Relation Between Endothelial Cell Apoptosis and Blood Flow Direction in Human Atherosclerotic Plaques

Olivier Tricot, MD; Ziad Mallat, MD, PhD; Christophe Heymes, PhD; Joël Belmin, MD; Guy Lesèche, MD; Alain Tedgui, PhD

**Background**—Blood flow characteristics influence endothelial cell apoptosis. However, little is known about the occurrence of endothelial cell apoptosis in human atherosclerosis and its relation to blood flow.

**Methods and Results**—A total of 42 human carotid atherosclerotic plaques were retrieved by endarterectomy; they were examined in the longitudinal axial direction. Plaques were included in this study when upstream and downstream parts were clearly visible, occlusion was absent, and immunostaining for luminal endothelium was present all along the plaque. Using these criteria, 13 plaques were processed for further immunohistochemical studies (using anti-CD31, anti-Ki-67, and anti-splicing factor antibodies) and in situ detection of apoptosis (terminal dUTP nick end-labeling and ligase assay). Eight plaques showed ≥1 apoptotic endothelial cell at the luminal surface. Quantitative analysis of endothelial cell apoptosis in these plaques showed a systematic preferential occurrence of apoptosis in the downstream parts of plaques, where low flow and low shear stress prevail, in comparison with the upstream parts (18.8±3.3% versus 2.7±1.2%, respectively, P<0.001). Endothelial cell apoptosis was barely detectable in plaque microvessels.

**Conclusions**—Our results suggest that in vivo local shear stress influences luminal endothelial cell apoptosis and may be a major determinant of plaque erosion and thrombosis. (Circulation. 2000;101:2450-2453.)

**Key Words:** blood flow ■ stress, mechanical ■ atherosclerosis ■ apoptosis ■ endothelium

High laminar blood flow is probably the most potent endogenous antiatherosclerotic factor, as illustrated by the focal distribution of atherosclerotic lesions in areas with low or turbulent flow.1-2 These areas are characterized by increased endothelial cell turnover rates,3 suggesting increased cell death. This view is supported by recent studies showing that endothelial cells cultured under static conditions undergo apoptosis, whereas normal levels of shear stress are protective.4-6 Taken together, these data suggest a mechanistic link between low shear stress, endothelial cell apoptosis, and the susceptibility to plaque development.7 Endothelial cell death by apoptosis may also participate in plaque disruption and thrombosis. Exposure of the subendothelium to blood flow promotes platelet aggregation and vaso spasms, and it was recently shown that apoptotic endothelial cells exhibit marked procoagulant activities and become proadhesive, even for nonactivated platelets.8,9

Despite the major potential implications of endothelial cell apoptosis in atherosclerotic plaque development and stability, little is known about the occurrence of endothelial cell apoptosis in human atherosclerosis and its relation to blood flow. We used the carotid endarterectomy technique, which preserves the en bloc plaque structure, to investigate the occurrence and distribution of endothelial cell apoptosis in human plaques examined in the longitudinal axial direction, upstream and downstream from the stenosis, where shear stress varies dramatically.10,11

**Methods**

A total of 42 human atherosclerotic plaques that were removed from 42 patients undergoing en bloc carotid endarterectomy were collected. The plaques were immediately fixed in 4% paraformaldehyde and frozen in mounting medium (OCT Compound, Miles Inc, Diagnostics Division). Blood flow direction was indicated on the mounting support. Longitudinal cryostat sections (5 to 6 μm) of the whole plaque were obtained in a direction parallel to the long axis of the artery. The upstream part of the plaque was defined as the area between the beginning of the plaque and the site of maximal stenosis. The downstream part was defined as the area between the site of maximal stenosis and the end of the plaque. Plaques were included in this study when upstream and downstream parts were clearly visible, occlusion was absent, and immunostaining for luminal endothelium was present all along the plaque. We excluded plaques with erosion or rupture from the main analysis because we could not determine whether the rupture occurred in vivo or during the surgical manipulation of the specimen. Thirteen plaques fulfilled the inclusion criteria mentioned above. Figure 1 shows a typical longitudinal section of a human carotid plaque.

Adjacent sections were stained for endothelial cells with an antibody against CD31 (clone JC/70A, DAKO, dilution 1:20), for proliferation with an antibody against Ki-67 (clone MIB-1, DAKO, dilution 1:100), and for RNA transcription with an antibody...
against “splicing factor” (clone SC-35, DAKO, dilution 1:100). Negative controls were performed with an irrelevant immunoglobulin.

In situ detection of apoptosis was performed using 2 different techniques: the TdT dUTP nick end-labeling (TUNEL) method and the in situ ligase assay. Negative controls were performed by omitting the enzyme TdT (for TUNEL) or the T4 DNA ligase (for the ligase assay); positive controls were performed by pretreating the sections with 10 U/mL DNase I for 20 minutes at 37°C.

An endothelial cell was considered apoptotic when the following criteria were fulfilled: positive immunostaining with anti-CD31 antibody, positive staining with the TUNEL technique, and negative immunostaining with anti-Ki-67 and anti-splicing factor antibodies. Luminal endothelial cells considered apoptotic were counted in the upstream and downstream parts of the plaques by an observer who was unaware of the blood flow direction. For each stain, 3 slides (3 tissue sections/slide) taken from different areas of the plaque were used for quantitative analysis.

The results are expressed as means ± SEM. A paired Student’s t test was used to compare the differences between the upstream and downstream parts of the atherosclerotic plaques.

Figure 1. Typical longitudinal section of a carotid plaque showing upstream and downstream parts.

Figure 2. Representative photomicrographs showing immunostaining with anti-CD31 antibody (A, D), staining with the TUNEL technique (B, E), and the ligase reaction (C, F) in the prestenotic (A through C) and poststenotic (D through F) parts of an atherosclerotic plaque. There was clear evidence for a preferential apoptosis of endothelial cells in the poststenotic area. Erythrocyte aggregates were frequently seen at the contact of apoptotic endothelial cells. To visualize the blue staining of the ligase reaction, counterstaining with hematoxylin was omitted.
downstream areas, and $P<0.05$ was considered statistically significant.

Results

Of the 13 plaques included in the main analysis, 8 showed $\geq 1$ apoptotic endothelial cell at the luminal surface. Endothelial cell apoptosis in these plaques showed a systematic preferential occurrence of apoptosis in the downstream part of the plaque (Figure 2). Results obtained with the ligase assay in 4 different plaques were very similar to those obtained with the TUNEL technique (Figure 2). We counted $360\pm 64$ luminal endothelial cells in the upstream parts of plaques and $331\pm 68$ cells in the downstream parts. Quantitative analysis of luminal endothelial cell apoptosis showed a markedly elevated number of apoptotic cells in the downstream parts of plaques compared with the upstream parts ($69.3\pm 16.0$ versus $10.4\pm 3.9$ cells, respectively). This corresponded to $18.8\pm 3.3\%$ apoptotic cells in the downstream areas and to $2.7\pm 1.2\%$ apoptotic cells in the upstream areas ($n=8$, $P<0.001$) (Table). To rule out any possibility of selection bias due to the exclusion of eroded or ruptured plaques, we analyzed the occurrence of apoptosis in the remaining luminal endothelial cells of these plaques ($n=20$). Again, we found the same pattern of distribution of apoptotic endothelial cells ($20.4\pm 3.9$ apoptotic cells in the downstream part versus $3.7\pm 1.2$ apoptotic cells in the upstream part, $P<0.001$).

Endothelial cell apoptosis was barely detectable in plaque microvessels, whether located in the upstream or downstream part of the plaque (Figure 3). Positive staining with anti-Ki-67 antibody was rarely observed in luminal endothelial cells. No staining with anti-splicing factor antibody was detected.

Discussion

Despite the large body of evidence suggesting that apoptosis is a crucial event in atherosclerosis,13–15 no specific study has directly examined the occurrence of endothelial cell apoptosis in human plaques and the potential mechanisms involved.

Using carotid human atherosclerotic plaques, which are validated models to study interactions between low or oscillatory shear stress and atherosclerosis,10,11 we provide the first in vivo evidence that blood flow directly influences endothelial cell survival or apoptosis in human atherosclerosis. Luminal endothelial cell apoptosis was observed in 60% of the plaques examined. In these plaques, we found a 7-fold increase in endothelial cell apoptosis in the downstream parts of plaques, where low shear prevails,10,11 in comparison with the upstream parts. The absence of apoptosis in 5 of the 13 plaques might be accounted for by counter-regulatory mechanisms of apoptosis, differences in local hemodynamics, or a short window of apoptotic death.

The lack of previous studies (including ones from our group) showing endothelial cell apoptosis in atherosclerotic plaques may be due to the use of transverse tissue sections taken almost exclusively from the most stenotic area of the plaque instead of the use of longitudinal tissue sections that allow for the examination of the entire plaque. Despite the abundance of plaque microvessels in both upstream and downstream parts, all sections showing microvessels were free of apoptosis, regardless of their location. In addition, we found that, contrary to the results in luminal endothelial cells, apoptosis within the plaque was significantly higher in the upstream part than in the downstream part.16 Taken together, these findings indicate that luminal endothelial cell apoptosis

<table>
<thead>
<tr>
<th>Counted endothelial cells</th>
<th>Upstream</th>
<th>Downstream</th>
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<tbody>
<tr>
<td></td>
<td>$360\pm 64$</td>
<td>$331\pm 68$</td>
</tr>
<tr>
<td>Apoptotic cells</td>
<td>$10.4\pm 3.9$</td>
<td>$69.3\pm 16.0$</td>
</tr>
<tr>
<td>Percentage of apoptotic cells</td>
<td>$2.7\pm 1.2$</td>
<td>$18.8\pm 3.3^*$</td>
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Values are expressed as means±SEM. $^*P<0.001$ ($n=8$).
does not reflect a general apoptotic process occurring down-
stream from maximal stenosis, but most likely depends on
local blood flow hemodynamics.

Our results agree with previous in vitro studies showing
that laminar shear stress protects endothelial cells from
apoptotic death.4,17,18 Several mechanisms have been pro-
posed to account for the antiapoptotic effects of laminar shear
stress, including upregulation of superoxide dismutase19 and
Akt-mediated activation of nitric oxide synthase, with subse-
quent inhibition of the caspase cascade.20

Our study suggests that relatively large areas of endo-
thelial erosion may occur in the distal part of atheroscle-
rotic plaques. Given the high procoagulant and proadhe-
sive potentials of apoptotic endothelial cells and the
propensity of denuded vessel segments to platelet aggrega-
tion and vasospasm, this may lead to lumen thrombosis
favoring plaque progression or the occurrence of acute
coronary syndromes. Recently, Farb et al21 identified
superficial endothelial erosion without plaque rupture as
an important predisposing substrate for lumen vessel
thrombosis, acute coronary syndromes, and sudden death.
Our results suggest that in vivo local shear stress influ-
ences luminal endothelial cell apoptosis and may be a
major determinant of plaque erosion and thrombosis.

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