Early Atherosclerosis Exhibits an Enhanced Procoagulant State

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Background—Thrombin generation in vivo may be important in regulating atherosclerotic progression. In the present study, we examined for the first time the activity and presence of relevant coagulation proteins in relation to the progression of atherosclerosis.

Methods and Results—Both early and stable advanced atherosclerotic lesions were collected pairwise from each individual (n=27) during autopsy. Tissue homogenates were prepared from both total plaques and isolated plaque layers, in which the activity of factors (F) II, X, and XII and tissue factor was determined. Microarray analysis was implemented to elucidate local messenger RNA synthesis of coagulation proteins. Part of each specimen was paraffin embedded, and histological sections were immunohistochemically stained for multiple coagulation markers with the use of commercial antibodies. Data are expressed as median (interquartile range [IQR]). Tissue factor, FII, FX, and FXII activities were significantly higher in early atherosclerotic lesions than in stable advanced atherosclerotic lesions. Endogenous thrombin potential and thrombin-antithrombin complex values consolidated a procoagulant profile of early atherosclerotic lesions (endogenous thrombin potential, 1240 nmol/L · min [IQR, 1173 to 1311]; thrombin-antithrombin complex, 1045 ng/mg [IQR, 842.6 to 1376]) versus stable advanced atherosclerotic lesions (endogenous thrombin potential, 782 nmol/L · min [IQR, 0 to 1151]; thrombin-antithrombin complex, 718.4 ng/mg [IQR, 508.6 to 1151]). Tissue factor, FVII, and FX colocalized with macrophages and smooth muscle cells. In addition, multiple procoagulant and anticoagulant proteases were immunohistochemically mapped to various locations throughout the atherosclerotic vessel wall in both early and advanced atherosclerotic stages.

Conclusions—This study shows an enhanced procoagulant state of early-stage atherosclerotic plaques compared with advanced-stage plaques, which may provide novel insights into the role of coagulation during atherosclerotic plaque progression. (Circulation. 2010;122:821-830.)

Key Words: atherosclerosis ■ hypercoagulability ■ immunohistochemistry ■ plaque ■ thrombosis

Atherosclerosis is widely recognized as a chronic inflammatory disease.1 Rupture of an atherosclerotic plaque is considered the predominant underlying cause of acute atherothrombotic events such as myocardial infarction, ischemic stroke, and vascular death. A close relation between blood coagulation and atherosclerosis2,3 is supported by studies revealing the presence of specific coagulation proteins within an atherosclerotic lesion. Tissue factor (TF) and factor (F) VII, of which the complex is the principal initiator of coagulation in vivo, are expressed on macrophages and vascular smooth muscle cells (SMC) within the arterial wall and atherosclerotic lesion.4,5 Both proteins potentially participate in multiple pathogenic processes such as migration and proliferation of SMC,6 inflammation, and angiogenesis.7 In addition to the single effects of each protein, the local interaction between macrophage/SMC-derived TF and FVII may provide a catalytic complex for subsequent generation of thrombin and fibrin, of which the latter is also detectable in atherosclerotic lesions.8 To the procoagulant condition of the atherosclerotic lesion may be further enhanced by the presence of various proinflammatory cytokines (eg, tumor necrosis factor-α, interleukin-10), which may downregulate local expression of anticoagulant proteins such as thrombomodulin and the endothelial protein C receptor on endothelial cells.11

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821
Clinical Perspective on p 830

Thrombin, a key enzyme in blood coagulation, may also play a critical role in many processes related to the development, progression, and atherothrombotic potential of atherosclerotic plaques. Direct evidence for the role of thrombin in the atherogenic process comes from experiments showing reduced progression of atherosclerosis in apolipoprotein E−/− mice on pharmacological inhibition of thrombin. Moreover, decreased expression of TF pathway inhibitor (TFPI) on an apolipoprotein E−/− background increased the atherosclerotic burden.

Because of the reported involvement of procoagulant and anticoagulant coagulation factors during plaque progression, we hypothesized that the overall distribution and activity of coagulation proteins in the arterial vessel wall correlate with the extent and progression of atherosclerotic lesions. Moreover, we hypothesized that the amount of thrombin that can be generated from atherosclerotic tissue homogenates depends not only on the amount of TF but also on the presence and activity of other coagulation proteins that either amplify or dampen thrombin generation. Hence, we studied the localization of all coagulation proteins, in addition to the TF/FVII complex, on histologically defined early and stable advanced atherosclerotic lesions. In addition to thrombin generation, we determined the procoagulant activity of several coagulation proteins in the same lesions.

Methods

Patient Characteristics and Tissue Specimens

The tissue specimens were obtained from the Maastricht Pathology Tissue Collection. Collection, storage, and use of tissue and patient data were performed in agreement with the Code for Proper Secondary Use of Human Tissue in the Netherlands (http://www.fmwv.nl). Both early atherosclerotic lesions (EAL) and stable advanced atherosclerotic lesions (SAAL) were collected pairwise from each corresponding individual (n=27) during postmortem dissection of the abdominal aortas within 8 hours of death. Autopsy specimens were obtained from adult men and women with an age range of 45 to 84 years (mean, 55 years). Clinical characteristics of the patients are provided in Table 1. The cause of death was diverse (eg, myocardial infarction, stroke). Individuals with sepsis or cancer were excluded. All tissue specimens were histologically evaluated on hematoxylin and eosin–stained sections (4 μm). Plaque subtypes were determined in compliance with the modified American Heart Association classification, based on morphological description, proposed by Virmani et al. Because one of the main goals in this study was to discriminate the overall prothrombotic potential of atherosclerotic lesions between early and advanced stages of development, we classified the plaques as follows: intimal thickening and xanthomas are uniformly termed EAL, whereas all types of stable advanced plaques are termed SAAL. Complicated lesions, including lesions with intraplaque hemorrhage, a surface defect, and/or thrombotic deposit, were not included in this study.

Preparation of Tissue Homogenates

A section of each of the collected specimens (27 EAL/27 SAAL, obtained in pairs from n=27) was snap-frozen on collection. Snap-frozen atherosclerotic tissues were freeze-dried for 3 days and pulverized, and subsequently the tissue powders were dissolved in 50 mmol/L N-octyl-β-D-glucopyranoside (Sigma-Aldrich) in HN buffer (25 mmol/L HEPES, 175 mmol/L NaCl, pH 7.7), vortexed, reduced progression of atherosclerosis in apolipoprotein E

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<th>Table 1. Patient Clinical Characteristics of Autopsy Cases From Which Pairs EAL and SAAL Were Obtained and Examined</th>
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Values are n (%) except age.

and centrifuged twice (10 minutes, 13 000 rpm). Total protein content of the tissue homogenates was spectrophotometrically determined with the use of the Biorad DC Protein Assay system according to the manufacturer’s instructions (Bio-Rad Laboratories B.V., Veenendaal, Netherlands). All samples were further diluted into a final concentration of 5 mg/mL.

Effect of Time Delay Between Death and Postmortem Examination on Coagulation Protein Activity

See Methods in the online-only Data Supplement.

EAL and SAAL Layer Preparation and Homogenization

See Methods in the online-only Data Supplement.

Thrombin Generation, Prothrombin, FX, and FXII Activity Assays, Thrombin-Antithrombin Complex Levels, TF Activity Assay, and TFPI Antigen Assay

The calibrated automated thrombogram (Thrombinoscope, the Netherlands) was used to determine the contribution of atherosclerotic tissue homogenates to thrombin generation in human plasma (in triplicate; interassay coefficient of variation <10%). For additional information, see Methods in the online-only Data Supplement.

Effect of Phospholipid Concentration on Thrombin Generation in Normal Arterial Vessel Wall Homogenates

See Methods in the online-only Data Supplement.

RNA Isolation and Quantification, Microarray Hybridization, and Data Analysis

See Methods in the online-only Data Supplement.

Immunohistochemical and Immunofluorescence Stainings and Immunohistological Evaluation

See Methods in the online-only Data Supplement.

Statistical Analysis

Data analysis was computed with SPSS, version 17.02 (SPSS Inc, Chicago, Ill) and Prism, version 5.00 (GraphPad Software Inc, San Diego, Calif). Results are expressed as median (interquartile range [IQR]). An exact-distribution Wilcoxon 2-sample test was used for all intraindividual comparisons. A 2-tailed P<0.05 was considered significant.
EAL Demonstrate a 3-Fold Increase in TF Activity Versus SAAL

To better appreciate the procoagulant potential of these 2 sets of atherosclerotic plaque homogenates, we assessed the activity of TF, which is known to be a pivotal trigger of coagulation in vivo. TF activity was ∼3-fold higher (0.036 pmol/mg [IQR, 0.017 to 0.055]) in EAL compared with SAAL (0.009 pmol/mg [IQR, 0.005 to 0.022]) (Figure 1D; Wilcoxon 2-sample test, 2-tailed exact P<0.05). Twenty-six of 27 EAL homogenates indicated elevated TF activity levels compared with their corresponding SAAL specimens.

Results

EAL Exhibit Higher Functional Activity of Key Coagulation Proteins Than SAAL Ex Vivo

We determined the dependence of the thrombin-generating potential of both EAL and SAAL on their prothrombin, FX, and FXII content. From all 54 specimens (27 pairs, EAL and corresponding SAAL) that we examined, prothrombin activity was detected in only 11 samples. From the latter 11 samples with detected activity, EAL specimens had significantly higher prothrombin activity at 0.0% (IQR, 0.0 to 7.761) compared with their paired SAAL at 0.0% (IQR, 0.0 to 0.0) (Figure 1A; Wilcoxon 2-sample test, 2-tailed exact P<0.05). The activity of FX revealed a similar trend, with a significant 3-fold upregulation in EAL at 0.276% (IQR, 0.164 to 0.536) compared with SAAL at 0.136% (IQR, 0.054 to 0.237) (Figure 1B; Wilcoxon 2-sample test, 2-tailed exact P<0.05). Furthermore, FXII also demonstrated significantly higher activity levels in EAL of 2.636% (IQR, 1.344 to 3.372) compared with levels in SAAL of 0.930% (IQR, 0.337 to 1.526) (Figure 1C; Wilcoxon 2-sample test, 2-tailed exact P<0.05).

EAL and SAAL Demonstrate a 3-Fold Increase in TF Activity in Vivo

To better appreciate the procoagulant potential of these 2 sets of atherosclerotic plaque homogenates, we assessed the activity of TF, which is known to be a pivotal trigger of coagulation in vivo. TF activity was significantly higher in EAL homogenates compared with the corresponding SAAL. FX activity was significantly elevated (∼3-fold increase) in EAL homogenates compared with SAAL homogenates; Wilcoxon 2-sample test, 2-tailed exact P<0.05. C, FXII activity comparison between EAL and SAAL, showing significantly higher levels in EAL (∼2-fold increase); Wilcoxon 2-sample test, 2-tailed exact P<0.05. D, TF activity is ∼3-fold higher in EAL homogenates than in SAAL homogenates; Wilcoxon 2-sample test, 2-tailed exact P<0.05. E, TFPI activity shows 1.6-fold increase in EAL F, TF/TFPI ratio in EAL (0.294 [IQR, 0.109 to 0.770]) and SAAL (0.174 [IQR, 0.117 to 0.257]); Wilcoxon 2-sample test, 2-tailed exact P<0.05.

Notably, the 8-hour window between death and postmortem collection did not significantly affect the activity of TF, FII, FX, and FXII in atherosclerotic lesions harvested at various time points: 0 (baseline), 2, 4, and 8 hours. No significant differences were found between the different time points of all tested proteins and also compared with baseline values (Figure 1 in the online-only Data Supplement), strongly suggesting that the postmortem values reflected actual coagulation activity in vivo.

Shift of the TF/TFPI Ratio Suggests an Increased Atherothrombotic Tendency in EAL

TFPI is a potent natural inhibitor of the TF-driven pathway of the coagulation cascade and also plays an important role in regulating inflammation. Furthermore, it has been shown that TFPI modulates thrombus formation in experimental models in vivo,16 primarily by attenuating the procoagulant activity and overexpression of TF.17,18 Therefore, we tested the levels of TFPI by utilizing a homemade enzyme-linked immunosorbent assay. An ∼1.6-fold significant increase in TFPI antigen levels was found in EAL compared with SAAL. EAL demonstrated TFPI activity equal to 0.089 nmol/L per milligram (IQR, 0.072 to 0.140), whereas SAAL showed 0.056 nmol/L per milligram (IQR, 0.030 to 0.088) (Figure 1E; Wilcoxon 2-sample test, 2-tailed exact P<0.05). Despite the higher levels of TFPI antigen in the EAL homogenates, in SAAL the TF/TFPI balance in the early lesions remained in favor of TF, shown by the higher TF/TFPI ratios in EAL homogenates (Figure 1F; Wilcoxon 2-sample test, 2-tailed exact P<0.05).

Enhanced Thrombin Generation in EAL

In the absence of TF and entirely dependent on the procoagulant molecular content in the tissue homogenate, all 27 EAL induced thrombin formation in normal pooled plasma, showing significantly higher values (1240 nmol/L · min
Thrombin generation assessed in paired EAL and SAAL and areas of normal aorta. EAL and SAAL show significantly higher thrombin generation levels compared with normal arterial vessels. These data consolidate the procoagulant state in early atherosclerosis but further indicate that the prothrombotic tendency in EAL is not dependent on variations in the cellular density/phospholipid content as a result of vessel wall structure alterations that occur on atherosclerotic progression. HAV indicates healthy arterial vessels.

**Figure 2.** Effect of phospholipid concentration on thrombin generation. Overall procoagulant activity of atherosclerotic plaque homogenates and normal vessels is shown, assessed by means of thrombin generation and/or TAT complexes. A, Influence of increasing phospholipid concentrations (1, 2, 3, 4, 5, 10, 20, 30, 40, and 50 μmol/L) on thrombin generation in normal pooled plasma triggered by 1 pM TF (without addition of plaque homogenates). Furthermore, thrombin generation in EAL and/or SAAL homogenates was established at a final phospholipid concentration of >4 μmol/L, thrombin generation is independent from additional phospholipids present in the measured sample (such as is present in the added plaque homogenate) (P value was calculated by repeated-measures ANOVA). Furthermore, thrombin generation in EAL and/or SAAL homogenates was established at a final phospholipid concentration of >4 μmol/L, irrespective of the plaque type studied. ETP indicates endogenous thrombin potential. B, Thrombin generation assessed in paired EAL and SAAL and areas of normal aorta. EAL and SAAL show significantly higher thrombin generation levels compared with normal arterial vessels. These data consolidate the procoagulant state in early atherosclerosis but further indicate that the prothrombotic tendency in EAL is not dependent on variations in the cellular density/phospholipid content as a result of vessel wall structure alterations that occur on atherosclerotic progression. HAV indicates healthy arterial vessels. C, TAT complex levels measured in paired EAL and SAAL homogenates.

[1Q1, 1173 to 1311] compared with SAAL (782 nmol/L·min [IQR, 0 to 1151]) (Figure 2B; Wilcoxon 2-sample test, 2-tailed exact \( P < 0.05 \)). Twenty-six EAL induced higher endogenous thrombin potential than their corresponding advanced atheromas. For the SAAL, 10 lesions did not trigger any thrombin generation.

Furthermore, EAL showed a significantly increased thrombin generation potential compared with areas of normal aorta obtained from the same individuals (263.3 nmol/L·min [IQR, 117.8 to 350.3]; Wilcoxon 2-sample test, 2-tailed exact \( P < 0.05 \)). Twenty-six EAL induced higher endogenous thrombin potential than their corresponding advanced atheromas. For the SAAL, 10 lesions did not trigger any thrombin generation.

Thrombin-Antithrombin Complex Levels Additionally Point to Higher Thrombin Generation in EAL Homogenates

Once generated, thrombin is inhibited on binding to antithrombin, thus forming a stable thrombin-antithrombin (TAT) complex. TAT complexes are considered a marker of in vivo intravascular thrombin generation; therefore, the main goal of this experiment was to assess whether there was an excess of activated FII generation in EAL in situ compared with their matched SAAL. The concentration of TAT complexes in EAL was significantly higher (1045 ng/mg [IQR, 842.6 to 1376]) compared with their paired SAAL homogenates (718.4 ng/mg [IQR, 508.6 to 1151]) (Figure 2C; Wilcoxon 2-sample test, 2-tailed exact \( P < 0.05 \)), confirming a more procoagulant state in EAL.

Layer-Selective Analysis of Coagulation Factor Activities Consolidated a More Procoagulant State of EAL Versus SAAL

To provide better insight into the procoagulant properties of the atherosclerotic lesions, we undertook a more selective, layer-specific analysis in which the potential procoagulant effects of the different vessel wall layers were studied. The activity of coagulation factors was analyzed in tissue homogenates prepared from tunica intima, media, and adventitia (histologically controlled anatomic separation; Figure 3A). All 3 layers were harvested in 42 specimens (21 pairs of EAL and SAAL from the original tissue collection). Endogenous thrombin potential values in all layers of EAL were found to be significantly higher (intima: 1489 nmol/L·min [IQR, 1353 to 1680]; media: 1734 nmol/L·min [IQR, 1256 to 1983]; adventitia: 1872 nmol/L·min [IQR, 1655 to 2171]) compared with the corresponding SAAL layers (intima: 437.9 nmol/L·min [IQR, 290.3 to 549.9]; media: 392.1 nmol/L·min [IQR, 219.7 to 680.9]; adventitia: 524.1 nmol/L·min [IQR, 394.1 to 787.7]) (Figure 3B; Wilcoxon 2-sample test, 2-tailed exact \( P < 0.05 \), all). This strongly pronounced procoagulant state of the EAL layers was additionally confirmed by significantly elevated prothrombin, FX, and FXII levels (Figure 3C to 3E). Intimal layers of both EAL and SAAL showed comparable TF activity, whereas TF was significantly increased in media and adventitia of EAL versus SAAL (Figure 3F). Although they demonstrated comparable activities in terms of TF, EAL intimal layers contained significantly higher TFPI levels (Figure 3G), yielding a significantly lower TF/TFPI ratio in EAL compared with SAAL. EAL and SAAL media layers did not significantly differ in TFPI levels, whereas TFPI in EAL adventitia was significantly higher compared with SAAL (Figure 3G). Tunica adventitia exhibited the most procoagulant phenotype of all vessel wall layers in terms of thrombin generation. Its values in both EAL and SAAL were significantly higher than those measured in tunica intima and media.

Gene Expression of Coagulation Genes in EAL Versus SAAL

To better explore to what extent and which coagulation proteins are expressed on the genome level within the arterial
vessel wall, gene expression profiles of EAL and SAAL were obtained with the use of microarray analysis. In a separate set of patients, early and advanced carotid lesions were collected from the same patient (at autopsy), and fold changes in gene expression were assessed by comparing the advanced lesions with the early lesions. The results indicated that several coagulation factor genes were expressed in both types of atherosclerotic lesions. After correction for multiple testing, 14 coagulation genes showed significant differential transcript levels between EAL and SAAL. Figure 4 demonstrates the relative miRNA levels, described as the SAAL/EAL ratio. Of these 14 differentially regulated genes, 6 were upregulated in EAL (expressed as fold change $< -1.0$), whereas 8 were upregulated in SAAL (expressed as fold change $> +1.0$) (Table I in the online-only Data Supplement). Fold changes ranged from $-1.13$ to $-2.96$ for the upregulated genes in EAL and from $1.08$ to $1.29$ for the upregulated genes in SAAL. Additional information is provided in Table I in the online-only Data Supplement.

Immunohistochemical Staining: EAL

In EAL, moderate (fibrinogen/fibrin, FIX, TFPI) to strong positivity for von Willebrand factor, FX, prothrombin/thrombin, protein S, and activated protein C (APC) was observed in the endothelial luminal cells, indicated by a sharp demarcation of the endothelial lining (Figure 5 and Table 2). In addition, a positive focal endothelial distribution for TF, FVII, FXII, FXI, kallikrein, and thrombomodulin was shown. Macrophages and foam cells stained intensely positive for TF, FVII, FX, prothrombin/thrombin, kallikrein, and FXI. Despite the fact that other coagulation proteins such as FXII, FIX, protein S, protein C, and APC were also expressed by macrophages and foam cells, their expression or immunoreactivity was either scarce or focal. Furthermore, EAL were characterized by TF, FVII, and FX expression throughout the SMC-rich intima. Medial SMC-associated FVII was located in the cytoplasm and not on the membrane. FXII and FII showed enhanced expression in medial SMC. TF, FVII, FX, fibrin, kallikrein, thrombomodulin, and TFPI were also asso-

Figure 3. Layer-selective coagulation factor activity analysis, presenting the procoagulant state of tunica intima, media, and adventitia in EAL vs SAAL. A, Hematoxylin and eosin–stained sections, demonstrating histologically controlled layer preparation and confirming the anatomy of the desired vessel wall layer. The activities of coagulation proteins were then tested in tunica intima, media, and adventitia, prepared from the harvested layers of paired EAL and SAAL: endogenous thrombin potential (ETP) (B); prothrombin (C); FX (D); FXII (E); TF (F); and TFPI (G). TF/TFPI ratio is demonstrated in H. *Statistical significance (Wilcoxon 2-sample test, 2-tailed exact $P<0.05$). INT indicates tunica intima; MED, tunica media; and ADV, tunica adventitia.
Associated with medial SMC, whereas FIX demonstrated a more patchy expression. Within the adventitia, the vasa vasorum externa showed positive staining for most of the studied factors, whereas the fibroblasts were positively associated with FX, prothrombin/thrombin, kallikrein, von Willebrand factor, and FXII.

An extensive overview of all single- and double-staining observations in EAL and SAAL is provided in Figure 5, Table 2, and Figure II in the online-only Data Supplement.

Immunohistochemical Staining: SAAL

In atherosclerotic tissues classified as SAAL, the endothelial luminal lining was moderately positive for FIX, thrombomodulin, APC, and von Willebrand factor, whereas FXI stained weakly positive (Figure 5). Some endothelial segments demonstrated a focal expression of the anticoagulant protein protein S. Moreover, all anticoagulant proteins (protein S, thrombomodulin, APC, and TFPI) were found to be associated with macrophages and foam cells. Furthermore, thrombomodulin, APC, and TFPI were also localized in the endothelial cells of the vasa vasorum and in endothelial cells of vessels sprouting into the lesions. Besides thrombomodulin, intimal and medial SMC contained most of the procoagulant proteins, as well as thrombin and fibrinogen/fibrin (Figure 5 and Figure II in the online-only Data Supplement). A slight focal association between FIX and XI with intimal SMC was observed. In contrast, SMC of the media stained moderately for FXII. Some of the medial SMC stained positive for FXI but also showed double positive staining for both CD68 and anti-smooth muscle actin, suggesting either transdifferentiation of SMC into foam-like cells or SMC outgrowth from mononuclear cells; the latter was reported...
recently to be a thrombin-promoted action.\textsuperscript{19} TF and FVII, FX, FXI, and FXII were either weakly or focally present on macrophages, and FXII was also found on some foam cells. FIX showed a pronounced focal distribution on both macrophages and foam cells. Some fibroblasts contained FX, FXII, and fibrin.

None of the SAAL from the current set of lesions showed a strong or even moderately positive staining for TF; this was also the case in the necrotic core. The necrotic core revealed a focal presence for most of the procoagulant proteins, except for thrombin, fibrin, and the anticoagulant APC, which stained weakly positive.

A broad histological evaluation is available in Figure 5, Table 2, and Figure II in the online-only Data Supplement.

**Immunofluorescence Staining: Colocalization of TF/FVII/FX with Macrophages and Vascular SMC**

Double immunofluorescence staining with CD68 for macrophages and anti–smooth muscle actin for SMC suggests that most of the macrophages and SMC were involved in the synthesis of TF, FVII, and FX. The formation of the ternary complex TF/FVII/FX is a potent trigger not only for coagulation (thrombin) but also for many proinflammatory cell-signaling pathways that are pivotal in cardiovascular disease. Therefore, we also examined the presence of these procoagulant proteins on macrophages and SMC by means of immunofluorescence staining on corresponding EAL and SAAL sections, which revealed that TF/FVII/FX colocalized with both macrophage/foam cells and SMC, suggesting a local system of thrombin generation, which may regulate pathological processes such as cell migration and inflammation. When EAL and SAAL are compared, colocalization is scarcer and more diffuse in SAAL, whereas EAL sections show brighter labeling and denser character (Figure 6).

**Discussion**

The present study shows that atherosclerotic plaques exhibit functional activity of many coagulation proteins (prothrom-
bin, FX, FXII, and TF) and represents the first study to demonstrate the presence and distribution of all coagulation proteins in both early and advanced human atherosclerotic plaques. We provide new data pointing to local synthesis of several coagulation proteins within the atherosclerotic vessel wall. Furthermore, we indicate a colocalization of key procoagulant proteins with SMC and macrophages, suggesting an active, cell-based coagulation network within the atherosclerotic plaque. Finally, the principal finding of this study is an enhanced procoagulant profile of EAL compared with SAAL homogenates, consolidated by an elevated thrombin generation potential and significantly increased TAT complex levels in early-stage atherosclerotic tissues. Thus, we provide novel evidence that may help in widening the thrombogenic spectrum of “high-risk” plaques and suggest that local coagulation factors may play an important role not only in contributing to the onset of atherothrombosis but also in contributing to progression of the atherosclerotic process.

In contrast to our expectations, these data reject the initial hypothesis that thrombin generation would positively correlate with progression of atherosclerosis. One possible mechanism that might explain the abundant presence and functional activity of coagulation proteins in the early stage of atherosclerosis is that many of the coagulation proteins help to propagate the atheromatous plaque by inducing multiple proatherogenic actions such as cellular adhesion, migration, angiogenesis, and inflammation.7,12 In addition to their prothrombotic nature, coagulation proteases induce cell proliferation,6 and the latter are of great importance in determining the stability of an atherosclerotic lesion. The abundance of almost all (intrinsic and extrinsic) coagulation proteins suggests that the generation of thrombin is an active process during atherogenesis, supporting a major role of thrombin (and possibly fibrin) in this condition. Moreover, the enhanced procoagulant state of EAL was additionally confirmed by the layer-selective analysis, which also revealed a significantly increased thrombomimetic phenotype for tunica adventitia that suggests local coagulation factors may play an important role not only in contributing to the onset of atherothrombosis but also in contributing to progression of the atherosclerotic process.

The pleiotropic effects of proteases such as thrombin and activated FX, as well as the cell growth–promoting effects of fibrin (and its split products), may also be evoked as part of a response to injury mechanism. This response action of blood coagulation is now well established in inflammatory conditions like sepsis. As a side effect of this process, the formation of fibrin may serve to protect the early lesions from rupture and contribute to plaque stability. In addition, a recent study demonstrates that hypercoagulability in transgenic mice promotes plaque stability.27

At the same time, the activity of coagulation proteases contributes to local inflammation and angiogenesis, and therefore the latter will eventually prevail over processes such as proliferation, thus compromising plaque stability. This proinflammatory state of the evolving plaque, including
increased apoptosis of SMC, gradual protein loss, and enhanced angiogenesis, will herald plaque evolution and greater vulnerability. Hence, EAL may be more stable because of more clotting activity, whereas SAAL may be more vulnerable because of instability. In the case of a plaque rupture, even relatively small amounts of TF and other proteins may still be highly thrombogenic, precipitating thrombus formation and cardiovascular events.

In conclusion, our findings provide substantial new data illustrating the close involvement of coagulation proteins in the entire process of atherogenesis. Whereas in the early lesions essentially all coagulation proteins, including those from the contact/intrinsic system, are readily detectable (possibly supporting plaque stability), on transformation to advanced lesions the amount and activity of these proteins diminish. The loss in coagulation activity, possibly due to increased inflammatory pressure, may reduce plaque stability and contribute to the risk of plaque rupture. These results point to various and specific functions of coagulation proteins in regulating progression of atherosclerosis and may provide novel insights into the genesis of atherothrombosis. These data also suggest ways to modulate atherogenesis and possibly reduce atherosclerosis that may eventually be clinically useful. The fact that new specific anticoagulant agents are being clinically tested underscores the necessity of further studies in this area.

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Disclosures

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References


**CLINICAL PERSPECTIVE**

Apart from their well-established role in coagulation, several hemostatic factors (eg, tissue factor/activated factor VII complex, activated factor X, thrombin) have been reported to evoke multiple proatherogenic events on a wide range of arterial wall constituents. While exploring the presence and distribution of all coagulation proteins in both early and advanced human atherosclerotic plaques, we found a colocalization of key procoagulant proteins with smooth muscle cells and macrophages, thus suggesting an active cell-based coagulation network within the atherosclerotic plaque. Furthermore, we provide new evidence pointing toward local synthesis of several coagulation factors within the atherosclerotic vessel wall. The principal finding of this study, indicating enhanced procoagulant activity of early atherosclerotic plaques versus stable advanced plaques, suggests a role for the hemostatic proteins and hypercoagulability in regulating the onset and progression of atherosclerosis. These findings may become clinically relevant in the new era of selective oral anticoagulants, in which such agents may have effects on the complex process of atherosclerosis beyond their direct antithrombotic action.
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SUPPLEMENTAL MATERIAL

SUPPLEMENTAL MATERIALS AND METHODS

EAL & SAAL Layer Preparation and Homogenization

A segment from each of the collected arterial samples was used for an anatomic separation into its three layers – tunica intima, media and adventitia. Once the separated layer strips were obtained, a section of each layer was examined (H&E staining) in order to confirm the desired layer anatomy. The remaining part of the layer specimen was homogenized according to the aforementioned technique and used for further coagulation factors activity analysis. Layer preparation was successful in 21 out of the 27 pairs of EAL and SAAL (42 out of 54 atherosclerotic specimens).

Effect of Time Delay between Death and Post-Mortem Examination on Coagulation Proteins Activity

To address the question about the effect of time on the stability of coagulation proteins activities, we obtained vital atherosclerotic lesions from patients undergoing carotid endarterectomies (Department of Vascular Surgery, Maastricht University Medical Center; n=4). Each specimen was divided into 4 proportionally equal longitudinal segments (consisting of the entire vessel wall/plaque structures) and was kept at 4°C in PBS solution, thus simulating post-mortem conditions. One segment per sample was collected per time point (baseline (0 hours), 2 hours, 4 hours and 8 hours), snap frozen, homogenized and then studied for the activities of TF, FII, FX and FXII as described in the “Materials and Methods” section.
**Thrombin Generation**

The Calibrated Automated Thrombogram (CAT, Thrombinscope, the Netherlands) was used to determine the contribution of atherosclerotic tissue homogenates to thrombin generation in human plasma (in triplicates; inter-assay CV<10%) We adapted the protocol from the recording of thrombin generation curves in platelet poor plasma as described previously\(^1\): thrombin generation was triggered in 80 µL of platelet poor pooled human plasma (University Hospital Maastricht, consisted of plasma from 80 healthy volunteers) by adding 15 µl of tissue homogenate (5 mg/mL total protein), 16 mM Ca\(^{2+}\) and 4 µM phospholipids to the reaction mixture (final concentrations, determined as optimal pre-analytical conditions for CAT method above which a threshold effect is observed\(^2^4\)). Endogenous thrombin potential (ETP, the area under the curve) was calculated from the thrombin generation curve using Thrombinscope software (Thrombinscope B.V., The Netherlands).

**Prothrombin, FX and FXII Activity Assays**

FII, FX and FXII activities in atherosclerotic tissue homogenates were assessed via modified thrombin generation assays by adapting the original protocol, as described\(^1\).

**Prothrombin Activity Assay**

The assay was performed in triplicates. The reaction mixture consisted of 80 µl FII-deficient or diluted plasma, in the presence of 10 µl tissue supernatant (final protein concentration of 0.4 mg/mL), 20 µl MP reagent (Thrombinscope B.V.) containing 4 µM phospholipds, 3 µl corn trypsin inhibitor (CTI, Hematologic Technologies, Inc., final concentration of 40 µg/mL), and 2 µl Active site inhibited seven (ASIS, final concentration of 25 nM). CTI was used to specifically inhibit activated FXII (FXIIa), whereas ASIS binds to TF, thereby suspending FVII/VIIa of forming a complex with the latter and blocking the extrinsic pathway-driven coagulation. The reaction was initiated by adding FluCa buffer containing Fluobuffer, 16 mM CaCl\(_2\), fluorogenic substrate and ecarin (from Echis carinatus venom, Sigma-Aldrich). Ecarin is used as a specific activator of prothrombin. Hence, thrombin generation was
completely dependent on the presence and activity of FII in the atherosclerotic lesion homogenate. A reference curve was prepared by serial dilution of FII-deficient plasma in human normal pooled plasma obtained from 80 healthy volunteers within the department of Internal Medicine, Maastricht University Medical Center. Fluorescence was read in an Ascent Reader (Thermolabsystems OY, Helsinki, Finland) equipped with a 390/460 filter set, and thrombin generation curves were calculated with the Thrombinscope software (Thrombinscope BV) as described previously.

**FX and FXII Activity Assay**
A method analog to the FII activity described above was implemented for assessing FX activity. FX-deficient plasma (Dade Behring) was utilized for the preparation of the standard curve and 5 µl Russell's viper venom factor X activator (RVV-X, Enzyme Research Laboratories Inc.) was added in the trigger mix as a specific activator of FX. Hence, thrombin generation was determined by the presence and activity of FX in the atherosclerotic lesion homogenate. For the FXII activity assay, FXII-deficient plasma (George King) was used for preparation of the standard curve and analysis of tissue homogenates. CTI was omitted from the reaction to allow for FXII dependent activation of thrombin generation. Kaolin (Sigma-Aldrich) was used as trigger at a final concentration of 400 µg/mL. The test was carried out in triplicates.

**Effect of Phospholipid Concentration on Thrombin Generation / Thrombin Generation in Normal Arterial Vessel Wall Homogenates**
Due to the different nature of the atherosclerotic lesions, we assumed that early and stable advanced plaques may also vary in phospholipid content as a result of altered cellular density, the latter normally observed upon atherosclerotic progression. Hence, we estimated that differences in the phospholipid levels in EAL (plaques higher in cellular density) and SAAL homogenates (plaques with more fibrotic and acellular character) may potentially influence thrombin generation, yielding an enhanced pro-thrombotic state in EAL in vitro. To address this matter, we first studied the influence of increasing
phospholipid concentrations (1, 2, 3, 4, 5, 10, 20, 30, 40 and 50 μM) on thrombin generation in normal pool plasma, triggered by 1pM TF (without addition of plaque homogenates). In addition, areas from normal abdominal aorta were harvested from the same individuals (n=27) from which the original set of atherosclerotic plaques was obtained. All tissue specimens were histologically evaluated and showed no signs of atherosclerosis development. Homogenates were prepared and thrombin generation was assessed as described.

**Thrombin-Antithrombin Complexes (TAT) Levels**

TAT complexes were determined in triplicates using a commercial ELISA kit (Cat.#TAT-EIA, Kordia, The Netherlands). This assay was performed in compliance with all manufacturer's directions, however, it was slightly adapted with respect to the use of tissue homogenates. Instead of plasma, the same amount of atherosclerotic homogenate was added per well (100 μl of diluted sample with a final concentration of 5 mg/mL total protein).

**Tissue Factor Activity Assay**

TF activities in tissue homogenates were determined in triplicates using a home-made activity assay. In brief, dissolved tissue homogenates with a concentration of 5 mg/mL total protein were diluted 160 times in HN-buffer. Samples were incubated for 10 minutes at 37°C in the presence of recombinant FVII (FVII) (Novo Nordisk, Bagsværd, Denmark), 0.2 mM 20/80 PS/PC vesicles, 1 U/mL Bovine FX (Sigma-Aldrich) and 100 mM Ca²⁺. The formation of FXa was then measured kinetically using the chromogenic substrate 2765 (Chromogenix, final concentration of 0.7 mg/mL diluted in 50 mM Tris-HCl, 175 nM NaCl, 30 mM Na₂EDTA, pH 7.4) by measuring the OD at 405 nm each 15 seconds, for 15 minutes at 37°C.
**TFPI Antigen Assay**

The levels of TFPI antigen in human EAL and SAAL were measured by the means of a home-made high-sensitive total TFPI immunoenzymatic method (ELISA). This assay was performed in triplicates using own monoclonal anti-TFPI K1 fragment antibody for capture and a specific HRP-conjugated monoclonal anti-TFPI K2 fragment antibody for detection (C. F. A. Maurissen, J.R., and T. M. Hackeng, manuscript in preparation).

**RNA Isolation and Quantification/ Microarray Hybridization and Data Analysis**

In a separate new set, consisting of paired early and advanced carotid lesions (n=4 pairs) collected from each patient upon autopsy, we studied which coagulation proteins show mRNA expression within the arterial vessel wall upon atherosclerotic progression. Total RNA was isolated using the guanidine isothiocyanate/CsCl method, followed by further purification and concentration using RNeasy mini columns (Qiagen, Hilden, Germany). RNA quantity and quality were determined using a nanodrop spectrophotometer (Witec AG, Littau, Switzerland) and a 2100 Bioanalyser (Agilent Technologies, Palo Alto, USA) respectively. Good quality RNA (RIN≥5), from both EAL and SAAL, was successfully collected. Double-stranded cDNA was synthesized from ~2.0 μg of total RNA using the One-Cycle Target Labeling Kit (Affymetrix, Santa Clara, CA, USA), and used as a template for the preparation of biotin-labeled cRNA using the GeneChip IVT Labeling Kit (Affymetrix, Santa Clara, CA, USA). Biotin-labeled cRNA was hybridized in duplicate to the HGU133 2.0 Plus Array (Affymetrix, Santa Clara, CA, USA), washed, stained with phycoerythrin-streptavidin conjugate (Molecular Probes, Eugene, USA), and the signals were amplified by staining with biotin-labeled anti-streptavidin antibody (Vector Laboratories, Burlingame, USA) followed by phycoerythrin-streptavidin. The arrays were laser scanned with the GeneChip Scanner 3000 (Affymetrix, Santa Clara, CA, USA) according to the manufacturer’s instructions. Data were saved as a raw image file and quantified using GCOS 1.2 (Affymetrix, Santa Clara, CA, USA). Rosetta Resolver Platform Version 4 (specifically developed for Affymetrix GeneChips) was used to correct for multiple testing and analyze differences in single gene expression.
**Immunohistochemical (IHC) & Immunofluorescence Stainings**

Paraffin sections (4 µm) were deparaffinized and washed 3 times in Tris-buffered saline (5 mmol/L Tris-HCl, pH 7.5, 140 mmol/L NaCl). Before application of the TM and FIX antibody, tissue sections were pretreated with pepsin (Sigma Chemical Company, St. Louis, MO, #7012) (1 mg/mL in 0.1 M HCl) at room temperature for 30 minutes, in order to increase the visibility.


For the mouse monoclonal antibodies, biotinylated sheep anti-mouse IgG (1:250, Amersham Life Science, # RPN-1001) was used as the secondary antibody, whereas power vision poly-AP anti-goat (Klinipath, Duiven, the Netherlands # DPVG-110 AP) was used as a secondary antibody for the polyclonal antibodies. After incubation with an alkaline phosphatase–coupled avidin-biotin complex (ABC complex, Dako), antibodies were visualized with an alkaline substrate kit (Vector SK-5100, Vector Laboratories, Inc). Sections were counterstained with hematoxylin (Klinipath, # 4085-9002,) and
mounted with imsol (Klinipath, # 7961,) and entellan (Merck # 7961,). In negative controls, incubation with primary antibody was omitted.

Double staining was performed to co-localize presence of coagulation factors with vascular SMC or MΦ. For this purpose, the single staining procedure was followed by secondary staining just before the hematoxylin counterstaining. Mouse anti-human CD68 (1:100, Dako # M 0814) and mouse anti-human ASMA (1: 500, Dako, # M 0814) were used to identify MΦ and SMC, respectively. Before application of the CD68 antibody tissue sections were pretreated with pepsin (1 mg/mL in 0.1 M HCl) at room temperature for 30 minutes. For visualization, biotinylated sheep anti-mouse antibody (Amersham, # RPN-1001), strept ABC-alkaline phosphatase (Dako, # K-0391) and Alkaline Phosphatase kit I (blue)(Vector Laboratories, Burlingame, Carlicifornia. #SK-5100,) were used.

Localization and co-localization of hemostatic proteases was further assessed by the use of single and double immunofluorescence staining on paired EAL and SAAL sections. The following secondary antibodies were utilized: Rabbit polyclonal anti-goat IgG - H&L (FITC-labeled, Abcam, ab6737, 1:200); goat polyclonal anti-mouse IgG - H&L (FITC-labeled, Abcam, ab6785, 1:200); rabbit polyclonal anti-goat IgG - H&L (Rhodamine-labeled, Abcam, ab6738, 1:200) and goat polyclonal anti-mouse IgG - H&L (Rhodamine-labeled, Abcam, ab6786, 1:200).

**Immunohistological Evaluation**

A semi-quantitative visual scoring system was used to evaluate the IHC staining. Two investigators (M.Y., S.N.), blinded with respect to the plaque phenotype, independently examined the specimens using light microscopy at 250x magnification. The intensity of the staining was ranked on an arbitrary scale as follows: - = Negative; + = Weak positive; ++ = Moderate positive, +++ = Strong positive; Focal (F) = Only certain cells stain, not all. For this purpose, fifteen random slides were analyzed for each of the stained coagulation proteins as per plaque type. The intra- and inter-observer variability was less than 10%.
SUPPLEMENTAL TABLES

Table S1: Differential expression of coagulation factor genes in EAL vs. SAAL.

Negative fold change values indicate up-regulation of coagulation factors expression in EAL, whereas positive values represent up-regulated genes in SAAL.
<table>
<thead>
<tr>
<th>Sequence Description</th>
<th>Relative mRNA Level - Ratio (SAAL/EAL)</th>
<th>Fold Change</th>
<th>P-value</th>
<th>Intensity - EAL</th>
<th>Intensity - SAAL</th>
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<td>Fibrinogen β-chain</td>
<td>0.88219</td>
<td>-1.13362</td>
<td>0.15738</td>
<td>15.07589</td>
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<td>FIX</td>
<td>1.17648</td>
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<td>0.00030</td>
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<td>FV</td>
<td>1.03517</td>
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<td>0.49269</td>
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<td>FVII</td>
<td>1.05108</td>
<td>+1.05108</td>
<td>0.32227</td>
<td>14.80140</td>
<td>14.98169</td>
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<td>FVIII, Procoagulant Component</td>
<td>0.88213</td>
<td>-1.13362</td>
<td>0.00965</td>
<td>21.98895</td>
<td>20.95785</td>
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<td>FVIII-associated (Intronic Transcript) 1</td>
<td>0.76292</td>
<td>-1.31076</td>
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<td>FX</td>
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<td>FXIII, A1 Polypeptide</td>
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<td>0.01157</td>
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<td>Heparin Cofactor II</td>
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<td>PAI-1, Member 1</td>
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<td>PAI-1, Member 2</td>
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<td>Plasminogen Activator, Urokinase</td>
<td>1.13254</td>
<td>+1.13254</td>
<td>0.00122</td>
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<td>Protein S (α)</td>
<td>1.20045</td>
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<td>0.00269</td>
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<td>Thrombomodulin</td>
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<td>-1.06691</td>
<td>0.31647</td>
<td>40.74794</td>
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<td>von Willebrand Factor</td>
<td>0.94222</td>
<td>-1.06132</td>
<td>0.28796</td>
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<td>α-2 Antiplasmin</td>
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<td>+1.15311</td>
<td>0.00439</td>
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<td>α-2-Macroglubulin</td>
<td>1.17901</td>
<td>+1.17901</td>
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<td>Marker, Positive Control</td>
<td>0.78588</td>
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<td>7.67398E-20</td>
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<td>Blank, Background Negative Control</td>
<td>0.90907</td>
<td>-1.10002</td>
<td>0.13188</td>
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SUPPLEMENTAL FIGURES

Figure S1:
Figure S2:

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FIGURE LEGENDS

Figure S1: Effect of Time Delay between Death and Post-Mortem Examination on (A) TF, (B) FII, (C) FX and (D) FXII activities in homogenized human atherosclerotic plaques (n=4)
Each dotted line, connecting 4 grey dots, represent one single atherosclerotic plaque (n=1), initially divided into 4 proportionally equal segments which were kept at 4ºC in PBS and harvested at different time points (0 hrs, 2 hrs, 4 hrs and 8 hrs) after autopsy (p values were calculated by using Repeated-measures ANOVA test). Thus, each dotted line also indicates the effect of time on the activity of the tested coagulation proteins.

Figure S2: Immunohistochemical (IHC) stainings - Localization and co-localization of coagulation proteases in human atherosclerotic plaques at a cellular level, 100x magnification. Positive staining is presented in red. (Images from both EAL and SAAL are shown.)
Legend: TF – Tissue Factor; FVII - Factor VII; FX – Factor X; FII/FIIa – Prothrombin/Thrombin; FXII – Factor XII; FXI – Factor XI; FIX – Factor IX; KLK – Kallikrein; TM – Thrombomodulin; PS – Protein S; APC – Activated Protein C; TFPI – Tissue Factor Pathway Inhibitor; TAT – Thrombin-Antithrombin Complex; vWF – von Willebrand factor; ECs – Endothelial Cells; MΦ – Macrophages; FC – Foam Cells; SMC – Smooth Muscle Cell

SUPPLEMENTAL REFERENCES


