Caspase-3 and Tissue Factor Expression in Lipid-Rich Plaque Macrophages
Evidence for Apoptosis as Link Between Inflammation and Atherothrombosis

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Background—Macrophages associated with arterial wall lipid deposition contribute to inflammatory processes. Tissue factor (TF) has been implicated in the thrombogenicity of atherosclerotic plaques. Intimal cells undergoing apoptosis have been postulated as a source for TF. However, there is only limited knowledge of cell type, plaque component, and conditions associated with TF expression and apoptosis. We examined the hypothesis that macrophages exposed to conditions of lipid-rich plaque undergo apoptosis and express TF.

Methods and Results—In human carotid (n=11005) and coronary (n=1006) atherosclerotic plaques, TF and caspase-3 mRNA and protein expression (evaluated by in situ hybridization and immunohistochemistry) were increased significantly in lipid-rich compared with fibrous plaque components (P<0.01) and correlated with high macrophage content (P<0.05). Double-labeling studies demonstrated colocalization of TF and active caspase-3. In hyperlipidemic mice, expression of TF and active caspase-3 was observed simultaneously and colocalized in neointimal macrophages after arterial injury. In neointima of normolipidemic animals, TF and active caspase-3 were absent after arterial injury. In monocytes cultured in the presence of oxidized LDL, strong induction and colocalization of TF and active caspase-3 were found compared with baseline (P<0.05). Both antigens were significantly decreased after cotreatment with a caspase inhibitor (P<0.05) and were absent in untreated control cells.

Conclusions—The expression of TF as the primary cell-associated activator of the coagulation pathway proves to be closely related to macrophages undergoing apoptosis in conditions of lipid-rich plaque, pointing to a key role of lipid content and inflammatory cell viability in determining plaque thrombogenicity. (Circulation. 2004;109:2001-2008.)

Key Words: lipids • inflammation • apoptosis • thrombosis

The atherogenic process is considered to be an exaggerated response of the vascular wall to injury characterized by chronic inflammation and fibrocellular proliferation. Hypercholesterolemia is one of the major risk factors for the development of atherosclerosis in both humans and animal models. LDL accumulates within the subendothelial space as a consequence of endothelial dysfunction. In the subendothelial space, LDL undergoes oxidative changes. The resulting oxidized LDL can serve as a proinflammatory agent, leading to the recruitment of monocytes to the arterial wall. In the early course of atherosclerosis, attraction of monocytes and their subsequent differentiation into macrophages may have a protective function by providing phagocytic capacity to remove the proinflammatory and cytotoxic oxidized LDL particles. However, progressive accumulation of macrophages and lipids leading to apoptosis and tissue factor expression in these inflammatory cells in more advanced stages of atherosclerosis could contribute to plaque progression, vulnerability, and thrombosis.

Tissue factor is a low-molecular-weight transmembrane glycoprotein that initiates the extrinsic coagulation cascade. Evaluation of coronary plaque tissue from atherectomy specimens of patients with unstable angina suggested a strong relationship between tissue factor content and macrophages in lipid-rich plaque areas. Studies of the relative thrombogenicity of different components of disrupted human atherosclerotic plaques have demonstrated that lipid-rich plaque tissue is >6 times more thrombogenic than fibrous plaque.
components. Furthermore, the use of a tissue factor pathway inhibitor significantly reduced the thrombogenicity of these plaques. This points to tissue factor as a possible key mediator of increased thrombogenicity in arterial lesions.

Apoptosis has been shown to occur in macrophages and smooth muscle cells of human and experimental atherosclerotic lesions. Recently, in vitro studies suggested that apoptotic cells might acquire procoagulant properties. Among these are the relocalization of phosphatidylserine to the outer cell membrane, offering an efficient catalytic surface for procoagulant enzyme activity and the expression of tissue factor.

In the present study, we hypothesized that macrophages undergoing apoptosis in lipid-rich plaque express tissue factor. This may indicate that lipid-induced macrophage apoptosis is an important link between cell-mediated thrombogenicity and inflammation in atherosclerosis.

To address this question, we investigated the spatial and temporal patterns of tissue factor and active caspase-3 expression and macrophage content in human and experimental atherosclerotic lesions, comparing lipid-rich versus fibrous plaque components in vivo. In addition, we investigated the effect of oxidized LDL on the expression of tissue factor and active caspase-3 in monocytes in vitro.

Methods

Human Specimens
Paraffin blocks of formalin-fixed atherosclerotic plaque tissue were obtained from carotid endarterectomy specimens (n=15) and epicardial atherosclerotic coronary arteries (n=6) from explanted hearts. Use of excess anonymous surgical pathology tissue was approved by the institutional review board.

Endothelial Denudation Injury in Mice
Male C57BL/6 mice aged 3 to 4 months (weight, 30±4 g), with apolipoprotein E (apoE<sup>−/−</sup>) or wild-type genotypes, were purchased from Jackson Laboratories (Bar Harbor, Maine) and housed at the Center for Laboratory Animal Sciences (Mount Sinai School of Medicine). Wild-type mice received standard rodent chow, and apoE<sup>−/−</sup> mice received a Western diet (Harlan Teklad) 1 week before arterial injury until they were euthanized, as described. Procedures and animal care were approved by the institutional animal care and use committee and were in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, Washington, DC, 1996). Two groups of hyperlipidemic apoE<sup>−/−</sup> and normolipidemic wild-type mice (n=10 per group) underwent femoral arterial injury as described. Plasma cholesterol levels were measured with a commercial assay at time of euthanization (Sigma).

Monocyte In Vitro Study
Human mononuclear cells from peripheral blood of healthy human subjects were isolated by Ficoll Paque (Pharmacia) density gradient centrifugation. A highly pure (>97%) monocyte population (CD14+) was obtained by MACS cell sorting (Miltenyi Biotec), as described. Cell viability was analyzed by trypan blue staining. The cells were
seeded at a density of $1 \times 10^6$ cells/mL in 8-well chamber slides (Nalgene Nunc) in RPMI 1640 (Gibco, Life Technologies) with 1% penicillin/streptomycin and 1% heat-inactivated FCS. Human LDL (Sigma) was CuSO$_4$-oxidized in vitro according to established protocols. Oxidation of LDL was verified by agarose gel electrophoresis. Cells were incubated for 2 hours at 37°C in 5% CO$_2$ and then treated with either oxidized LDL (75 µg/mL) or staurosporine (0.5 µg/mL) (Calbiochem) alone or in combination with the general caspase inhibitor Z-VAD-FMK (20 µg/mL) (Calbiochem) and CuSO$_4$. Oxidation of LDL was verified by agarose gel electrophoresis. Cells were incubated for 2 hours at 37°C in 5% CO$_2$ and then treated with either oxidized LDL (75 µg/mL) or staurosporine (0.5 µg/mL) (Calbiochem) alone or in combination with the general caspase inhibitor Z-VAD-FMK (20 µg/mL) (Calbiochem). Treated and untreated cells were incubated for 2, 4, and 6 hours under the same conditions. To exclude any proapoptotic effects not directly related to oxidized LDL, we also tested the effects of native LDL and oxidized albumin on apoptosis induction in cultured monocytes.

**Histology and Immunostaining**

Sections from human atheroma and mouse femoral arteries were stained by the combined Masson’s elastin method. Immunohistochemistry was performed on serial sections for tissue factor (polyclonal antihuman soluble tissue factor; gift from Dr Yale Nemerson, Mount Sinai School of Medicine, New York, NY)16, activated caspase-3 fragment (R&D Systems; 1:150), and CD-68 (R&D Systems; 1:100). Primary antibodies were detected with horseradish peroxidase (HRP)-streptavidin/biotin (Biogenex) and counterstained with hematoxylin. In negative controls, primary antibody was substituted with the appropriate control IgG. Detection of apoptosis via active caspase-3 immunostaining was validated by immunofluorescence double labeling with antibodies specific for PARP (Enzyme Systems, 1:100; Promega Corp; 1:200) and terminal deoxynucleotidyl transferase-mediated dUTP biotin nick-end labeling (TUNEL) testing (Roche Molecular Biochemicals) (data not shown), as published.17

Cultured cells were fixed in 4% paraformaldehyde for 30 minutes and stained with the use of fluorescein isothiocyanate (FITC)-labeled secondary antibodies (Jackson Immuno Laboratories) and nuclear DAPI counterstaining.

For double labeling, secondary antibodies with FITC and rhodamine or HRP and alkaline phosphatase were used. Negative control experiments with the use of appropriate IgG were performed in all experiments.

**Morphometry**

Masson’s trichrome stain was used to identify the following 2 plaque components: (1) fibrous tissue (tissue composed of densely stained collagen ranging from sparse cellularity to abundant numbers of smooth muscle cells); and (2) lipid-rich plaque areas (atheromatous gruel containing foam cells, cellular debris, and cholesterol clefts without evidence of robust connective tissue matrix). In each plaque component, the percentage of cells expressing tissue factor, active caspase-3, and CD-68 was quantified by computer-assisted planimetry (Image-Pro-Plus 3.0.1 software). Expression of tissue factor and active caspase-3 in experimental arterial lesions and cultured monocytes was also measured morphometrically.

**In Situ Hybridization for Tissue Factor and Caspase-3 mRNA**

In situ hybridization was performed on human carotid atheroma with digoxigenin-labeled riboprobes on the basis of published sequences for tissue factor and caspase-3 (GenBank).18 Sense probes were used as negative controls. Detection and visualization of bound riboprobes were performed as described.18

**Protein Extraction and Western Blot**

Expression of the p17 fragment of caspase-3 protein and expression of the 89-kDa fragment of PARP were evaluated by Western blot analysis. Briefly, cell pellets and tissue of carotid atheroma were sonicated and lysed for 30 minutes on ice in lysis buffer supplemented with a mixture of proteinase inhibitors (Sigma). The samples were cleared by microcentrifugation (14,000 rpm, 30 minutes, 4°C) and assessed for protein concentration. One hundred microliters of protein per sample was electrophoresed in 18% (p17/caspase-3) and 10% (PARP) SDS-PAGE and electroblotted onto nitrocellulose membranes. After incubation in 1% casein-blocking buffer (Bio-Rad), the membranes were exposed to antibodies against p17 active caspase-3 (R&D) and against PARP (Roche). After the membranes were washed, the appropriate secondary peroxidase-labeled antibody was applied for 1 hour at room temperature. The bound antibodies were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech).

**DNA Extraction and DNA Agarose Gel Electrophoresis**

DNA from cultured cells was extracted with the use of a commercially available kit (Roche Molecular Biochemicals), and equal amounts from treated and untreated cells were analyzed on a 1% agarose gel.

**Statistical Analysis**

Results are expressed as mean±SEM. For data analysis, SPSS/PC+ software was used. After we tested for normal distribution and equality
of variances with Levene’s F test, we used the independent sample t test to compare variables. Correlation coefficients were determined by Pearson’s test. Probability values were 2-tailed and corrected for ties. Probability values <0.05 were considered significant.

Results

Tissue Factor and Caspase-3 in Human Atheroma

Both tissue factor and active caspase-3 antigen expression of intimal cells were significantly increased in lipid-rich compared with fibrous plaque tissue (31±3% versus 9±1% [P<0.01]; 20±2% versus 5±1% [P<0.01], respectively) (Figures 1 and 2). The number of CD-68–positive macrophages was very high in lipid-rich compared with fibrous plaque tissue (55±4% versus 2±2%; P<0.01) (Figures 1 and 2).

Tissue factor and caspase-3 mRNA transcripts were detected in areas close to the atheromatous core of carotid plaque in nearly identical locations on serial sections (Figure 3). In fibrous parts and in the media, tissue factor and caspase-3 mRNA were absent.

A nearly complete colocalization of tissue factor and active caspase-3 signals was found in lipid-rich plaque areas (Figure 4, panel 1, D to F and H). Of note, active cellular apoptosis was confirmed by colocalization of p17 active caspase-3 and PARP with the use of immunofluorescence and by Western blot, demonstrating cleavage products of both caspase-3 (p17) and PARP (p89) in protein extracted from lipid-rich plaque (Figure 4, panel 2, A through I). Tissue factor and active caspase-3 expression showed a highly significant correlation in lipid-rich areas of individual atheroma (r=0.72, P<0.01). A significant correlation between CD-68, active caspase-3, and tissue factor expression was also found (r=0.61, P<0.01; r=0.58, P<0.05).

Tissue Factor and Caspase-3 in Neointima of Mice

Simultaneous expression of tissue factor and active caspase-3 was observed in neointima of hyperlipidemic mice (1500±350 mg/dL plasma cholesterol) at 2 and 4 weeks and showed a significant positive correlation (r=0.81, P<0.01). Both antigens were nearly absent in the neointima of normolipidemic animals (36±5% versus 1±1% [P<0.01]; 32±4% versus 1±1% [P<0.01], respectively) (Figures 5 and 6). Active caspase-3 and tissue factor antigen were associated with the presence of foam cell macrophages in lipid-rich neointima (49±6% versus 1±1%; P<0.01) (Figures 5 and 6).
6), and colocalization of tissue factor and active caspase-3 signals was found (Figure 4, panel 1, G).

**Tissue Factor and Caspase-3 in Monocytes In Vitro**

In cultured monocytes treated with oxidized LDL, active caspase-3 expression increased gradually from 0% of cells at baseline to 2±2% at 2 hours, 58±3% at 4 hours, and up to 90±3% at 6 hours (P<0.05) (Figure 7, panels 1 and 2). Untreated cells showed no active caspase-3 expression at any time point (P<0.05) (Figure 7, panels 1 and 2). DNA laddering present after 6 hours of treatment confirmed cellular apoptosis (Figure 7, panel 1, M). Expression of tissue factor increased from a baseline of 8±1% of cells to 16±3% at 2 hours, 45±13% at 4 hours, and 77±13% at 6 hours of oxidized LDL treatment (P<0.05) (Figure 7, panels 1 and 2). Untreated cells did not express tissue factor at 6 hours (P<0.05) (Figure 7, panels 1 and 2). Cells treated with staurosporine demonstrated a similar increase in active caspase-3 (85±7%) and tissue factor expression (69±2%) at 6 hours (Figure 7, panel 2). Double labeling demonstrated true coexpression of active caspase-3 and tissue factor in single cells (Figure 4, panel 1, A to C).

Coincubation of monocytes with oxidized LDL and the general caspase inhibitor Z-VAD-FMK significantly decreased active caspase-3 (90±3% versus 33±3%; P<0.05) and tissue factor expression (77±13% versus 6±6%; P<0.05) at 6 hours (Figure 7, panel 2). Monocytes cotreated with staurosporine and caspase inhibitor also showed decreased expression of active caspase-3 (85±7% versus 3±1%; P<0.05) and tissue factor (69±2% versus 30±5%; P<0.05) at 6 hours (Figure 7, panel 2). Results after addition of a negative control of caspase inhibitor to oxidized LDL were identical to those with oxidized LDL treatment alone (data not shown). Treatment of cells with native LDL or oxidized albumin did not induce monocyte apoptosis (data not shown).

**Discussion**

We report that apoptotic cell death of macrophages is linked to the expression of the tissue factor molecule in lipid-rich plaque tissue. The present study shows that apoptosis of intimal cells was increased up to 4-fold in lipid-rich compared with fibrous plaque tissue of human coronary and carotid atheroma (Figure 2). Concordantly, tissue factor expression in lipid-rich plaque tissue was shown to be increased >3-fold over fibrous plaque (Figure 2). The spatial
distribution of active caspase-3 and tissue factor immunostaining in serial sections suggests colocalization of these antigens in CD-68-positive macrophages in lipid-rich plaque area (Figure 1). The specificity of active caspase-3 antibody labeling was corroborated by the selective detection of p17 active caspase-3 fragment in a Western blot of protein extracted from lipid-rich plaque (Figure 4, panel 2). Colocalization of active caspase-3 and cleaved PARP in macrophages of lipid-rich plaque further indicates activation of the apoptotic program (Figure 4, panel 2).

In addition, active caspase-3 and tissue factor protein were consistently demonstrated to colocalize in intimal cells in vivo at the site of foam cell and lipid accumulation (Figure 4, panel 1). The expression of active caspase-3, tissue factor, and CD-68 in lipid-rich plaque showed significant positive correlations when quantified by histomorphometry. These data demonstrate that apoptosis of macrophages exposed to the microenvironment of lipid-rich plaque tissue is associated with the expression of tissue factor.

This finding raises the intriguing question of whether both processes are linked and occur in response to stimuli present in lipid-rich plaque. We observed that the synthesis of caspase-3 mRNA as key effector molecule, albeit not a marker of the execution phase of apoptosis, occurs simultaneously with the synthesis of tissue factor mRNA in macrophages bordering the lipid-rich atheromatous core (Figure 3). Macrophages may already synthesize the tissue factor molecule for a longer time and then finally start to express caspase-3, preparing them to undergo apoptosis. Alternatively, macrophages may start both processes simultaneously, becoming tissue factor positive as they undergo apoptosis. Knowing which of these 2 possibilities occurs in lipid-rich plaque tissue will help to decide the important question of whether lipid-induced macrophage apoptosis in atherosclerotic lesions plays either a beneficial or detrimental role in thrombotic disease progression.

To answer this question, we studied neointima formation in conditions of hyperlipidemia and normolipidemia in a mouse model of arterial injury over a well-defined time period. We found that strong expression of active caspase-3 and tissue factor of up to 32% and 36% of neointimal cells, respectively, occurred simultaneously in neointima of mice with very high plasma cholesterol levels (mean, 1500 mg/dL) (Figures 5 and 6). In neointima of normolipidemic mice, active caspase-3, tissue factor expression, and macrophages were nearly absent. Confirming the findings in human atheroma, active caspase-3 and tissue factor in neointima of hyperlipidemic mice also showed strong colocalization on foam cells/macrophages (Figure 4, panel 1). The parallel initiation of both processes in macrophages could potentially be explained by signals present in lipid-rich plaque tissue activating the Fas-receptor pathway (leading to apoptosis) and nuclear factor-κB (resulting in tissue factor expression).
To further assess the link between macrophage apoptosis and tissue factor expression, we performed in vitro experiments with human peripheral blood monocytes. In monocytes treated with oxidized LDL for 6 hours, active caspase-3 and tissue factor expression increased simultaneously from a very low baseline up to 90% and 77% of cells, respectively (P < 0.05) (Figure 7), and coexpression of both determinants in single cells was observed (Figure 4, panel 1). Interestingly, monocytes coincubated with oxidized LDL and a general caspase inhibitor showed a significant decrease in the expression of active caspase-3 from 90% to 33% (P < 0.05) and of tissue factor from 77% to 6% of cells at 6 hours (P < 0.05) (Figure 7, panel 2).

In summary, the present data derived from human atherosclerotic disease specimens and in vitro experiments with human peripheral blood monocytes provide evidence that oxidized LDL induces apoptosis in macrophages and coexpression of tissue factor and active caspase-3. The high incidence of apoptotic events in macrophages exposed to lipid-rich plaque conditions and their coexpression with tissue factor molecule suggest a pathobiological link between apoptotic events in macrophages and their expression of tissue factor, as demonstrated in the present study.6 The direct modulation of macrophage apoptosis, with the ultimate goal to prevent apoptotic cell death of macrophages inside the plaque and to allow these inflammatory cells to leave the atherosclerotic lesion alive, could be a future therapeutic strategy. This could be achieved by pharmacological intervention with caspase inhibitors or by selective gene delivery of antiapoptotic genes to macrophages.23,24 However, further study of these approaches in animal models and assessment of the treatment effect on tissue factor and caspase activity are necessary.

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References
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