A 2-Step Mechanism of Arterial Thrombus Formation Induced by Human Atherosclerotic Plaques

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Objectives
The aim of this study was to understand the initial mechanism of arterial thrombus formation induced by vulnerable human atherosclerotic plaques to re-assess and improve current antithrombotic strategies.

Background
Rupture of atherosclerotic plaques causes arterial thrombus formation that might lead to myocardial infarction and ischemic stroke. Atherothrombosis is considered as an inseparable tangle of platelet activation and coagulation processes, involving plaque components such as tissue factor (TF) and collagen as well as blood-borne TF and coagulation factor XIIa (FXIIa). A combination of anticoagulants and antiplatelet agents is the present treatment.

Methods
Human atheromatous plaque material was exposed to blood or blood components at physiological calcium/magnesium concentration. Platelet aggregation and coagulation were measured under static and arterial flow conditions by state-of-the-art microscopic and physiological techniques. Plaque TF, plaque collagen, FXIIa, and platelet glycoprotein VI (GPVI) were specifically inhibited.

Results
Plaques induced thrombus formation by 2 discrete steps. The rapid first phase of GPVI-mediated platelet adhesion and aggregation onto plaque collagen occurred within 1 min. The second phase of coagulation started after a delay of >3 min with the formation of thrombin and fibrin, and was driven entirely by plaque TF. Coagulation occurred only in flow niches provided by platelet aggregates, with no evidence for a role of blood-borne TF and FXIIa. Inhibition of GPVI but not plaque TF inhibited plaque-induced thrombus formation.

Conclusions
The major thrombogenic plaque components—collagen and TF—induce platelet activation and coagulation, respectively, in 2 consecutive steps. Targeting specifically the first step is crucial and might be sufficient to inhibit atherothrombus formation. (J Am Coll Cardiol 2010;55:1147–58) © 2010 by the American College of Cardiology Foundation

Rupture of vulnerable atherosclerotic plaques causes thrombus formation leading to acute myocardial infarction, ischemic stroke, and peripheral arterial occlusive disease (1–4). Arterial thrombus formation after plaque rupture is considered as reciprocally-amplifying, inseparable processes of platelet activation and coagulation (5). Optimal treatment requires a combination of anticoagulants and antiplatelet agents with the profound negative prospect of bleeding risk in these patients (6–8).

Systemic and local factors are critical for atherothrombosis. Patients with cardiovascular disease often have increased platelet reactivity and coagulation (9,10), and pro-thrombotic substances are present in human atherosclerotic plaques that are not found in the intima of healthy arteries (11–14). Abundant evidence exists that a main pro-thrombotic substance in human atherosclerotic plaque is tissue factor (TF), which is expressed on apoptosis-derived microparticles and present mainly in the lipid-rich core (3,15). Tissue factor—through the formation of thrombin—induces platelet activation and stimulates fibrin formation. In lipid-rich human atherosclerotic plaques, direct platelet agonists have also been identified: lysosphosphatidic acid mediating platelet shape change, and morphologically diverse collagen type I and type III structures inducing platelet adhesion and aggregation under arterial flow through binding to platelet collagen receptors, including glycoprotein VI.

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(GPVI) (14,16,17). The von Willebrand factor has an essential role in mediating platelet adhesion to collagen fibers and atherosclerotic plaques and supporting subsequent platelet aggregation on these matrixes at high shear conditions (11,18).

The formation of a thrombus after plaque rupture is triggered by thrombogenic plaque substances that set in motion an avalanche of aggregating platelets and fibrin strand formation, growing and accelerating as it proceeds. We reasoned that identifying the critical trigger(s) of plaque-induced thrombus formation will improve current antithrombotic strategies, which are so far directed mainly against the avalanche itself, i.e., platelets (fibrinogen receptor antagonists) with their positive feedback activation loops (aspirin, P2Y 12 antagonists) and thrombin (heparins, bivalirudin) but not their triggers.

In the present investigation, we studied atheromatous human plaques, exposed them to blood and blood components, and applied and optimized state-of-the-art microscopic and physiological techniques. We studied the kinetics of atherothrombosis and addressed the following main questions: 1) Is plaque TF—through the formation of thrombin—involves in plaque-induced platelet adhesion and aggregation? 2) Is collagenous matrix of plaques—via its activation of platelets and/or coagulation factor XII (FXII)—involved in plaque-induced thrombin and fibrin formation? We obtained a surprisingly simple answer to these questions: mainly that plaque collagen induces platelet adhesion and aggregation, whereas plaque TF induces blood coagulation.

Methods

Reagents and antibodies. Collagen (Horm) was obtained from Nycomed Pharma (Munich, Germany). Sodium citrate was from Fresenius Kabi Deutschland (Bad Homburg, Germany); heparin, Triton X-100, bovine serum albumin (essentially fatty acid free), mepacrine, and collagenase (type VII from Clostridium histolyticum) as well as mouse immunoglobulin G (IgG)1 and rabbit IgG were obtained from Sigma (Taufkirchen, Germany). Glutaraldehyde was purchased from Serva, (Heidelberg, Germany). The GPVI receptor blocking single-chain variable fragment antibody 10B12 and its control 2D4 (anti-HLA type A2) was selected from the Cambridge Antibody Technology (now MedImmune Cambridge, Cambridge, United Kingdom) phage display libraries as described previously (19). Anti-collagen type I and anti-collagen type III antibodies were obtained from Rockland Immunochemicals (Gilbertsville, Pennsylvania). Anti-TF antibody was purchased from American Diagnostica (Pfungsstadt, Germany); corn trypsin inhibitor (CTI) was purchased from Calbiochem (San Diego, California), and lipitated TF (Recombiplasmin) was purchased from Instrumentation Laboratory (Kirchheim, Germany). For thrombin generation assays the fluorogenic substrate Z-GGR-AMC (Bachem, Bubendorf, Switzerland) was dissolved to a concentration of 5 mmol/l in buffer containing 10% dimethyl sulfoxide; as buffer we used 20-mmol/l HEPES, 150-mmol/l sodium chloride, with 60-mg/ml bovine serum albumin (Lot A-7030, Sigma, Taufkirchen, Germany), pH 7.35.

Human atheromatous plaques. Atherosclerotic plaques were obtained from patients who underwent endarterectomy of the carotid artery with en bloc preservation of the tissue specimen (20). Patient consent was obtained and approved by the Ethics Committee of the Faculty of Medicine of the University of Munich. Plaque specimens were immediately shock frozen at −80°C after surgical removal. The atheromatous plaques macroscopically visible by their yellowish color were dissected under sterile conditions from other regions of the atherosclerotic tissue (21). Calcified plaques were discarded. The plaques were characterized by histological analysis as atheroma with a thin fibrous capsule (17,18,22). They were homogenized and processed as described (17,18). The plaque concentration was adjusted to 100 mg wet weight/ml. Plaques were obtained from a total of 45 patients.

Plaque collagen and TF localization. The collagen immunostaining of paraffin sections of atheromatous plaques and of plaque homogenates was done as described (17,18,20). For TF-immunostaining of plaque tissue sections and homogenates, an anti-TF antibody (No. 4509, American Diagnostica) was used. Immunofluorescence microscopy was performed with a Zeiss confocal fluorescence microscope (LSM510 META) and software.

Blood sampling and platelet preparation. In accordance with the Helsinki protocol, informed consent was obtained from healthy blood donors that had not taken any platelet- or coagulation-relevant medication 14 days before blood-drawing. Platelet-rich plasma (PRP) was prepared from citrate (0.313% wt/vol final concentration) anticoagulated blood by centrifugation for 20 min at 180 g. For platelet-poor plasma (PPP) the PRP was additionally centrifuged for 10 min at 1,000 g. For platelet-free plasma (PFP) the PRP was centrifuged for 5 min at 4°C and at 6,000 g, and subsequently the supernatant was centrifuged at 10,000 g.

Platelet aggregation and blood coagulation in whole blood and PRP. Platelet aggregation stimulated by collagen, atheromatous plaque, or TF was measured turbidimetrically with stirred PRP (1,100 rpm) at 37°C in a LABOR aggregometer (Fa. Fresenius, Bad Homburg, Germany) or measured via impedance in stirred (1,000 rpm) diluted blood by using the Multiplate system (Dynabyte Medical, Munich, Germany) as described by our group recently (23). Calcium chloride (CaCl2) (12 mmol/l) and magnesium chloride (MgCl2) (0.1 mmol/l) were added to obtain phys-
iological plasma concentration of these divalent cations, and to observe clotting. The concentration of calcium (Ca^{2+}) was 1.23 ± 0.08 mmol/l and that of magnesium (Mg^{2+}) was 0.51 ± 0.23 mmol/l (n = 5) as measured with the electrolyte analyzer CRT-8 (Nova Biomedical, Roedermark, Germany). Recombinant lipitated TF showed dose-dependent (0.05 to 1.00 ng/ml) clotting under these conditions. Clotting was detected in both aggregometers as a sudden vertical deflection in the aggregation curve.

The kinetics of fibrin formation was also determined by thrombelastography (Rotem, Pentapharm GmbH, Munich, Germany).

**Thrombin generation in human PRP and PFP.** Measurements of thrombin generation with the Thrombogram method (24) were performed with a ThermoLabsystems Ascent reader (Helsinki, Finland) coupled to the Thrombinscope software developed by Synapse (Utrecht, the Netherlands). Collagen, TF, or atheromatous plaque was incubated together with human PRP (300,000 platelets/µl) or PFP and buffer (20-mmol/l HEPES, 5-g/l bovine serum albumin, 0.14-mol/l sodium chloride, 0.6-mmol/l MgCl₂, pH 7.35). Thrombin measurement was started through the addition of fluo buffer (20-mmol/l HEPES, 60-µg/ml bovine serum albumin, pH 7.35) containing the fluorescent thrombin substrate Z-GGR-AMC (400 mol/l) and CaCl₂ (12 mmol/l). Measurements were performed in triplicate.

**Inhibition of plaque TF and collagen-platelet GPVI interactions.** Plaque (0.5 mg) was incubated with polyclonal anti-TF antibody (1 µg) for 10 min at 37°C or rabbit IgG as control. Platelet collagens type I and III were blocked with specific polyclonal antibodies (0.5 µg/µl), again with rabbit IgG as control. To disrupt the collagenous structures in the plaque, atheromatous plaque (2 mg/ml) was incubated for 24 h at 37°C with collagenase (0.6 µg/µl) dissolved in 50 µl TESCA buffer (50-mmol/l TES, N-Tris[hydroxymethyl]methyl-2-amino-ethan-sulfonic acid, 0.36-mmol/l CaCl₂) containing in addition 9-mmol/l CaCl₂. Control incubations were with TESCA buffer plus 9 mmol/l CaCl₂ alone. Platelet GPVI receptor was inhibited by anti GPVI–single-chain variable fragment 10B12 (50 µg/ml) or control (anti-HLA type A2) (19).

**Analysis of platelet adhesion and thrombus formation in flowing whole blood.** Atheromatous plaque homogenate was immobilized onto glass coverslips as described (17,18,21). The height of the plaque coating was <5 µm. Glass coverslips were also coated with 30 µl of lipitated TF (0.3 µg/ml and 0.9 µg/ml) and fibrous collagen (5 µg/ml). The coated surfaces were perfused (syringe pump; Harvard Apparatus, Holliston, Massachusetts) with citrated blood after addition of CaCl₂ and MgCl₂ in a parallel plate flow chamber at a wall shear rate of 1,500 s⁻¹. The chamber was mounted on the stage of an upright microscope (Axioskop 2 plus, Carl Zeiss, Germany), and blood flow and platelet deposition were observed and recorded in real-time with a CCD camera (AVT-BT 71, AVT Horn, Aalen, Germany) and a DVD video recorder (Panasonic, LQ-MD 800, AVT Horn). We used bright field and reflection interference contrast microscopy. Both required no labeling of platelets, whereas for fluorescence microscopy the platelets were stained with 10-µmol/l mepacrine. Reflection interference contrast microscopy allowed us to visualize platelet spreading. By combining bright field microscopy and fluorescence microscopy, platelet adhesion and aggregation could be discerned from the plaque coating. During each flow experiment, representative images (fields of view) of the perfused surface were recorded and later digitized and analyzed off-line with the Matrox Inspector software package (Matrox Electronics Systems, Ltd., Dorval, Quebec, Canada). In each field the areas covered by plaques and the respective aggregates attached to them were measured and related as percentage “plaque covered with platelet aggregates.” Single platelet adhesion was easily discernible and was not included in the measurements. Online Video 1 was prepared with Adobe Premiere (Adobe Systems, San Jose, California).

**Statistical analysis.** Results are shown as mean ± SD from n experiments. Statistical significance was calculated by paired and unpaired Student t test or Mann-Whitney U test, where appropriate. Differences were considered significant when p (2-tailed test) was <0.05.

**Results**

Characterization of collagen type III-positive matrix structures and TF in atheromatous plaques. Atheromatous plaques containing both lipid-rich core and capsule were used in the present study. Figure 1A shows representative light micrographs of plaque sections after staining with specific anti-collagen type III and anti-TF antibodies. It is seen that TF-positive structures and collagenous matrix are not colocalized. The TF was mainly found in macrophages, foam cells, and smooth muscle cells in the fibrous cap, often at the transition from cap to core. Endothelial cells were TF-negative. Plaque homogenates containing all potential thrombogenic substances were used for further experimentation. Figure 1B shows fluorescence micrographs of plaque homogenates after coating onto glass coverslips and staining with specific anti-collagen type III and anti-TF antibodies. It can be seen that TF-positive structures and collagenous matrix are not colocalized. The TF was mainly found in macrophages, foam cells, and smooth muscle cells in the fibrous cap, often at the transition from cap to core. Endothelial cells were TF-negative.
plaque-induced clotting times in PPP to 21 ± 3.9 min (n = 10), close to control values in the absence of plaque (>25 min). Together these results show that TF is present in the fibrous cap and lipid core of the plaques studied and that TF is the main determinant of clotting in PPP. The mean concentration of active TF in atheromatous plaques was calculated as 62 pg/mg, which is similar to the TF antigen content described in the literature in atherectomy samples of human coronary atherosclerotic plaques (range 0.03 to 0.05 ng TF/mg wet weight) (25) and to our own immunoblot data with anti-TF antibody (data not shown).

**Kinetics of plaque-induced platelet aggregation and clot formation under static conditions: comparison with collagen and TF.** In citrated PRP re-adjusted to physiological Ca\(^{2+}\) and Mg\(^{2+}\) concentrations, both plaque and collagen induced platelet shape change and aggregation first (within the first 1 min) and then clotting—after a delay of several minutes. Both platelet stimuli caused almost identical platelet aggregation in PRP with regard to kinetics and peak amplitude, reached after 2 to 3 min. Coagulation was observed after maximal platelet aggregation (Fig. 1A). The lag times of clotting stimulated with plaque and collagen were similar (4.7 ± 0.9 min and 5.9 ± 1.1 min, respectively; n = 6, p > 0.05). Recombinant lipidated TF induced platelet aggregation only in the presence of Ca\(^{2+}\) and Mg\(^{2+}\) followed immediately by clotting (Fig. 2A). Spontaneous clotting of Ca\(^{2+}\)-readjusted and Mg\(^{2+}\)-readjusted PRP started after 15 ± 4 min (n = 4).

In whole blood with reconstituted Ca\(^{2+}\) and Mg\(^{2+}\), platelet aggregation after plaque and collagen stimulation also occurred before clot formation. Clot formation started at 3.8 ± 1.3 min (n = 5) with plaque stimulation and 4.7 ± 1.5 min (n = 7) with collagen stimulation (Fig. 2B).

**Mechanism of plaque-induced platelet aggregation.** Platelet-induced platelet aggregation was completely dependent on the interaction of collagenous plaque structures with platelet GPVI as observed previously after plaque-stimulation of isolated platelets or platelets in anticoagulated blood (17). It was completely prevented by: 1) pre-treatment of plaques with either collagenase or a GPVI-Fc fusion protein or anti-collagen type I and type III antibodies; or 2) incubation of PRP with anti-GPVI antibodies (Fig. 3A) (and data not shown). After blockade of platelet GPVI the kinetics of plaque-induced aggregation and clotting resembled the...
pattern observed after stimulation with TF (Fig. 2A). Moreover, inhibition of thrombin with hirudin or inhibition of plaque TF did not affect plaque-stimulated aggregation of PRP and blood (see the following text). These results indicate that the rapid first phase of plaque-mediated platelet aggregation is mediated by interaction of collagenous type I and type III plaque structures with platelet GPVI and not by locally generated thrombin.
Mechanism of plaque-induced thrombin and fibrin formation.

We pre-incubated plaque homogenates with a polyclonal anti-TF antibody in a concentration sufficient to block clotting triggered by 0.5 ng/ml TF. Such inhibition of plaque TF had no effect on platelet aggregation but prolonged clotting time from 5.7 ± 1.6 min to 10 ± 1.6 min in PRP (n = 4, p < 0.003) (Fig. 3B).

Similarly, in whole blood, pre-incubation of plaque with a specific anti-TF antibody delayed plaque-stimulated coagulation from 4.2 ± 0.5 min (plaque + rabbit IgG control) to 10.5 ± 2.6 min (n = 3; p < 0.05) but had no effect on platelet aggregation (Fig. 3C) (data not shown). Thus, plaque TF seemed to be crucial for coagulation but not for platelet aggregation in both PRP and blood.

Next, the kinetics and quantity of thrombin formation was measured in PRP and PFP stimulated by plaque, TF, and collagen (Fig. 4). We used a final concentration of recombinant lipilated TF of 0.1 ng/ml, corresponding to our determinations of plaque TF (see preceding text). Pre-incubation of plaque or TF with an anti-TF antibody significantly delayed the lag time and time to peak of thrombin formation in PRP after stimulation (Figs. 4A and 4B). In contrast, collagenase treatment, which increased the lag time of thrombin formation drastically from 20.5 ± 2.1 min to 38.4 ± 11.1 min after collagen stimulation (p < 0.001) (Fig. 4C), had no influence on the rate of thrombin formation with plaque stimulation (Fig. 4A; and data not shown). This indicates that only plaque TF but not platelet activation by collagenous plaque constitu-
ents is critical for thrombin formation after exposure of PRP to plaque. In PFP, anti TF-antibody treatment of plaque entirely abolished thrombin formation, whereas collagenase treatment of plaques had no effect, indicating the specificity of these treatments (data not shown). These results suggest that collagen matrix is not required to support the activity of TF. In addition, immunofluorescence microscopy of plaque homogenates demonstrated no colocalization of TF with collagen structures (data not shown).

To corroborate the results from nephelometry and thrombin generation measurements, we also analyzed the clotting time in PPP and PRP as well as whole blood by using thrombelastography (Rotem; Pentapharm). Plaque-induced coagulation in PPP, PRP, and whole blood after similar lag times: 9.2 ± 0.4 min, 9.1 ± 4.1 min, and 9.0 ± 3.8 min, respectively (n = 9 plaques). Blockade of plaque TF by pre-incubating plaques with an anti-TF antibody drastically prolonged the plaque-induced clotting times in PRP and blood to 21 ± 3.9 min and 18.0 ± 3.2 min (n = 10 for PRP; n = 12 for blood), respectively, close to control values in the absence of plaque.

Blood-borne TF did not play a role in plaque-stimulated coagulation for the following reasons: the inhibition of plaque-induced clotting with the anti-TF antibody: 1) caused a similar delay of clotting in PRP and blood; 2) depended on the incubation time of plaque with the anti-TF antibody; and 3) did not occur when the anti-TF antibody was added directly to the blood. Furthermore, we could not confirm a role of blood-borne TF in collagen-stimulated clot formation as reported previously (26). In the presence of CTI to inhibit the FXII-dependent coagulation pathway, collagen used at a concentration (2.5 μg/ml) that induced maximal platelet aggregation similar to plaques induced fibrin formation in blood only after a long lag time not different from control (data not shown).

Together these results indicate that plaque TF and not blood-borne TF is critical for fibrin formation after plaque stimulation of blood.

**Effect of FXII inhibition on collagen-, plaque-, and TF-induced thrombin and fibrin formation.** After plaque-induced stimulation of PRP and blood, thrombin might additionally be generated by the “intrinsic” FXII-initiated coagulation cascade. However, we observed that inhibition of FXII with CTI, which delayed spontaneous and collagen-induced clotting in stirred PRP and whole blood, had no significant effect on plaque-induced clotting times and platelet aggregation (Table 1, Fig. 2B).

Similarly, with collagen stimulation, CTI delayed the lag time of thrombin formation from 17.1 ± 2.9 min to 26.4 ± 4.4 min and the time to peak from 22.7 ± 3.2 min to 32.2 ± 4.2 min (n = 3); this was not seen after plaque stimulation (data not shown).

**Kinetics and mechanisms of atherothrombosis under arterial blood flow conditions.** On both, collagen- and plaque-coated surfaces, the kinetics of aggregation were indistinguishable beginning within the first minute after start of flow (0.8 ± 0.3 min; n = 5) and continuing by...
further recruitment and aggregation of oncoming platelets (Fig. 5, Online Video 1) (data not shown). A firmly adherent layer of spread platelets at the bottom of the aggregates was detectable 5 min after start of flow and gave a firm base against detachment. On the contrary, on the TF-coated surface first fibrin fibers formed, which were oriented parallel to the flow direction, and only after this fibrin layer had formed, platelets started to adhere and aggregate thereupon (Fig. 5G).

Once large platelet aggregates on the plaque-coated surface were generated, fibrin fibers developed at the bottom of the aggregates in flow niches and protruded from there radially outward (Figs. 5C to 5E, Online Video 1). On collagen- and plaque-coated surfaces, fibrin polymerization at the aggregates started 5 to 6 min after the beginning of flow. No fibers were detectable in the aggregate-free areas of the perfused surface. On TF (0.3 ng) coated surfaces, a radial outgrowth of fibrin fibers at the bottom of the platelet aggregates appeared as a second wave of fibrin formation 4 min after flow start (Fig. 5H).

Incubation of blood obtained from 5 different donors with the anti-GPVI antibody 10B12 inhibited plaque-induced platelet adhesion and aggregate formation on average by 78% after 5 min of blood flow. The degree of inhibition varied between blood donors. In blood donor 3, even after 15 min, no platelet aggregates and hence no fibrin formation were observed after anti-GPVI antibody pre-incubation of blood (data not shown). In contrast, pretreatment of plaques with anti-TF antibody showed no significant effect on the time of platelet aggregate formation (data not shown) and on platelet aggregate coverage of the plaque surface 3 and 5 min after start of flow (Fig. 6B, top). Fibrin formation time, however, was drastically prolonged in comparison with controls (Fig. 6B, bar diagram). After plaque TF inhibition, 2 of 5 plaques did not induce any fibrin fibers 15 min after start of flow.

Inhibition of FXII did not change the kinetics of platelet adhesion and aggregation or the size of aggregates on all 3 surface coatings, and on plaque and TF surfaces, CTI did not significantly change the lag time of fibrin formation. On plaque surfaces, fibrin was detected after 5.4 ± 1.4 min (n = 5) without CTI and after 6.2 ± 1.2 min (n = 3; p > 0.05) with CTI. Only on collagen surfaces, CTI completely prevented fibrin formation: in the absence of CTI, fibrin fibers were detected after a delay of 5.6 ± 0.4 min after start of flow; in the presence of CTI fibrin formation could not be detected on collagen up to 15 min after start of flow (n = 5; p < 0.003). These results under flow are concordant with the results under static conditions and indicate a purely FXII-dependent pathway for fibrin formed on collagen but not on plaque surfaces.

### Discussion

Our study addressed the mechanism and kinetics of atherothrombosis after rupture of human atherosclerotic plaques. Under static and under arterial flow conditions, plaque-induced thrombosis proceeded in 2 distinct steps. Collagenous plaque components rapidly (within the first minute) induced platelet adhesion and aggregation through activation of GPVI. Thrombin was not formed in this initial phase and played no role in platelet activation. Only after a delay of several minutes (under flow after 5 min) was thrombin and fibrin formation observed, driven entirely by plaque TF. Fibrin fibers were observed only at sites where platelet aggregates had formed. The key role of collagen/GPVI interaction in inducing atherothrombosis was demonstrated by the drastic inhibition of plaque-induced thrombus formation by blocking platelet GPVI. In contrast inhibition of thrombin or plaque TF did not inhibit plaque-induced platelet adhesion and aggregation.

Our results of the early phase (first 15 min) of plaque-induced thrombus formation are in partial conflict with earlier studies of Badimon et al. (27), who found that local inhibition of plaque TF reduced deposition of both platelets and fibrin after 5 min of perfusion. In our study, plaque TF was found to be critical only for fibrin formation but not for platelet adhesion and aggregate formation. The discrepancies between the earlier study and our own results are explained by several experimental differences: 1) the source and processing of human plaque material; 2) the type of blood anticoagulation and perfusion model used; and 3) the analysis of thrombus formation. In short, we used fresh lipid-rich carotid plaque material immediately frozen in liquid nitrogen. The atheromatous plaque material containing the cap was gently homogenized, to expose all the thrombogenic plaque components to the blood under static and defined shear conditions. It is likely that after plaque rupture (or after plaque damage by percutaneous coronary intervention) components of both the cap and core will be exposed to circulating blood (1,2). Finally, thrombus formation under flow was analyzed by microscopic real-time...
Visualization. In the former study, specimens of human aorta were obtained at necropsy; the tissue caps were removed from the atherosclerotic plaques, thereby possibly eliminating collagenous platelet-activating matrix components; an extracorporeal method of perfusing heparinized pig blood by an arteriovenous shunt was used; and end point measurements (after 5 min) of deposition of $^{111}$In-labeled platelets and $^{123}$I-labeled fibrinogen were performed.

On collagen, we found that coagulation was activated through the intrinsic (factor XIIa) pathway, confirming previous and recent studies (28–30). In contrast, coagulation induced by plaques was entirely driven by the TF-

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**Figure 5** Platelet Adhesion, Aggregation, and Fibrin Formation of Flowing Blood Exposed to Plaque and TF

Citrated blood readjusted to physiological calcium ($Ca^{2+}$) and magnesium ($Mg^{2+}$) concentrations was perfused over plaque or TF coatings at a shear rate of 1.500 s$^{-1}$. (A to D) Images representative of 5 experiments. (A) Plaque homogenate at the beginning of blood flow, yellow autofluorescence. (B) Plaques with attached platelet aggregates; specific fluorescence in green. (C) Bottom view of platelet aggregate attached to plaque demonstrating spread platelets (reflection interference contrast microscopy [RICM], black region, arrowheads), and fibrin fibers emanating radially from aggregate (arrows). (D) Scanning electron micrograph of platelet aggregate on atherosclerotic plaque and radially oriented fibrin fibers that predominate at the bottom of the aggregate. Of note, regions of the glass surface not coated with plaque material appear devoid of fibrin fibers. Large platelet aggregates on plaque (E) with radial outgrowth of fibrin fibers and (F) without fibrin formation after pretreatment of plaque with an anti-TF antibody. (G) Fibrin fibers oriented parallel to flow streamlines formed on TF coated surface after initiation of blood flow; no platelets are adherent yet. (H) After platelets aggregate on the fibrin coating, then a second wave of radial fibrin outgrowth occurs at the base of aggregates. BF = bright field microscopy; other abbreviations as in Figure 2.
Citrate blood readjusted to physiological Ca\(^{2+}\) and Mg\(^{2+}\) concentrations was perfused over plaque-coated surfaces for 15 min at 37°C with a shear rate of 1,500 s\(^{-1}\). Plaque pooled from different patients was used. (A) Blood was pretreated with the anti-GPVI antibody 10B12 or control antibody. Representative flow images 5 min after start of flow; plaque material is visualized by autofluorescence (light-grey), platelets by mepacrine fluorescence (white; *). Bar: 50 μm; below, bar diagram, results of experiments with 5 blood donors. (B) Plaque was pretreated with anti-TF antibody or rabbit IgG; left, platelet coverage 3 and 5 min after start of flow; +, platelets; o, plaque. Right, bar diagram of the effects of anti-TF antibody or rabbit IgG on platelet aggregate formation 5 min after start of flow (left) and on fibrin formation time; values are mean ± SD (n = 5 with plaques of different patients and blood of different donors). *p < 0.003 treatment versus control. Abbreviations as in Figure 2, 3, and 6.
triggered extrinsic pathway and over rode the factor XII activation, resulting in fibrin formation several minutes before the collagenous matrix or other plaque components could become operative via the intrinsic pathway. This is further corroborated by previous results, which showed that fibrin formation on TF-expressing cellular surfaces is initiated by TF/factor VIIa–dependent direct activation of factor X, whereas on collagen surface this occurs via the intrinsic pathway (31).

Although TF is accepted as the primary initiator of coagulation, plaque TF exposure alone does not determine whether or not clotting will occur under flow. Our results under flow conditions show fibrin formation only subsequent to plaque-induced platelet activation and no fibrin formation in the absence of platelets (data not shown). This is in agreement with the current model of coagulation separating an initiation and a propagation phase of thrombin formation on activated platelets. Thus, plaque TF expression combined with support of prothrombinase assembly on activated platelets is much more efficient in initiating coagulation than TF alone (32). Platelets provide the surface on which most of the thrombin required for clot formation is generated, and coagulation proceeds when TF is brought into close proximity to activated platelets. This could explain why we observed fibrin fibers radiating from platelet aggregates.

The unaltered platelet adhesion and aggregation on TF-blocked plaque indicates that thrombin generated by plaque TF is not involved in these platelet responses. Under neither static nor arterial flow conditions did thrombin, which was formed later by plaque TF activity, play a role in the early phase of plaque-induced platelet adhesion and aggregate formation. Thrombin, however, might be important in the later phase of plaque-induced thrombosis and, by inducing platelet secretion and phosphatidylserine exposure, provide a pro-coagulant platelet surface helping in the assembly and activation of clotting factors.

The orientation of fibrin fibers polymerizing in flow closely follows the direction of adjacent flow streamlines even in complex 3-dimensional flow geometry (33). The radial fiber orientation at the bottom of the platelet aggregates indicated flow niches of almost zero flow (an optimal condition for fibrin polymerization) adjacent to the high shear regions outside the aggregates, as can be clearly seen by the movement of red cells (Online Video 1). In addition, on the TF-coated surface the second wave of aggregate–fostered fibrin formation suggests a role for phosphatidylserine exposure on the surface of activated platelets (34). In an ex vivo flow model over collagen it was shown that adherent platelets formed the nuclei for fibrin fiber formation and that large platelet thrombi contained immuno-detectable fibrin solely at the thrombus base (35).

Recent studies in mice indicate an important role for “blood-borne” TF in arterial thrombus formation after vascular injury in vivo (36). Blood-borne TF is membrane bound on microparticles from various cellular origins (36) and is also thought to enhance thrombosis after plaque rupture (37). Our results clearly show that plaque TF and not blood-borne TF determines plaque-induced coagulation. However, our study was carried out with blood from healthy volunteers, and the situation could be different in cardiovascular patients with risk factors (smoking, diabetes, hypercholesterolemia) having higher blood thrombogenicity perhaps due to circulating TF. This possibility cannot be excluded. However, at present, it is not clear whether active TF is present or not in blood (38).

Current optimal treatment of patients with acute arterial thrombosis requires a combination of anticoagulants and antiplatelet agents. The most serious adverse effect of this combined antithrombotic therapy is bleeding, which can counteract the beneficial therapeutic effect (8). Moreover, the antiplatelet drugs currently available (aspirin, P2Y12 antagonists) do not effectively inhibit plaque-induced platelet activation under arterial flow conditions (18). Our recent study shows that binding of von Willebrand factor to platelet glycoprotein Ib is critical for plaque-induced platelet adhesion and aggregate formation under arterial flow conditions (1,500/s), giving further support to target glycoprotein Ib as novel antithrombotic strategy (11,18,39). Ongoing clinical trials in patients with atherothrombosis target according to the present study the 2nd step of plaque-induced thrombus formation: factor Xa inhibitors, oral thrombin inhibitors, and thrombin receptor (protease-activated receptor) antagonists. The results of the rivaroxaban (factor Xa inhibitor) dose–finding study in patients with acute coronary syndrome show that the combination of anticoagulants in high dose with dual platelet inhibition drastically increases the bleeding risk without showing major clinical benefit (40).

Study limitation. This is an ex vivo study exposing homogenates of human atheromatous plaques to blood of healthy persons and not an in vivo study after plaque rupture of cardiovascular patients.

Conclusions

Our finding that thrombi develop sequentially on atherosclerotic plaques in arterial flow—first, GPVI-dependent platelet activation; and second, plaque TF-mediated fibrin formation in flow niches provided by the growing platelet aggregates—implies that the first GPVI-dependent step could be a promising therapeutic target for the prevention of atherothrombosis after plaque rupture. Notably, animal experiments have shown that the bleeding time was only moderately increased after depletion of platelet GPVI (41). In contrast, a strategy directed to inhibit TF in plaque or blood, or FXIIa is not supported by the present study.

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REFERENCES


31. For a supplementary video, please see the online version of this article.

Key Words: aggregation • atherothrombosis • collagen • fibrin • glycoprotein VI • plaque • platelet • tissue factor • thrombin.