstract

Aim: **The study aimed to examine the hypothesis that advanced plaques with calcification are more stable (lower proportion of lipid component and higher proportion of fibrous tissue) compared to plaques without calcification.**

Methods: **Carotid endarterectomy (CEA) specimens from 141 consecutive patients were studied. The specimens were analyzed histologically for fibrous tissues, smooth muscle cells, macrophage, lymphocyte, hemorrhage and lipid, according to the methods of European Carotid Plaque Study Group; plaques were also graded according to American Heart Association (AHA) consensus and its modification. Clinical data was recorded and the plasma concentrations of cholesterol and inflammatory markers were measured.**

Results: **Thirty five out of 141 plaque specimens were identified to have advanced atherosclerosis (type V according to AHA criteria) and these were analyzed further. There were 29 type Va (non-calcified) plaques and 6 type Vb (calcified) plaques. Calcified plaques had significantly less lipid than non-calcified plaques ($p < 0.0001$): the mean percentage of lipid for non-calcified and calcified plaques was 61.29% and 23.48%, respectively. Also calcified plaques had more fibrous tissue than non-calcified plaques ($p = 0.004$): the mean percentage of fibrous tissue for non-calcified and calcified plaques was 23.74% and 59.37%, respectively ($P<0.0001$). The 6 calcified plaques showed no inflammatory cell infiltrate and did not exhibit thin fibrous cap atheroma which are the characteristics indicating high risk for plaque rupture.**

Conclusion: **Calcified plaques had significantly less lipid and more fibrous tissue than non-calcified plaques. These findings might suggest indirectly that plaque calcification is a marker of plaque stability. This may be a useful clinical tool to identify asymptomatic carotid stenosis patients with high risk plaques, which could improve benefit of CEA.**

Key words: **calcification, carotid, plaque, atherosclerosis, instability**

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Introduction

Carotid stenosis is a cause of stroke. Carotid endarterectomy (CEA) can reduce stroke risk in patients especially those with symptomatic severe carotid stenosis[1]. However the benefit of CEA in asymptomatic patients is still controversial, because in these patients CEA shows only marginal benefit[2,3]. It is estimated that 85 patients with asymptomatic severe carotid stenosis need to be operated on to prevent one disabling stroke or death in one year[2], whereas six patients with symptomatic severe carotid stenosis need to be operated on to prevent one stroke in one year[1]. Therefore, an ability to identify asymptomatic carotid stenosis patients who have a high risk plaque (i.e. vulnerability to rupture, known as plaque instability) could improve the efficacy of CEA in asymptomatic patients and could reduce the number of unnecessary operations in this group of patients.

In common with coronary plaques, unstable carotid plaques have a high lipid content, a thin fibrous cap, a high inflammatory cell content, and increased protease activity[4]. Calcification is a feature of advanced plaques, but the relation between calcification and other plaque components remains uncertain and the relation of calcification to plaque stability is not clear. Here, we investigated association between calcification and other plaque features characteristic of plaque instability.

Materials and Methods

The study was approved by the Southampton and South West Hampshire Research Ethics Committee, and written informed consent was obtained from all patients recruited. This was a prospective study and 141 consecutive patients undergoing CEA were recruited and their medical history was recorded[5]. Patients' records were also checked. On pre-operative visits venous blood samples were taken into vacutainer (r) tubes containing 0.12 ml of 15% EDTA. Plasma was isolated by centrifugation at 2500 rpm for 10 minutes and then stored at -70 ˚C. The plasma concentration of high sensitivity C-reactive protein (hs-CRP) was measured by an immunoturbidimetric technique, using a commercially available kit, made by Wako Laboratories and available from Alpha Laboratories (Eastleigh, UK). Inter- and intra-assay coefficients of variation were 6.4% and 3.4% respectively. Plasma concentrations of soluble E-selectin (sE-selectin) were measured using Quantikine ELISA kits from R & D Systems Europe (Abingdon, UK). The limit of detection was 0.1 ng/mL; inter- and intra-assay coefficients of variation were less than 10% and 5%, respectively. Plasma soluble intercellular adhesion molecule-1 (sICAM-1) and soluble vascular cell adhesion molecule-1 (sVCAM-1) concentrations were measured using Cytoscreen ELISA kits from BioSource (Nivelles, Belgium). Limits of detection were 0.04 ng/mL (sICAM-1) and 0.5 ng/mL (sVCAM-1). Inter- and intra-assay coefficients of variation were less than 5% for both assays. Total plasma cholesterol and low-density lipoprotein concentrations were measured using commercially available, enzyme-based diagnostic kits (Sigma Chemical Co., Poole, UK).

Specimens of carotid plaque obtained during CEA were immediately washed with saline in operating theatre. Serial transverse 2 mm sections were taken. These sections were then labeled alphabetically, starting from the distal end of the internal carotid artery and ending at the common carotid artery. Alternate sections of the plaque were cut from each block. We fixed sections in formaldehyde and then embedded them in paraffin wax and stained with haematoxylin and eosin (H & E). These stained sections were viewed with a microscope under 10 x magnification in random order; they were graded blindly and independently by KR and PJG, using criteria of American Heart Association (AHA) concensus[6] and its modification[7]. Also the amount of different plaque components (histomorphometry) was measured according to the European Carotid Plaque Study Group guidelines, which were developed and validated in our unit for a previous study of carotid atherosclerosis[8].

The AHA classification has six grades or types[6]: type I (initial lesion); type II (early lesion or fatty streak); type III (intermediate lesion or preatheroma); type IV (atheroma or atheromatous plaque); type V (fibroatherma or fibrotic lesion); and type VI (lesion with surface defect, hemorrhage, thrombotic deposit, or a combination of these). The modification of this classification entails a series of descriptive grades of increasing severity: pathological intimal thickening (smooth muscle cells in the matrix with areas of extracellular lipid accumulation but no necrosis or thrombus); fibrous cap atheroma (a well-formed necrotic core with an overlying fibrous cap; no

thrombus); thin fibrous cap atheroma (a thin fibrous cap infiltrated by macrophages and lymphocytes with rare smooth muscle cells and an underlying necrotic core; no thrombus); erosion (luminal thrombosis); plaque rupture (fibroatheroma with disruption; luminal thrombus communicating with necrotic core); and calcified nodule and fibrocalcific plaque[7]. For the histomorphometry measurement, the contribution of fibrous tissue, lipid, hemorrhage, macrophages, lymphocytes, smooth muscle cells, and new blood vessels was measured volumetrically with a standard light microscope with a reticule, which superimposed a grid of many equally-sized squares on the field of view[8]. This was done by identifying and recording for each field of view the type of tissue at the 36 reticule points (intersections of two reticule lines). The field of view was scanned carefully to ensure that no areas of the section were missed or counted more than once. From 6 to 20 fields were examined to cover the surface area of each section. The percentage contribution of each constituent was calculated. Inter-observer agreement of histomorphometry was 92%.

Statistical analysis

Normally distributed ordinal data were compared using Student's t-test, and non-normally distributed data were compared using the Mann-Whitney Rank sum test. Categorical data were compared by Chi-square test or Fisher's exact test. A p value of less than 0.05 was considered significant.

Results

35 out of 141 plaque specimens were identified to have severe atherosclerosis (type V according to AHA criteria) and these were analysed further. They were classified as 29 type Va (non-calcified) plaques and 6 as type Vb (calcified) plaques. The mean period between symptom and surgery was 41 days. There were no statistically significant differences between patients with non-calcified and calcified plaques in baseline characteristics, blood lipid concentrations or plasma inflammatory markers (Tables 1 and 2). In an analysis of histomorphometry (plaque composition) (Table 3), calcified plaques had significantly more fibrous tissue and less lipid than non-calcified plaques. The mean percentage of fibrous tissue for non-calcified and calcified plaques was 23.74% and 59.37%, respectively, while the mean percentage of lipid for non-calcified and calcified plaques was 61.29% and

Table 1 Baseline characteristics of patients undergoing carotid endarterectomy according to whether the AHA type V plaque was calcified or non-calcified

Risk factors	Non-calcified plaque (n = 29)		Calcified plaque (n = 6)		P value
Mean age, y (SEM)	70.21 (1.9)		69.00 (4.0)		0.79
Male, n (%)	16	(55.2)	2	(33.3)	0.40
Hypertension, n (%)	20	(69.0)	3	(50.0)	0.36
Diabetes, n (%)	3	(10.3)	2	(33.3)	0.20
Current smoker, n (%)	24	(82.8)	5	(83.3)	1.00
Mean body mass index, kg/m^2 (SEM)	25.43 (3.4)		25.92 (1.7)		0.76
Coronary artery disease,n (%)	9	(31.0)	3	(50.0)	0.15
Peripheral artery disease, n (%)	3	(10.3)	1	(16.7)	1.00
Clinical history					
Asymptomatic	8	(27.6)	2	(33.3)	0.44
Amaurosis fugax	3	(10.3)	2	(33.3)	
Transient ischemic attack	13	(44.8)	0		
Stroke	5	(17.2)	2	(33.3)	
Severe stenosis (70-99%)	24	(82.8)	5	(83.3)	0.85
Eversion carotid endarterectomy (%)	8	(27.6)	1	(16.7)	1.0

SEM, Standard error of the mean; n = total number

Table 2 Blood lipid and inflammatory marker concentrations in patients according to whether the AHA type V plaque was calcified or non-calcified

	Non-calcified plaque (n = 29)	Calcified plaque (n = 6)	P value
LDL, mmol/L	1.97 (0.1)	2.67 (0.8)	0.23
Total cholesterol, mmol/L	4.80 (0.2)	5.90 (1.0)	0.35
hs-CRP (mg/L)	6.02 (1.8)	19.16 (13.2)	0.37
sICAM-1 (ng/mL)	310.93 (15.0)	343.10 (53.6)	0.42
sVCAM-1 (ng/mL)	693.01 (51.5)	683.28 (87.9)	0.93
sE-selectin (ng/mL)	36.32 (4.3)	46.95 (7.3)	0.24

Data are mean with SEM shown in parentheses. n = total number
LDL, low-density lipoprotein; hs-CRP, high-sensitivity C-reactive protein; sICAM, soluble intercellular adhesion molecule; sVCAM, soluble vascular adhesion molecule; sE-selectin, soluble E selectin

Table 3 The percentage of the content of fibrous tissue, lipid, hemorrhage, lymphocyte, macrophage, smooth muscle cells, and new blood vessels according to whether the AHA type V plaque was calcified or non-calcified

Plaque component	Non-calcified plaque (n = 29)	Calcified plaque (n = 6)	P value
Fibrous tissue	23.74 (3.5)	59.37 (7.6)	< 0.0001
Lipid	61.29 (3.7)	23.48 (8.1)	0.004
Hemorrhage	1.30 (0.4)	0.52 (0.4)	0.38
Lymphocytes	3.88 (1.4)	1.05 (0.6)	0.35
Macrophages	1.11 (0.6)	0	0.38
Smooth muscle cells	2.28 (0.7)	7.67 (6.3)	0.43
New blood vessels	0.07 (0.03)	0.04 (0.03)	0.61

Data are mean percentage with SEM shown in parentheses, n = total number of plaques in each type

Table 4 The number and percentage of plaque morphology of non-calcified and calcified plaques according to the modified AHA classification

	Non-calcified plaque (n = 29)	Calcified plaque (n = 6)	P value
Fibrous cap atheroma	2 (6.9)	0	< 0.0001
Thin fibrous cap atheroma	27 (93.1)	0	
Calcified nodule	0	4 (66.7)	
Fibrocalcific plaque	0	2 (33.3)	

Data are number of plaque with percentage shown in parentheses, n = total number of plaques in each type

23.48% respectively. Calcified plaques showed no macrophage infiltration and did not exhibit thin fibrous cap atheroma which are the characteristics indicating high risk for plaque rupture (Table 4).

Discussion

Here we show that calcified plaques have more fibrous tissue and less lipid content than non-calcified plaques. It is well known that plaques with a thin fibrous cap and a large lipid core are at high risk for rupture, increasing likelihood of thrombosis and stroke[7]. We found that calcified plaques showed no macrophage infiltration and no calcified plaques were classified as thin fibrous cap atheromas. It was considered that thin fibrous cap atheromas are most likely to rupture[7]. Together, our findings indicate that calcification is associated with plaque stability. In a previous study, Wahlgren et al. showed that calcified plaques had fewer macrophages in the fibrous cap

than non-calcified plaques[9]. Shaalan et al. investigated plaque calcification by CT scan and plaque characteristics by immunohistochemistry[10]. They found calcified plaques to have a less inflammatory character and to be associated with fewer ischemic symptoms than non-calcified plaques. Similarly Nandalur et al. reported that the proportion of carotid plaque calcification is associated with plaque stability in patients with carotid stenosis and they proposed plaque calcification of 45% of the total volume according to CT scan that might represent a clinically useful cutoff[11]. Li and colleague reported the importance of the location of calcification in plaques by magnetic resonance imaging study. They found when calcification locates in fibrous cap especially in thin fibrous cap, it creates a very high stress concen-tration on plaque and consequently increases risk of plaque rupture[12,13]. Also a recent systematic review found symptomatic plaques have a lower degree of calcification than asymptomatic plaques[14]. Our study adds novel information on the relationship between plaque calcification and plaque instability in terms of the morphologic characteristics i.e. no thin fibrous cap atheroma in calcified plaque. Overall, our findings suggested that calcification is associated indirectly with stable plaques.

The mechanisms of the association between calcification and plaque stability are not clear. Our study together with others found that calcified plaque had fewer macrophages[9]. It is recognised that macrophages play a role which makes plaques more unstable through many machanisms namely excessive inflammatory cytokine and matrix metalloproteinases production[15,16], apoptosis[17] and potent initiators of thrombosis[18].

Plaque calcification may be applied to select patients with asymptomatic carotid stenosis who have high risk for strokes. Calcification can be identified by many available investigations such as ultrasound, CT scanning and magnetic resonance imaging[19]. If the significance of calcification in this study is confirmed, by a prospective randomized controlled trial, patients with asymptomatic carotid stenosis with calcification might need medical treatment alone. Also calcification might be a marker to monitor the effectiveness of medical treatment to make plaques more stable, such as by lipid lowering agents or by antihypertensive drugs. Interestingly, Zhao et al. reported that lipid lowering agents not only reduce the amount of lipid in the plaque but increase plaque calcium[20].

Although we believe our data is valid, the sample size was small, meaning that the number of calcified plaques was small (n = 6), which was prone to have type II error in the analyses. However, our study result is still consistent with other studies in terms of inflammatory cells and plaque stability.[9-11] Also the mean duration between symptom and surgery was 41 days, that might have time to heal plaque. This might partly explain no correlation between symptom and plaque stability criteria was found.

Conclusion

Plaques with calcification demonstrate less lipid and more fibrous components, compared to plaques without calcification. These findings might suggest indirectly that plaque calcification is a marker of plaque stability. This needs to be tested in bigger studies. If that is the case, this will benefit enormously because plaque calcification is readily identified on non-invasive testing such as ultrasound and this may be a useful clinical tool to identify patients with lower risk plaques.

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