Increased Endothelial Cell Apoptosis in Symptomatic High-grade Carotid Artery Stenosis: Preliminary Data

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Objective. Endothelial cell (EC) apoptosis has been associated with thrombus formation on an eroded atherosclerotic plaque surface. Alongside plaque rupture, it may constitute another mechanism of plaque destabilisation. We investigated whether EC apoptosis also may be involved in plaque destabilisation in high-grade internal carotid artery (ICA) stenosis.

Methods. We compared the degree of EC apoptosis in carotid endarterectomy specimens from n = 38 patients undergoing surgery for high-grade ICA stenosis (>70%); n = 19 clinically asymptomatic; n = 19 symptomatic). The total number of endothelial cells (ECs) and apoptotic cells were determined using CD31 immunohistochemistry and the TdT dUTP nick end-labeling (TUNEL) method respectively.

Results. Overall, EC apoptosis was a rare finding. The median percentage of apoptotic ECs was 0.0% (0.0–0.7%) in asymptomatic and 0.5% (0.0–7.3%) in symptomatic plaques (p = 0.015, Mann-Whitney U test). No difference was observed between ruptured and unruptured plaque (0.0% [0.0–6.0%] vs 0.0% [0.0–5.7%]; p = 0.446).

Conclusions. Our results indicate that TUNEL-detected EC apoptosis is rare in carotid plaque from patients with >70% stenosis.

Keywords: Apoptosis; Atherosclerosis; Carotid arteries; Stenosis.

Introduction

In high-grade ICA atherosclerotic stenosis, the most common pathological feature associated with symptom development is plaque surface disruption.1–4 However, the surfaces of a substantial percentage (29–67%) of plaque specimens taken from symptomatic patients were grossly intact, suggesting that there are other mechanisms that can also cause thromboembolism.3–7 Endothelial denudation (i.e. plaque erosion) has often been observed in carotid and coronary atherosclerotic lesions.8 The mechanisms behind this have not yet been completely understood, but could include an increased rate of EC apoptosis.9

Therefore we investigated the extent of EC apoptosis in patients with unstable, recently symptomatic, and patients with stable, asymptomatic high-grade ICA stenosis.

Methods

Patients

A total of 38 surgical inpatients on a list to undergo carotid endarterectomy for high-grade ICA stenosis (>70% according to the NASCET criteria10) were included prospectively in the study (see Table 1). A large proportion of these patients (32/38) had also been involved in a recently published study.11 All patients gave written informed consent. The study was approved by the ethics review committee of the University Hospital of Frankfurt.

Patients were classified as symptomatic if they reported retinal or cerebral transient ischaemic attacks or minor ischaemic strokes attributable to the high-grade ICA stenosis <60 days prior to surgery.

Preoperative treatment was similar for both symptomatic and asymptomatic patients (see Table 1).11

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Antiplatelet medication and oral anticoagulants were stopped at least six days prior to surgery. On the day of surgery, all patients exhibited normal routine coagulation parameters. Five minutes before clamping, they received anticoagulation with 100 IU heparin/kg bodyweight. This was then antagonised with 50 IU protamine/kg bodyweight ten minutes after declamping.

Histological sampling

All carotid plaques were excised en bloc. In the majority \((n = 33)\) of cases, the eversion technique was used so that the residual lumen was not incised; in the remaining cases, longitudinal arteriotomy was performed, providing plaque exclusively from the ICA, which could then be used for further analyses. After removal, the plaque was immediately fixed in 4% formalin, decalcified, and sectioned transversely at 2 mm intervals. Each 2 mm tissue block was embedded separately in paraffin. The quantitative analysis was based on all blocks derived from each individual plaque. The mean number \((\pm SD)\) of blocks examined per plaque was \(7.2 \pm 3.6\). The total number of blocks examined was \(279\). From each series of blocks, \(3 \mu m\) sections were obtained. Plaque rupture was defined as an intimal defect larger than \(1000 \mu m\) in width, exposing the necrotic core of the atheromatous plaque.\(^{11}\)

Immunohistochemistry

The endothelial layer was visualised by staining with CD31 (Dako Diagnostics). Sections were incubated with the primary antibody (diluted 1:200), followed by a biotinylated secondary antibody (Amersham Life Sciences) and an avidin and biotinylated horseradish peroxidase macromolecular complex (Vectorstain ABC Kit), and then visualised by 3,3 diaminobenzidine (see Fig. 1A).

In situ detection of apoptotic ECs

For the in situ detection of ECs, the TUNEL method was used according to the protocol described in our

\[\text{Table 1. Clinical characteristics of asymptomatic and symptomatic patients with high-grade ICA stenosis}\]

<table>
<thead>
<tr>
<th></th>
<th>Asymptomatic ((n = 19))</th>
<th>Symptomatic ((n = 19))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (mean (\pm SD)), years</td>
<td>70 (\pm 7)</td>
<td>62 (\pm 9)</td>
</tr>
<tr>
<td>Number of male patients</td>
<td>15</td>
<td>12</td>
</tr>
<tr>
<td>Luminal narrowing (mean (\pm SD), %)</td>
<td>88 (\pm 5)</td>
<td>79 (\pm 9)</td>
</tr>
<tr>
<td>Time elapsed since last ischaemic symptom:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(&lt;30) days, %</td>
<td>82</td>
<td></td>
</tr>
<tr>
<td>(30–60) days, %</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>Risk factors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arterial hypertension, %</td>
<td>70</td>
<td>61</td>
</tr>
<tr>
<td>Smoking, %</td>
<td>57</td>
<td>53</td>
</tr>
<tr>
<td>Diabetes, %</td>
<td>37</td>
<td>27</td>
</tr>
<tr>
<td>Hypercholesterolaemia*, %</td>
<td>35</td>
<td>36</td>
</tr>
<tr>
<td>Current medication</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antihypertensives, %</td>
<td>54</td>
<td>58</td>
</tr>
<tr>
<td>Lipid lowering drugs, %</td>
<td>22</td>
<td>14</td>
</tr>
<tr>
<td>Oral anticoagulants, %</td>
<td>4</td>
<td>10</td>
</tr>
<tr>
<td>Antiaggregants, %</td>
<td>65</td>
<td>70</td>
</tr>
</tbody>
</table>

\*Low density lipoprotein cholesterol >160 mg/dl, fasting.
\^Medication was routinely stopped at least six days prior to surgery.

\[\text{Fig. 1. Anti-CD31 staining of the endothelial cell (EC) layer in a high-grade internal carotid artery stenosis (ICA) from a symptomatic patient (A). Adjacent plaque section is used for TUNEL staining, revealing some positive ECs (black arrows in B). Vital endothelial cells stained are marked with blue arrows. Note that erythrocyte aggregation was frequently associated with EC apoptosis.}\]
previous study.\textsuperscript{11} We selected adjacent sections for immunohistochemistry and TUNEL staining (see Fig. 1B).

Quantification of EC apoptosis

Images of all adjacent plaque sections stained according to the above-mentioned methods were digitised at a magnification of 40× (using analySIS, Soft Imaging System, Muenster, Germany). ECs were considered apoptotic if they showed positive immunostaining with anti-CD31 antibody and positive TUNEL staining in an adjacent section. Slices stained with anti-CD31 were taken from each section of the plaque to determine the total number of ECs per plaque section. The cells were counted independently by two examiners (FT, CL) and the values given represent the mean of the two sets of results. To measure the degree of EC apoptosis, we first calculated the percentage of apoptotic ECs per plaque section, and then worked out the mean percentage of apoptotic ECs in each plaque by calculating the mean from all consecutive sections.

Statistical analyses

The quantitative values were compared using the non-parametric Mann-Whitney U test. As we performed consecutive statistical tests, \( \alpha \)-adjustment was applied according to the modified Bonferroni procedure. All quantitative analyses were performed blinded to clinical data.

Results

The major findings are shown in Table 2. Overall, EC apoptosis was a rare finding. Nevertheless, we found a significantly higher median mean percentage of apoptotic ECs in symptomatic than in asymptomatic patients \((p = 0.015)\). However, there was scant difference between the median average percentage of apoptotic ECs in ruptured and unruptured plaques. In the sub-group of unruptured plaques \((n = 21)\), the median average percentage of apoptotic ECs was 0.0 (0.0–3.0) and 0.3 (0.0–6.0) in asymptomatic and symptomatic patients respectively (not shown in Table 2).

Discussion

In this study although apoptotic ECs were rare, we found a marginally higher percentage of apoptotic ECs in unruptured symptomatic plaque causing high-grade ICA stenosis.

Despite the potential implications of EC apoptosis in advanced atherosclerosis, no study investigating the relationship between the degree of EC apoptosis and clinically relevant thromboembolic events has yet been published. However, increased rates of EC apoptosis have been observed in atherosclerotic ICA plaque,\textsuperscript{12} particularly downstream from the point of maximal stenosis. Local haemodynamics contribute largely to EC turnover and apoptotic death,\textsuperscript{10,13} and low shear stress due to turbulent flow downstream from the point of maximal stenosis appears to enhance EC apoptosis in this area of the plaque.\textsuperscript{12} Surprisingly, this potential mechanism of EC apoptosis does not explain the significant differences we identified between symptomatic and asymptomatic patients, as the degree of stenosis did not differ between the two groups (see Table 1). It is therefore likely that there are other mechanisms that promote EC apoptosis in symptomatic plaques.

Some studies\textsuperscript{14,15} have shown that increased expression of phosphatidylserine causes apoptotic ECs to become procoagulant and thus enhance the binding of non-activated platelets. Another study\textsuperscript{16} suggests that it can also boost circulating tissue factor activity, which contributes largely to blood thrombogenicity. Furthermore, in vivo induction of EC apoptosis can lead to thrombus formation and denudation of the endothelial layer.\textsuperscript{11} Intraluminal thrombus formation over an eroded atherosclerotic plaque surface without signs of plaque rupture is associated primarily with extensive endothelial denudation, which is not seen in advanced human atherosclerotic plaques without

| Table 2. Apoptosis of endothelial cells in ICA plaques in relation to clinical (asymptomatic/symptomatic) or morphological (unruptured/ruptured) features of plaque instability. Values given are medians and ranges, * Mann-Whitney U test plus \( \alpha \)-adjustment \((P < 0.05/3 < 0.017)\) indicates significant findings |
|-----------------|-----------------|--------|-----------------|-----------------|--------|
|                 | Asymptomatic \((n = 19)\) | Symptomatic \((n = 19)\) |        | Unruptured \((n = 21)\) | Ruptured \((n = 17)\) |        |
| Total number of apoptotic endothelial cells | 0 (0–4) | 2 (0–19) | 0.016* | 0 (0–18) | 0 (0–19) | 0.427 |
| Total number of vital endothelial cells | 295 (218–1137) | 506 (133–1272) | 0.191 | 456 (133–1137) | 323 (218–1272) | 0.612 |
| Median mean percentage of apoptotic endothelial cells per plaque | 0.00 (0.0–3.0) | 0.7 (0.00–6.0) | 0.015* | 0.00 (0.0–6.0) | 0.00 (0.0–5.7) | 0.446 |
thrombus formation.\textsuperscript{6,17} EC apoptosis may therefore represent an early stage in the development of thrombosed plaque.

This study has several limitations: First, we used transverse sections from the stenotic part of the plaque instead of longitudinal sections, thus presumably missing parts of the vessel located further downstream or upstream from the point of maximal stenosis. This, however, would be the case for both groups. The relative number of apoptotic ECs was surprisingly low, but in line with previous studies investigating EC apoptosis in carotid atherosclerosis. Tricot et al.\textsuperscript{12} found \(\leq 1\) apoptotic EC in 5 out of 13 plaques analysed. The mean percentage of apoptotic ECs was 2.7 ± 1.2 and 18.8 ± 3.3 in the upstream and downstream parts of the plaques respectively. Second, TUNEL positivity usually indicates a late stage of the apoptotic process. Finally, the number of plaques used was few and differences observed are marginal.

Since TUNEL-detected apoptosis was such a rare event in the high grade carotid plaques studied, it may not be a useful tool to probe the endothelial mechanisms that contribute to plaque stability.

Acknowledgments

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References