In Vivo Induction of Endothelial Apoptosis Leads to Vessel Thrombosis and Endothelial Denudation
A Clue to the Understanding of the Mechanisms of Thrombotic Plaque Erosion

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Background—The mechanisms of thrombosis on plaque erosion are poorly understood. We examined the potential role of endothelial apoptosis in endothelial erosion and vessel thrombosis.

Methods and Results—Segments of New Zealand White rabbit femoral arteries were temporarily isolated in vivo. One artery was incubated with staurosporin for 30 minutes, whereas the contralateral artery was incubated with saline and served as control. Three days later, thrombosis was evaluated angiographically and histologically. TUNEL score in the endothelial layer was significantly increased in staurosporin-treated arteries compared with controls (2.43 ± 0.30 versus 0.93 ± 0.44, respectively; \( P = 0.001 \)). Large areas of endothelial denudation were detectable in staurosporin-treated vessels, whereas endothelium integrity was almost preserved in the saline group. Vessel thrombosis occurred in 58% of staurosporin-treated arteries (7 of 12) but in only 8% of saline-treated segments (\( P < 0.01 \)). Immunoreactivities for tissue factor, platelets, and fibrin were detectable within the thrombus. Addition of ZVAD-fmk (0.1 mmol/L) significantly reduced the occurrence of thrombosis (1 of 7 arteries or 14%, \( P = 0.04 \)). These results were confirmed in balloon-injured atheromatous arteries.

Conclusions—In vivo induction of endothelial apoptosis leads to both vessel thrombosis and endothelial denudation. Endothelial apoptosis may be a critical step in the transition from a stable endothelialized plaque to plaque erosion and thrombosis. (Circulation. 2004;109:2503-2506.)

Key Words: endothelium • apoptosis • atherosclerosis • thrombosis

Besides thrombotic plaque rupture, Virmani et al. identified noninflammatory plaque erosion as responsible for almost 30% to 40% of thrombotic coronary sudden death. In this case, thrombus is formed on a denuded endothelial plaque surface and is in direct contact with activated smooth muscle cells. Despite continuous progress in the understanding of the risk factors that predispose to thrombotic plaque erosion, including the potentially important role of blood thrombogenicity, the pathophysiological mechanisms leading to both superficial endothelial denudation and formation of a platelet/fibrin-rich thrombus remain poorly understood. Recently, we have reported a significant increase in the occurrence of apoptotic death in luminal endothelial cells located in low-shear-stress areas of advanced human carotid atherosclerotic plaques. Because apoptotic endothelial cells promote thrombin generation and platelet adhesion, we hypothesized that in vivo occurrence of endothelial apoptosis may initiate thrombus formation and lead, as expected, to endothelial denudation (by detachment of apoptotic cells), reproducing the 2 major features of thrombotic plaque erosion. This hypothesis was tested in segments of rabbit femoral arteries incubated with staurosporin or placebo.

Methods

Experimental Protocol
New Zealand White rabbits (n = 12; CEGAV, St Mars d’Egremne, France) were anesthetized with xylazine (5 mg/kg) and ketamine (35 mg/kg). Ligatures were used to isolate a femoral artery segment (1 cm long) that was punctured proximally by a 27-gauge needle and incubated randomly with either staurosporin (10^{-5} mol/L) on one side (n = 12) or saline on the contralateral side (n = 12). After 30 minutes of incubation, femoral segments were washed twice with saline and reexposed to the circulating blood for 3 days. An additional 4 rabbits were used, and femoral segments (n = 8) were preincubated for 10 minutes with ZVAD-fmk (0.1 mmol/L), a broad inhibitor of caspases, before the 30-minute staurosporin incubation. Animal procedures received institutional approval and conformed to...
the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health.

In additional experiments, the staurosporin-versus-saline studies were performed on atherosclerotic-like lesions induced in femoral arteries of rabbits (n=16) by the combination of air desiccation and high-cholesterol diet (30 days), as previously described.8

Assessment of Vessel Thrombosis and Immunohistochemistry

Arteries underwent fixation with 4% buffered paraformaldehyde and were embedded in OCT medium and stored at −70°C. Each femoral artery was cut in serial sections (8 to 10 μm), at sites 1 to 2 mm apart, from the proximal to the distal end. At least 8 sections per animal were used for immunostaining. Thrombus was defined histologically as an adherent, fibrin-positive intraluminal material responsible for partial or total lumen occlusion. Sections were stained with the following antibodies: a monoclonal anti-CD31 antibody (Dako), a monoclonal anti-rabbit tissue factor (TF) antibody (American Diagnostica), a monoclonal anti-GP1b antibody (Beckman Coulter), a monoclonal anti-macrophage antibody (RAM-11; Dako), a biotinylated rabbit antibody against human fibrin (Dako), or a biotinylated antibody against mouse caspase-3 (Cell Signaling).

In situ detection of apoptotic cells was performed using TUNEL. A semiquantitative apoptosis score (0 through 3) evaluating the extent of apoptosis in the endothelial lining was established by a blinded investigator, as follows: 0 indicated no or barely detectable staining; 1, weak positive staining; 2, moderate limited staining; and 3, strong diffuse staining. Areas where endothelial cell lining was absent (negative CD31 staining) were considered as resulting from apoptosis-induced endothelial denudation and were counted as apoptotic (score 3). In addition, we have quantified the absolute number of endothelial cells (total and apoptotic) per arterial section in staurosporin-treated vessels with (n=7) or without (n=7) ZVAD-fmk. A thrombotic score was also used to evaluate the severity of the thrombotic response, as follows: 0 indicated no thrombosis; 1, a thrombus with less than 50% of lumen vessel obstruction; 2, between 50% and 75%; and 3, more than 75%.

Statistical Analysis

Results are expressed as mean±SEM. Differences in thrombus formation between groups were compared using the χ² test. One-way ANOVA was used to identify group differences with regard to the extent of TUNEL staining.

Results

We first examined the occurrence of thrombus in staurosporin-treated animals. We found a 7-fold increase in vessel thrombosis after staurosporin treatment compared with controls (7 of 12 arterial segments or 58.3% versus 1 of 12 or 8.3%, respectively; P<0.01) (Figure 1). Complete occlusion occurred in 43% of thrombosed vessels (3 of 7). Nonocclusive thrombi occupied 35% to 63% of the lumen vessel area. Thrombi were rich in fibrin (Figure 1) and stained positive for GP1b and TF (Figure 1). Staurosporin treatment was associated with an increase in TUNEL positivity in luminal endothelial cells compared with controls (2.43±0.30 versus 0.93±0.44, respectively; P=0.001). In addition, staurosporin-treated vessels showed large areas of endothelial denudation, whereas endothelial integrity was preserved in control segments (Figure 2), indicating a contribution of apoptosis to endothelial denudation. TUNEL-positive endothelial cells were detected at the interface between the thrombus and the vessel wall (Figure 2), suggesting a relationship between induction of apoptosis and thrombus formation. TUNEL staining was confirmed by positive staining against active caspase-3 (data not shown). TF staining in thrombosed vessels was diffuse within the thrombus but could also be seen in nondamaged endothelial cells (Data Supplement Figure). Addition of ZVAD-fmk (0.1 mmol/L; n=8) to staurosporin-treated vessels significantly inhibited both the extent of endothelial apoptotic score (1.25±0.25,
The mechanisms leading to the 2 major characteristics of plaque erosion, ie, endothelial denudation and platelet/fibrin-rich thrombotic occlusion, are still poorly understood. The fibrin component of the thrombus is intriguing. Indeed, in the absence of deep injury, subendothelial active TF is barely detectable and would not lead to the formation of a fibrin-rich adherent thrombus. The second enigma is endothelial denudation per se. It is unlikely that this process occurs before thrombus formation, because large areas of spontaneous endothelial denudation, such as those reported in thrombosed plaque erosion, are not seen even in advanced human atherosclerotic plaques. Therefore, one has to assume the presence of a process leading to the formation of a platelet/fibrin-rich thrombus on a nondenuded luminal endothelium. We hypothesized that endothelial apoptosis may be a clue to the understanding of both thrombus formation and endothelial denudation.

Several authors have shown that apoptotic cells become procoagulant in part because of increased expression of phosphatidylserine. In addition, apoptotic endothelial cells show a marked increase in the binding of nonactivated platelets and could contribute to platelet incorporation within the thrombus. In the present study, we have shown that induction of vascular endothelial cell apoptosis led to platelet/fibrin-rich thrombus formation and endothelial denudation. Both processes were significantly inhibited by addition of a broad caspase inhibitor. Although caspase activation may be involved in processes other than apoptosis, our findings that ZVAD-fmk reduced both endothelial TUNEL positivity and denudation suggest a role for endothelial apoptosis in the complex pathophysiological process leading to thrombotic erosion in the present study.

Kolodgie et al recently reported a specific accumulation of hyaluronan and CD44 at sites of plaque erosion. This finding fits well with our hypothesis given the increased propensity of endothelial cells to apoptosis when cultured on hyaluronan substrates. Moreover, significant apoptosis of luminal endothelial cells has been reported in advanced human atherosclerotic plaques, and a finding compatible with an initiating role for endothelial apoptosis in plaque erosion. Apoptotic endothelial cells and microparticles exposing phosphatidylserine could play a significant role in the activation of circulating TF, which has been identified as a major contributor to blood thrombogenicity. Interestingly, increased levels of circulating endothelial-derived microparticles have been reported in patients with unstable angina or myocardial infarction, suggesting a contribution of endothelial apoptotic injury to these acute processes. Taken together, these results provide evidence for a central role of endothelial apoptosis in the process leading to plaque erosion. It should be noted, however, that our results do not exclude the contribution of other nonapoptotic forms of endothelial injury or distinct prothrombotic mechanisms to plaque erosion (ie, activation/apoptosis of circulating blood cells, a prothrombogenic subendothelial surface, or hyperlipidemia). Moreover, because ZVAD-fmk was added before apoptosis induction, no therapeutic implications can be inferred from our results. Finally, caution is needed before extrapolating

Discussion

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these results, obtained in a rabbit model, to a more complex chronic atherosclerotic disease.

In conclusion, in vivo induction of endothelial cell apoptosis leads to both thrombus formation and endothelial denudation and may be a critical factor in the transition from a stable plaque phenotype to thrombotic plaque erosion.

Acknowledgments
This work was supported by grants from Fondation de France and Fondation pour la Recherche Médicale, France, and by grant OPAL from Sanofi-Synthelabo.

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_Circulation_. 2004;109:2503-2506; originally published online May 17, 2004;
doi: 10.1161/01.CIR.0000130172.62481.90

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2004 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

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http://circ.ahajournals.org/content/109/21/2503

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