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 ± 4% vs. 42 ± 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. 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.

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%. 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. In the advanced stages of carotid stenosis, a low concentration of vascular smooth muscle cells (VSMCs), and an increased concentration of macrophages and inflammatory cells in the fibrous cap weakens the plaque, causing plaque rupture. In addition, failed or delayed clearance of apoptotic cells can result in the chronic inflammation that characterises carotid stenosis. The in vivo clearance of apoptotic cells is also found to enhance the release of anti-inflammatory cytokines. 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. Annexin A1 was originally identified in leucocytes as a glucocorticoid-inducible protein that inhibits phospholipase A2, thus preventing the formation of pro-inflammatory eicosanoids and it has been shown to mimic the anti-inflammatory actions of glucocorticoids in many experimental models.

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. 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. 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. 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 (>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 and modified for our laboratory scanner. 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. 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 amaurosis 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
later embedded in paraffin for immunohistochemical study, while the other part was rapidly frozen in liquid nitrogen and then stored at −80 °C until further processing for ribonucleic acid (RNA) extraction.

**Serum sample collection**

Clotted bloods were prepared conventionally from each patient at the time of presentation, aliquoted and stored at −80 °C until the enzyme-linked immunosorbent assay (ELISA) was performed.

**Immunohistochemistry for Annexin A1**

Annexin belongs to a family of structurally related calcium- and phospholipid-binding proteins that includes Annexin A1, A2 and A5. The anti-inflammatory effects are mainly attributed to the multifaceted Annexin A1, while Annexin A5 is found to be an apoptotic factor. Preliminary staining results found no Annexin A2 or A5 protein in either type of plaque. Therefore, in the present study, we only focussed on Annexin A1. The 5-μm-thick paraffin-embedded plaque sections were cleared and rehydrated by sequential immersions in three changes of xylene, followed by graded ethanol and distilled water. For antigen retrieval, tissues were heated by microwave treatment in 0.01 M citrate buffer for 90 s. After cooling, slides were incubated with anti-Annexin A1 (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA) at a 1:200 dilution for 60 min, and subsequently incubated with the peroxidase-labelled secondary antibody (Dako, Carpinteria, CA, USA). Sections were detected by the Dako EnVision Visualization System (Dako, Carpinteria, CA, USA) with diaminobenzidine used as the chromogenic substrate. Staining development was stopped by immersion in tap water and counterstained with Lillie haematoxylin (Merck, Darmstadt, Germany). All slides were then dehydrated and cleared by sequential immersion in gradient ethanol and xylene. Negative control was always performed by omitting primary antibody. All stained slides were viewed under light microscope (Olympus, Leeds Precision Instruments) and pictures were taken with an imaging program.

**RNA extraction**

All plaque specimens were pulverised under liquid nitrogen, and total RNA was extracted using the TRIzol LS Reagent (GIBCO BRL, Rockville, MD, USA).

To evaluate the quality of RNA, absorbency at 260/280 nm was determined by spectrophotometry. Approximately 1 μg of each sample was loaded onto the agarose gel and resolved by electrophoresis. The gel was stained with ethidium bromide. The 18S and 28S bands were visualised using ultraviolet (UV) light.

**Reverse transcriptase-polymerase chain reaction (RT-PCR)**

Two micrograms of total RNA of each sample was used to synthesise the first-strand complementary DNA (cDNA) with the SuperScript Preamplification System for First Strand cDNA Synthesis Kit (GIBCO BRL, Rockville, MD, USA). Then, 1 μl of the reverse transcriptase (RT) product was used as the template to amplify specific Annexin A1 fragments. The polymerase chain reaction (PCR) conditions were optimised individually for each gene studied, and the cycle number for PCR was adjusted to 30, so that the reactions fell within the linear range of product amplification. The expression of the housekeeping gene, β-actin, was used as an internal control. The RT-PCR product was analyzed by electrophoresis on a 2% agarose gel and confirmed by sequencing using an Automatic Sequencer. Signal intensities were quantified using densitometry (Bio-Rad Laboratories, Hercules, CA, USA). The Annexin messenger RNA (mRNA) level was quantified by the intensity ratio of the target signal to the β-actin control under the same PCR reaction conditions. The Annexin A1 primer sequences of the PCR were 5′-TAAGCGAAACAATGCACAGC-3′ and 5′-CAAGCGTTCCGAAAATCTC-3′ with a product size of 331 bp. The β-actin primer sequences were 5′-GCCATCTCTGCCTGGAGCTGCT-3′ and 5′-GTGAGACCTGGGCGTCAGCAGC-3′, with a product size of 181 bp.

**Detection of inflammatory macrophages**

To investigate the inflammatory macrophage status of the studied plaques, the corresponding sections were also incubated with human anti-macrophage (Dako, Carpinteria, CA, USA) for 1.5 h at 37 °C and detected by the same staining procedure. Representative microscopic fields of the plaque specimens were chosen under low-power light microscopy. To avoid underestimating the quantity of macrophages in the plaque specimens due to regional variation, only areas with the largest number of macrophages were chosen. The number of positive-stained macrophage cells was measured for 10 contiguous 200× high-power fields for each sample. The overall mean of macrophage cells for each specimen was determined as macrophages per high-power field.

**Measurement of inflammatory cytokines**

Serum levels of inflammatory cytokines including interleukin (IL)-1β, IL-6, IL-8 and IL-10 of the study patients were measured by standard ELISA kits (Quantikine Human IL-1β, IL-6, IL-8 and IL-10m R & D Systems Ltd, Abington, UK).

**Statistical analysis**

Continuous data (age) were expressed as a mean, and range and categorical data were expressed as percentages. The intima media thickness (IMT) GSM and GSM standard deviation (homogenicity), the degree of stenosis and the common and internal carotid artery lumen values were normally distributed and compared for asymptomatic and symptomatic patients using Student’s unpaired t-test (SPSS14.0, Inc., Chicago, IL, USA). Comparison of Annexin A1 expression between the two groups of plaques was also carried out using Students T-test. The Pearson correlation test was used to assess the correlation between echogenicity, macrophage numbers and Annexin A1 gene expression. Differences at p < 0.05 were considered significant.
Results

Characteristics of the patients

In this study, 10 and 24 patients were classified as asymptomatic and symptomatic patients, respectively. Carotid plaque imaging and quantification of plaque echogenicity was feasible in all patients. The characteristics of all patients are summarised in Table 1. There was no significant difference between asymptomatic and symptomatic patients in terms of gender, age and atherosclerotic risk factors, except the co-existence of ischaemic heart disease.

Table 1  Demographics and atherosclerosis risk factors of 34 carotid endarterectomy (CEA) patients.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Asymptomatic Patients (n = 10)</th>
<th>Symptomatic Patients (n = 24)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>10</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>Age (mean)</td>
<td>72.07</td>
<td>69.5</td>
<td></td>
</tr>
<tr>
<td>Age (range)</td>
<td>48–82</td>
<td>57–77</td>
<td></td>
</tr>
<tr>
<td>Male (%)</td>
<td>70%</td>
<td>75%</td>
<td></td>
</tr>
<tr>
<td>Smoking (%)</td>
<td>50%</td>
<td>50%</td>
<td></td>
</tr>
<tr>
<td>Hypertension (%)</td>
<td>70%</td>
<td>75%</td>
<td></td>
</tr>
<tr>
<td>Diabetes Mellitus (%)</td>
<td>30%</td>
<td>41.7%</td>
<td></td>
</tr>
<tr>
<td>Hyperlipidaemia (%)</td>
<td>50%</td>
<td>58.3%</td>
<td></td>
</tr>
<tr>
<td>Ischemic Heart Disease (%)</td>
<td>20%</td>
<td>75%</td>
<td></td>
</tr>
<tr>
<td>Peripheral arterial occlusive disease (%)</td>
<td>2%</td>
<td>16.7%</td>
<td></td>
</tr>
</tbody>
</table>

Characteristics of plaques and carotid artery walls

The ultrasonic plaque characteristics are stated in Table 2. The homogeneity of the plaque did not differ significantly between the two groups. The echogenicity of the studied carotid plaques was widely distributed, ranging from more echolucent to downright echogenic (range from 93 to 155). The symptomatic patients’ plaques were echolucent (mean GSM of 103) than those of asymptomatic patients (mean GSM of 126, p = 0.022). They also exhibited a higher degree of luminal stenosis (85% vs. 71.4%, p = 0.018).

Table 2  Plaque characteristics, carotid arterial lumen and wall thickness in patients with asymptomatic and symptomatic carotid stenosis disease.

<table>
<thead>
<tr>
<th>Carotid artery Parameter</th>
<th>Asymptomatic patients (n = 10)</th>
<th>Symptomatic patients (n = 24)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IMT (mm)</td>
<td>0.94 ± 0.3</td>
<td>1.02 ± 0.15</td>
<td>0.628</td>
</tr>
<tr>
<td>CCA lumen (mm)</td>
<td>5.33 ± 0.25</td>
<td>5.87 ± 0.48</td>
<td>0.172</td>
</tr>
<tr>
<td>ICA lumen (mm)</td>
<td>4.74 ± 0.4</td>
<td>4.92 ± 0.57</td>
<td>0.169</td>
</tr>
<tr>
<td>Stenosis (%)</td>
<td>71.4 ± 3.87</td>
<td>85 ± 6.33</td>
<td>0.018</td>
</tr>
<tr>
<td>GSM (echogenicity)</td>
<td>126 ± 7.18</td>
<td>103 ± 7.9</td>
<td>0.022</td>
</tr>
<tr>
<td>GSM-SD (homogenicity)</td>
<td>0.97 ± 2.7</td>
<td>46 ± 2.17</td>
<td>0.066</td>
</tr>
</tbody>
</table>

Quantification of macrophages

Morphometric analysis demonstrated that there were significantly more macrophages in symptomatic plaques as compared with asymptomatic plaques (Table 3, p = 0.01). This difference persisted even after the macrophage numbers were normalised to the plaque area (6 ± 1.11 vs. 15.4 ± 2, p < 0.01). The GSM score correlated with numbers of macrophages (Pearson coefficient r = −0.52, p < 0.05). More macrophages were therefore found in the more echolucent plaques.

Table 3  The number of macrophages found in asymptomatic and symptomatic plaque specimens.

<table>
<thead>
<tr>
<th>Type of plaque Specimens</th>
<th>n</th>
<th>No. of Macrophage</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) Asymptomatic</td>
<td>10</td>
<td>15 ± 8.5</td>
<td>–</td>
</tr>
<tr>
<td>(2) Symptomatic</td>
<td>24</td>
<td>20.5 ± 5</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Expression of Annexin A1 protein

Annexin A1 protein was expressed in all atherosclerotic plaques, particularly the plaques from asymptomatic patients. Fig. 1 shows the representative immunohistochemical staining pictures of Annexin A1 in asymptomatic plaques. In the control experiment, the Annexin A1 antibody was omitted in the staining procedures (×40) (Fig. 1 (a)). Prominent Annexin A1 immunoreactivity was expressed in asymptomatic plaques (×40) (Fig. 1(b)). Fig. 1 (c) shows Annexin A1 expression in symptomatic plaques (×100) and Fig. 1(d) shows the higher magnification image (×100) of Annexin I expressed in asymptomatic plaques.

Expression of Annexin A1 mRNA

Fig. 2 shows the representative gel photograph of Annexin A1 expression in both types of tissues. Fig. 3 shows the summary of the relative expression of Annexin A1 in all plaques specimens after normalisation with the β-actin expression from the same mRNA template. The plaque expression levels of the Annexin A1 gene in asymptomatic patients were significantly higher than symptomatic patients (87 ± 4% vs. 42 ± 6.2%, p < 0.001). The Annexin A1 gene was found to be inversely correlated with the numbers of macrophage detected in plaques (r = −0.46, p < 0.05) (Fig. 4).

Measurement of inflammatory cytokines

Among all studied cytokines levels (IL-1β, IL-6, IL-8 and IL-10) detected in the serum samples, only IL-6 was significantly increased in the serum of symptomatic patients as compared with that of asymptomatic patients (Table 4, 230 ± 15 vs. 90 ± 20 pg ml⁻¹, p < 0.001).

Discussion

The present study demonstrated for the first time that Annexin A1 is strongly expressed within the plaques obtained.
from patients with advancing carotid stenosis, illustrating the relationship between Annexin A1 and advanced atherosclerosis. Although atherosclerotic plaque instability is determined by multiple factors, the inflammatory pathways have a particularly significant role.21 Annexin A1 has been suggested to play an important part in anti-inflammation by engulfing apoptotic cells and allowing safe post-apoptotic clearance of dead cells.22 It also blocks the release of inflammatory mediators by directly inhibiting cytosolic phospholipase A214 and the expression and activity of other inflammatory mediators such as cyclooxygenase,15 IL-6,23 IL-10 and inducible nitric oxide synthase (iNOS).24 Thus, the higher Annexin A1 expression in plaques obtained from asymptomatic patients as compared with that in symptomatic patients may act as a local protective factor in response to the inflammatory stimulus that releases in the plaques. This action is hypothetically beneficial in stabilising the plaques in carotid stenosis patients.

Atherosclerosis is a chronic inflammatory disease occurring within the artery wall. Macrophages are an inflammatory feature of plaques and are found to be involved in all stages of plaque development. We stained macrophage cells in the plaque specimens, rather than studying the macrophage serologically. As most studies detected the macrophages in

**Figure 1** Immunohistochemical staining of Annexin I protein in all atherosclerotic plaques. In the control experiment, the Annexin I antibody was omitted in the staining procedures (A). More pronounced Annexin I, was found particularly in asymptomatic plaques (B) than symptomatic plaques (C). Figure D showing the higher magnification picture (×100) of Annexin I expressed in asymptomatic plaques.

**Figure 2** Representative RT-PCR gel photos showing the Annexin A1 mRNA expression in both asymptomatic and symptomatic plaque specimens. Higher Annexin A1 expression was found in asymptomatic plaques as compared with symptomatic plaques.

**Figure 3** The histogram shows the Annexin A1 mRNA expression expressed as means ± S.D of the 34 studied plaques. The statistical difference was $p < 0.001$ when comparing the two types of plaques.
patients’ blood as a biomarker measurement rather than the direct measurement of macrophage in the plaque for indicating the inflammation status, the current immunohistochemical analyses become a more direct and promising indication of inflammatory events in the carotid plaques, as the systemic release of inflammatory factors is probably not an exact reflection of vascular cell levels. Our findings are consistent with those of Pilarczyk et al. and Seshiah et al. which show that macrophages are found to a greater extent in symptomatic plaques than in asymptomatic plaques. Macrophages can weaken plaque stability by secreting matrix metalloproteinases and releasing inflammatory cytokines. The accumulation of macrophages has also been correlated with plaque ulceration and thrombosis and the degree of stenosis, and it would shift the balance of VSMC survival vs. apoptosis. These phenomena further confirm that there is an intense inflammatory response in our symptomatic plaques that may lead to plaque instability.

Inflammation and apoptosis are both critical factors related to the atherosclerotic process and plaque instability. Our results of the anti-inflammatory Annexin A1 expression with the Pearson correlation coefficient \( r = 0.501, p = 0.009 \), indicating the inflammation status, become a more direct and promising indication of inflammatory events in the carotid plaques, as the systemic release of inflammatory factors is probably not an exact reflection of vascular cell levels. Our findings are consistent with those of Pilarczyk et al. and Seshiah et al. which show that macrophages are found to a greater extent in symptomatic plaques than in asymptomatic plaques. Macrophages can weaken plaque stability by secreting matrix metalloproteinases and releasing inflammatory cytokines. The accumulation of macrophages has also been correlated with plaque ulceration and thrombosis and the degree of stenosis, and it would shift the balance of VSMC survival vs. apoptosis. These phenomena further confirm that there is an intense inflammatory response in our symptomatic plaques that may lead to plaque instability.

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present study is only an observation study. Thus, no causal relationship between enhanced Annexin A1 and atherothrombotic events in advanced carotid plaques can be found.

Our results of Annexin A1 expression levels with respect to symptomatology and echogenicity can provide an insight into the morphological correlation of plaque instability. Higher Annexin A1 expression was probably triggered to act as a protective factor against a series of inflammatory events to stabilise the plaques and prevent plaque rupture. This could explain partly why echogenic plaques in advanced stenosis patients without neurological symptoms have higher Annexin A1 expression. However, this is a single-centre study with relatively less patients involved. Recruiting more patients or conducting multicentre studies would be particularly useful to further validate the present observation. Hope future studies can be warranted to reveal new molecular targets for therapeutic applications in stabilising atherosclerotic plaques.

Conflict of Interest/Funding

None.

References


