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Annexin A1 Expression in Atherosclerotic Carotid Plaques and its Relationship with Plaque Characteristics

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Abstract *Objective:* Annexin A1, a calcium and phospholipid-binding protein, is an important endogenous modulator of inflammation. Whether this regulatory role extends to atherosclerosis is unknown. The aim of this study is to investigate the genetic and protein expression of Annexin A1 in carotid endarterectomy specimens from patients with significant carotid stenosis.
Materials and methods: The echogenicity of atherosclerotic plaques was determined by ultrasound prior to carotid endarterectomy (CEA) in 34 consecutively recruited patients with carotid stenosis exceeding 70%. The Annexin A1 messenger RNA and protein expression of the corresponding plaques obtained from those patients were analyzed by reverse transcriptase polymerase chain reaction (RT-PCR) and the immunohistochemical method respectively. Results were analyzed with respect to plaque characteristics and symptomatic disease.
Results: There were 25 males and 9 females, with a mean age of 68.8. Ten patients were asymptomatic. The symptomatic patients' plaques were more echolucent (mean grey scale median (GSM) of 103) than those of asymptomatic patients (mean GSM = 126, $p = 0.022$). The Annexin A1 protein was constitutively expressed in all plaques, and Annexin A1 gene expression was statistically higher in patients with asymptomatic disease compared with those with neurological symptoms ($87 \pm 4\%$ vs. $42 \pm 6.2\%$; $p < 0.001$, unpaired *t*-test). The GSM score was positively correlated with Annexin A1 levels in patients with high-grade carotid artery stenosis ($r = 0.501$, $p = 0.009$).
Conclusions: This is the first study to suggest that high Annexin A1 expression may have a stabilising effect in asymptomatic patients with less echolucent atherosclerotic plaques. Since atherosclerosis is an inflammatory process, we further postulate that Annexin A1 may play an essential role in preventing plaque complications or disease progression.

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Cerebrovascular accidents or transient ischaemic attacks are due to microemboli generated from carotid atherosclerotic plaques.[1] Thus, the stability of the plaque dictates whether the patient will develop symptoms. Specifically, echolucent plaques that are lipid rich tend to develop complications and may rupture to contribute to symptomatic neurological events independent of the degree of stenosis.[2]

To date, carotid endarterectomy (CEA) is one of the most scrutinised surgical procedures, and it has been shown by multicentre trials to be effective in the prophylaxis against strokes, if the degree of stenosis is greater than 70%.[3–6] The biological examination of CEA specimens may therefore provide invaluable information regarding the cellular and molecular events leading to plaque rupture.

Immunohistochemical studies of plaque have shown that one of the major determinants of plaque rupture is fibrous cap inflammation.[7] In the advanced stages of carotid stenosis, a low concentration of vascular smooth muscle cells (VSMCs),[8] and an increased concentration of macrophages and inflammatory cells in the fibrous cap weakens the plaque, causing plaque rupture.[9] In addition, failed or delayed clearance of apoptotic cells can result in the chronic inflammation that characterises carotid stenosis.[10] The *in vivo* clearance of apoptotic cells is also found to enhance the release of anti-inflammatory cytokines.[11] Thus, phagocytosis of apoptotic cells probably plays a pivotal role in the resolution of inflammation that may stabilise the atherosclerotic plaque.

Annexin A1 belongs to a group of calcium-dependent, phospholipid-binding proteins that have been implicated in diverse cellular roles, including the control of inflammatory responses, membrane fusion, and cell differentiation and proliferation.[12,13] Annexin A1 was originally identified in leucocytes as a glucocorticoid-inducible protein that inhibits phospholipase A2, thus preventing the formation of pro-inflammatory eicosanoids,[14] and it has been shown to mimic the anti-inflammatory actions of glucocorticoids in many experimental models.[15]

Annexin A1 is also found to be a powerful phagocytotic protein that can dampen inflammatory responses by allowing safe post-apoptotic clearance of dead cells.[16] The Solito et al. research group also found that Annexin A1 may inhibit leucocyte migration by impairing neutrophil and monocyte adhesion to vascular endothelium, thus inducing apoptosis of inflammatory cells. All these effects contribute to the potent anti-inflammatory action exerted by both Annexin A1 and its induced proteins.[14] More recent research indicated that Annexin A1 has been indirectly linked to atherogenesis through the engulfment of apoptotic bodies present in the coronary atherosclerotic plaque.[17] The author suggested that the engulfment of apoptotic cell is the factor involved in the development and progression of atherosclerosis. However, Annexin A1 involvement in human atherosclerosis development, particularly in carotid stenosis, remains unknown.

Given the potential anti-inflammatory effect of Annexin A1 that could stabilise carotid plaques, we hypothesise that enhanced Annexin A1 protein expression in human asymptomatic plaques will stabilise carotid plaques via an anti-inflammatory mechanism. Thus, its expression may be beneficial as an endogenous defence with respect to the inflammatory stimuli that are released in the plaque. To test this hypothesis, carotid plaque specimens from both asymptomatic and symptomatic patients were examined using molecular biological approaches and immunohistochemical analysis. The association of plaque echolucency and detection of macrophages with Annexin A1 gene expression levels was also investigated. We expect this study to provide more biochemical information on carotid plaques. Understanding the role of the powerful anti-inflammatory actions of endogenous Annexin A1 compounds may be a useful tool in the development of potential therapeutics for resolving atherosclerotic carotid stenosis.

Methods

Patient recruitment

Between June 2003 and June 2009, 34 consecutive patients (25 male, nine female), who were attributed as having a high-grade ($\geq$70%) atherosclerotic stenosis of carotid artery that was revealed ultrasonically and who underwent CEA, were recruited for the study. The study protocol was approved by the institutional review committee and informed consent was obtained from the patients.

Carotid plaque ultrasonic characteristics

Carotid plaque characteristics were studied by ultrasonography before CEA surgery in all consecutive patients. The corresponding carotid plaque echogenicity was measured by a method described previously,[18] and modified for our laboratory scanner.[19] The plaque was outlined and the distribution of the grey levels within the plaque was assessed. The digitised images were converted to grey scale, and the level normalised with a 256 greyscale range with respect to blood = 0–5, maximal white according to a linear reference scale = 256. This would give a greyscale value of the adventitia of approximately 230. The plaque outline was mapped manually using commercial image processing software (Adobe Photoshop 5.0) and the greyscale histogram was calculated. Data regarding the grey scale median (GSM), and its standard deviation (homogenicity), were obtained from the histogram.

Carotid plaque collection

The corresponding plaques were excised at the time of CEA surgery in a sterile fashion without damage to the plaque surface. Ulcerated plaques and intraplaque haemorrhage are likely to contribute to symptomatic disease.[20] Patients with evidence of a >70% stenosis in the common or internal carotid arteries on duplex ultrasound and who gave a history of cerebrovascular symptoms (such as stroke, transient ischaemic attacks or amarousis fugax) prior to the carotid duplex ultrasound examination were classified as symptomatic. No haemorrhaging was found in any of the studied specimens. Five of the symptomatic plaques with an ulcerated luminal surface were excluded to maintain the homogeneity of the plaque specimens. All plaques were divided into two parts at the most prominent site of the plaque. One part was fixed for 24 h in buffered formalin and