Clinical Investigation and Reports

Shed Membrane Microparticles With Procoagulant Potential in Human Atherosclerotic Plaques
A Role for Apoptosis in Plaque Thrombogenicity

Ziad Mallat, MD; Bénédicte Hugel, PhD; Jeanny Ohan, PhD; Guy Lesèche, MD; Jean-Marie Freyssinet, PhD; Alain Tedgui, PhD

Background—The specific role of apoptosis in human atherosclerosis remains unknown. During apoptotic cell death, phosphatidylserine exposure on the cell surface confers a high tissue-factor (TF)–dependent procoagulant activity.

Methods and Results—In this study, we examined the role of apoptotic cell death in the promotion of plaque thrombogenicity. TF expression and its relation to apoptosis was analyzed in 16 human atherosclerotic plaques by the use of immunohistochemical techniques. The presence of shed membrane apoptotic microparticles was analyzed in extracts from 6 human atherosclerotic plaques and 3 underlying arterial walls. The microparticles were captured by annexin V and their amounts estimated with respect to their phospholipid content by use of a prothrombinase assay. The prothrombogenic potential of the microparticles was further assessed by the measurement of total and microparticle-dependent TF activity in the extracts. The cell origin of the microparticles was determined after capture by specific antibodies. We were able to detect marked TF expression in the plaques in close proximity to apoptotic cells and debris, suggesting a potential interaction between TF and the apoptotic cell surfaces. High levels of shed membrane apoptotic microparticles were detected in extracts from atherosclerotic plaques but not in the underlying arterial walls (29.5±3.7 nmol/L phosphatidylserine equivalent versus 1.3±0.4 nmol/L, respectively, P<0.02). The microparticles were mainly of monocytic and lymphocytic origin and retained 97±2% of total TF activity, indicating a direct causal relationship between shed membrane microparticles and procoagulant activity of plaque extracts.

Conclusions—These results indicate that shed membrane microparticles with procoagulant potential are produced in human atherosclerotic plaques. Apoptosis could be a critical determinant of plaque thrombogenicity after plaque rupture.

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Key Words: apoptosis ■ thrombosis ■ atherosclerosis

Apoptosis is a process of cell death that occurs during the development and progression of human atherosclerotic plaques.1–3 Much interest has focused on the regulation of apoptosis in atherosclerosis and plaque-derived cells,1,3,6–8 but only speculations have been raised about the potential role, beneficial or harmful, of this local process of cell death in atherosclerosis. Death of macrophages and T lymphocytes by apoptosis, a process with no or limited inflammatory response, might be viewed as beneficial. On the other hand, apoptotic death of smooth muscle cells, which may weaken the fibrous cap and contribute to plaque rupture, can be considered as harmful. However, there is no direct evidence to support these speculations. Recent studies have shown that apoptotic cells and microparticles exhibit marked procoagulant activity.9–14 This consequence of apoptosis is potentially deleterious in atherosclerosis because it might enhance plaque thrombogenicity after plaque rupture and lead to acute ischemic events and infarction.

Defined components of human atherosclerotic plaques are highly thrombogenic15,16 and express high levels of tissue factor (TF).16–20 Active TF is also present in atherosclerotic plaques.21,22 However, the exact mechanism(s) responsible for the enhanced TF activity and the enhanced prothrombogenic properties of certain plaque components are still poorly understood. TF activity is operational on the surface of cell membranes and it is highly dependent on the presence of anionic phospholipids, chiefly phosphatidylserine.23–27 Interestingly, phosphatidylserine is redistributed to the external membrane layer during apoptotic cell death28 and confers a procoagulant activity to the apoptotic cell surface.9,10 Recently, we have shown11,29 that shed membrane microparticles arising from cell fragmentation during apoptosis also retain procoagulant properties. However, it is not known whether shed membrane microparticles are produced in atherosclerotic plaques or whether they are implicated in the

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local activation of TF. This study was undertaken to examine (1) TF expression in apoptotic regions of human atherosclerotic plaques, (2) the presence of shed membrane microparticles in human atherosclerotic plaques, and (3) the procoagulant potential of these microparticles. In this study, we provide direct evidence that shed membrane apoptotic microparticles are produced in human atherosclerotic plaques and retain membrane-associated procoagulant activities. Apoptosis may therefore play a major role in the amplification of acute thrombotic events after plaque rupture.

Methods

Materials
Twenty-two human atherosclerotic plaques, removed from 20 patients undergoing carotid endarterectomy and 2 patients undergoing resection of abdominal aortic aneurysm, were collected. Sixteen plaques were processed for immunohistochemical studies. A piece from the most stenotic area of each arterial specimen was immediately placed for 2 hours in fresh 4% paraformaldehyde, then transferred to a 30% sucrose-PBS solution before being embedded in paraffin. Other adjacent segments of the stenotic area were snap frozen in optimal cutting temperature tissue processing medium (O.C.T. Compound, Miles Inc., Diagnostics Division) with liquid nitrogen and stored at −80°C for cryostat sectioning. For each specimen, several 5- to 6-μm sections were obtained for histological analysis, in situ detection of apoptosis, and immunohistochemical studies.

Six freshly removed advanced (complicated) human atherosclerotic plaques and 3 underlying, macroscopically normal, arterial walls were used for extraction of apoptotic microparticles.

In Situ Detection of Apoptotic Cell Death
In situ detection of apoptotic cells was performed on cryostat sections by the use of terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling of fragmented DNA (TUNEL method), as previously described. To enhance the specificity of the staining, prefixation time was abrogated and treatment of sections with proteinase k was omitted.

Immunohistochemistry
Cryostat sections were incubated with 1:10 normal horse serum for 30 minutes at room temperature, washed once in PBS, then incubated with a primary mouse monoclonal antibody against TF (anti-human TF, type 2, Calbiochem) at a dilution of 5 µg/mL. After they were washed in PBS, the slides were incubated with a secondary biotinylated horse anti-mouse IgG (Vector Laboratories, Inc.) at a dilution of 1:200. Immunostains were visualized with the use of avidin-biotin peroxidase (brown staining) or alkaline phosphatase (red color) visualization systems (Vectastain ABC Kit, standard and elite, Vector Laboratories). For negative controls, adjacent sections were stained with mouse IgG instead of the primary antibody. For double staining with TUNEL, sections were first stained with the specific anti-TF antibody as described above before they were processed for in situ detection of apoptosis by use of TUNEL.

Extraction of Apoptotic Microparticles From Arterial Tissue
Six freshly removed complicated atherosclerotic plaques were abundantly rinsed in different cold HBSS solutions supplemented with glucose, amino acids, and antibiotics (100 IU/mL penicillin and 100 µg/mL streptomycin). Under a sterile hood and on ice, the atherosclerotic plaque was rapidly separated from the underlying arterial wall and minced (200 mg of tissue from each plaque). The microparticles were released from the minced plaque by use of a previously described enzymatic digestion procedure for extraction of plaque-derived cells. The released cells were pelleted at 500g and the supernatant centrifuged at 12 000g for 1 minute. The remaining supernatant containing the microparticles was stored at −80°C for further analysis. The underlying, and macroscopically normal, arterial wall (200 mg of tissue) from 3 of these plaques was processed in an identical manner and served as control.

Detection of Apoptotic Microparticles
Microparticles were captured by immobilized annexin V as previously described. In brief, annexin V was biotinylated (annexin V ) and then insolubilized onto streptavidin-coated microtitration plates. After incubation for 30 minutes at room temperature, the plates were washed 3 times with TBS-CaCl2. Plates or underlying arterial wall supernatant was then added and remained in contact with insolubilized annexin V for 30 minutes at room temperature in the presence of 10 mmol/L CaCl2 added to HBSS. After the plates were washed, their anionic phospholipid content was determined by prothrombinase assay. In the assay, the blood clotting factor and calcium concentrations (factor Xa, factor V(a), prothrombin, and CaCl2) were determined to ensure that phosphatidylserine is the rate-limiting parameter of the reaction. After a 2-hour incubation at 37°C, the reaction was stopped by addition of 5 mmol/L EDTA. After addition of Chromozym TH, a chromogenic substrate for thrombin, linear absorbance changes were recorded at 405 nm by the use of a microtitration plate reader equipped with kinetics software. Results were expressed as nanomolar phosphatidylserine equivalent by reference to a standard curve constructed by the use of liposomes of defined composition.

Antigenic Capture and Characterization of Released Particles
Biotinylated antibodies (anti-CD11a, anti-CD4, and anti-CD8) were insolubilized onto streptavidin-coated microtitration plates. After incubation for 30 minutes at room temperature, the plates were washed 3 times with TBS-CaCl2. Plaque or underlying arterial wall supernatant was then added and incubated for 2 hours at room temperature before the plates were again washed 3 times with TBS-CaCl2. Microparticle detection was achieved by prothrombinase assay as described above. Background values obtained with irrelevant IgG were subtracted from those measured with specific monoclonal antibodies (mAbs).

Determination of Total and Microparticle-Dependent TF Activity
TF was measured in the whole supernatants before and after removing shed membrane microparticles by ultracentrifugation at 400 000g, 90 minutes, 20°C. TF activity was determined through its ability to promote the activation of factor X by factor VII(a) in the presence of CaCl2, exactly as described by Satta et al. The reaction proceeded for 30 minutes at 37°C and was stopped by addition of an excess of EDTA. Chromogenic substrate for factor Xa, S-2765, was added and the change in absorbance at 405 nm versus time was immediately recorded by the use of a microtitration plate reader equipped with kinetics software. Results were expressed as nanomolar of factor Xa generated in the assay by reference to a standard curve constructed with known amounts of factor Xa.

In control experiments, inhibition of TF was fully achieved by a specific mAb added at a final concentrations of 1 µg/mL and 6 µg/mL just before factors VII and X.

Statistical Analysis
Results are expressed as mean±SEM. Comparisons between groups were made by use of the Mann-Whitney U test. P<0.05 was considered statistically significant.

Results

Association of TF and Apoptosis in Human Atherosclerotic Plaques
We first examined the relation between TF expression and apoptosis in 16 human carotid and abdominal aortic athero-
sclerotic plaques. We consistently detected high levels of TF expression in acellular regions of plaques. Analyzing serial adjacent sections and double-stained sections, we found that these acellular areas were also strongly positive for TUNEL (Figure A), suggesting that they are the result of extensive apoptotic cell death. Positive staining for TUNEL in these acellular areas suggests that they might be rich in shed membrane apoptotic microparticles stemming from apoptotic cells. Therefore, TF may be present in close contact with phosphatidylserine in these areas, a condition that would considerably enhance its activity.23–25

Apoptosis also occurs in cellular regions of atherosclerotic plaques.2–7 Analysis of serial adjacent sections and double-stained sections showed that there is significant TF expression in these apoptotic regions (Figure B and C). Interestingly, significant extracellular staining for TF was observed in these areas (Figure B and C), suggesting that TF is released from the cell, possibly in apoptotic microparticles, during cell death. No staining was observed in sections probed with irrelevant IgG (Figure D).

**Presence of Shed Membrane Microparticles With Procoagulant Potential in Atherosclerotic Plaques**

On the basis of these results, we hypothesized that shed membrane microparticles bearing phosphatidylserine may be produced in the plaque, and that these microparticles may retain significant TF activity and therefore be highly thrombogenic. To examine the presence of microparticles in human atherosclerotic tissue, 6 fresh atherosclerotic plaques were obtained after carotid endarterectomy and were enzymatically digested by use of an already described procedure for isolation of plaque-derived cells.31 Underlying arterial walls of 3 of these plaques served as controls. Levels of shed membrane microparticles were then determined with respect to their procoagulant phospholipid content by a prothrombinase assay. We detected high levels of phosphatidylserine-
Levels of Shed Membrane Particles and Total TF Activity in 6 Human Atherosclerotic Plaques and 3 Underlying Arterial Walls

<table>
<thead>
<tr>
<th></th>
<th>Plaques (n=6)</th>
<th>Underlying Arteries (n=3)</th>
<th>P</th>
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<tbody>
<tr>
<td>Particles</td>
<td>29.5±3.7</td>
<td>1.3±0.4</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>Total TF activity*</td>
<td>8.3±4.3</td>
<td>0.9±0.1</td>
<td>&lt;0.03</td>
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(See Methods.)

*97±2% of total TF activity was lost after removing the microparticles by ultracentrifugation.

bearing microparticles in the 6 atherosclerotic plaques but very low levels in the 3 underlying arterial walls (29.5±3.7 nmol/L phosphatidylserine equivalent [23.2 to 45.9 nmol/L] versus 1.3±0.4 nmol/L [0.6 to 1.8 nmol/L], respectively, P<0.02) (Table).

To further evaluate the prothrombogenic properties of the shed membrane microparticles, we determined TF activity in supernatants before and after the removal of the microparticles by ultracentrifugation. We found that total TF activity was significantly elevated in the extracts of plaques in comparison with those of underlying arterial walls (8.3±4.3 nmol/L of generated factor Xa [2.8 to 25.6 nmol/L] versus 0.9±0.1 nmol/L [0.7 to 1.1 nmol/L], respectively, P<0.03) (Table). Interestingly, the removal of microparticles from plaque supernatants by ultracentrifugation resulted in 97±2% loss of TF activity, indicating that almost all TF activity of plaque extracts was associated with shed membrane microparticles.

Cell Origin of Shed Membrane Microparticles

To identify particle-associated antigens, extracts of plaques were incubated with corresponding insolubilized mAbs. Smooth muscle cells, macrophages, and T lymphocytes are the 3 major cell types present in the plaque. Rare mast cells are also encountered. However, we and others have shown that macrophages and lymphocytes form the bulk of apoptotic cells in the plaque. Therefore, in an attempt to characterize the cell origin of the particles, anti-CD11a, anti-CD4, and anti-GP Ib mAbs were used. There were no particles bearing GP Ib in the extracts. Most particles recovered from plaques were of monocytic and lymphocytic origin (19.2±13.4 nmol/L phosphatidylserine equivalent and 10.9±2.8 nmol/L phosphatidylserine equivalent after capture by anti-CD11a or anti-CD4, respectively), a finding that reflects the cell types undergoing apoptosis in the plaque. No direct comparison between capture by annexin V and mAbs could be done because of different preincubation times and affinities for the respective ligands. However, these experiments establish that particles from supernatants of plaques bear a proportion of antigens found on the corresponding cell surfaces.

Discussion

Apoptosis is a common event that occurs in atherosclerosis. However, no direct evidence has been provided for a beneficial or a harmful role for this process in plaque development and progression. In this study, we examined the role of in situ apoptotic cell death in the promotion of plaque thrombogenicity.

TF is a key element in the initiation of the coagulation cascade. In the normal vessel, TF expression is restricted to the adventitia and contributes to vessel repair after vessel injury. Enhanced TF expression and activity have been reported in human atherosclerotic plaques, and it is now believed that the increase of TF activity is a major determinant of plaque thrombogenicity. However, TF activity is highly dependent on the presence of phosphatidylserine, and it has recently been shown that apoptotic cell death results in phosphatidylserine exposure on the cell surface conferring a potent procoagulant activity to the cell. Therefore, we hypothesized that plaque thrombogenicity may be greatly influenced by the occurrence of apoptotic cell death.

We detected substantial expression of TF in TUNEL-positive areas of plaques. Although the TUNEL method, in some instances, may reveal processes other than apoptosis, we have adapted the technique to enhance its specificity for the detection of apoptosis. In addition, we have previously shown that in the human atherosclerotic plaque, TUNEL-positive cells colocalize with caspase 3, which is characteristic of apoptosis.

Extracellular expression of TF in the apoptotic areas suggested to us that it was probably released in microparticles during cell death. The finding of a close association between TF expression and apoptosis in acellular and cellular regions of atherosclerotic plaques suggested the possibility of synergy between TF and apoptotic cell membranes, rendering them highly thrombogenic.

To confirm this hypothesis, we searched for the presence of shed membrane particles with procoagulant potential in atherosclerotic plaques. In this study, we show that a large proportion of phosphatidylserine-bearing microparticles is produced in the atherosclerotic plaque. A number of arguments indicate that these microparticles most likely originated from apoptotic cells: (1) Several groups have shown that apoptosis occurs in the atherosclerotic plaque and may contribute to the development of the acellular lipid core. (2) Stimuli, like pro-inflammatory cytokines, that lead to cell death in the atherosclerotic plaque are potent inducers of apoptosis, and TUNEL-positive labeling in the plaque is highly associated with caspase expression, leading to the suggestion that necrosis in the plaque is a late secondary process due, in part, to the accumulation of unremoved apoptotic bodies. (3) We have previously shown that phosphatidylserine-bearing microparticles stem from surface blebs of apoptotic cells and their amount is correlated to the extent of apoptosis. (4) Phosphatidylserine exposure on the external layer of the cell membrane and shedding of membrane microparticles are early events of apoptosis and are produced even when secondary necrosis does not occur.

Interestingly, we found that these plaque-derived apoptotic microparticles account for almost all the TF activity of the plaque extracts, indicating a direct causal relationship between their presence and TF activity. This suggests that shed membrane apoptotic microparticles may play a major role in
the initiation of the coagulation cascade. On the other hand, most of these microparticles originated from macrophages and lymphocytes that are known to be abundant at sites of plaque rupture.\textsuperscript{13,14} We therefore propose that apoptotic death of a proportion of these cells may greatly enhance the thrombus formation accelerating vessel occlusion and could lead to acute infarction.

In addition to their direct effect in promotion and amplification of the coagulation cascade, the apoptotic particles bearing CD11a may also act in a variety of intercellular adhesion processes and may be responsible for dissemination of the procoagulant potential to sites remote from the microenvironment of their formation.\textsuperscript{14} They may also provide the substrate for secretory phospholipase A\textsubscript{2}, leading to the generation of lysophosphatidic acid, a potent mediator of the inflammatory reaction and a potent platelet agonist.\textsuperscript{38} Furthermore, such microparticles may play an important role in transcellular lipid metabolism by their concentrated delivery of bioactive lipids.\textsuperscript{39}

Once plaque material is exposed to circulating blood after plaque rupture or erosion, vascular thrombosis may ensue and may culminate in vascular occlusion, precipitating acute ischemic syndromes.\textsuperscript{40} Procoagulant potential of the exposed material is therefore a key determinant of plaque thrombogenicity and hence of the extent of vascular thrombosis and luminal narrowing. Our results provide strong evidence that apoptotic cell death in the atherosclerotic plaque is a major determinant of plaque thrombogenicity and could offer novel therapeutic strategies for preventing thrombus formation on plaque rupture.

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