Local Inhibition of Tissue Factor Reduces the Thrombogenicity of Disrupted Human Atherosclerotic Plaques

Effects of Tissue Factor Pathway Inhibitor on Plaque Thrombogenicity Under Flow Conditions

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Background—Plaque disruption and subsequent thrombus formation lead to acute coronary syndromes and progression of atherosclerotic disease. Tissue factor (TF) appears to mediate plaque thrombogenicity. Tissue factor pathway inhibitor (TFPI) is the major physiological inhibitor of TF. This study analyzes the role of TF on thrombogenicity of disrupted human atherosclerotic plaques and the therapeutic possibilities of its specific inhibition.

Methods and Results—Human atherosclerotic and normal arterial segments were exposed to heparinized blood at flow conditions modeling medium-grade coronary stenosis in the Badimon perfusion chamber. The antithrombotic effects of the specific inhibition of plaque TF was assessed by reduction in the deposition of radiolabeled platelets and fibrin(ogen) and immunohistochemical analysis of perfused arteries. TF activity was inhibited by both recombinant TFPI and a polyclonal antibody against human TF. Human lipid-rich plaques were more thrombogenic than less advanced atherosclerotic plaques. Specific inhibition of TF activity reduced plaque thrombogenicity, inhibiting both platelet and fibrin(ogen) deposition (580 versus 194 platelets $\times 10^6$/$cm^2; P, 0.01, and 652 versus 172$\times 10^{12}$ molecules of Fg/cm$^2; P<0.05$, respectively) and thrombosis (immunohistochemistry).

Conclusions—This study documents the key role of TF activity in acute arterial thrombosis after atherosclerotic plaque disruption and provides evidence of the benefit of blocking plaque TF activity. Therefore the inhibition of the TF pathway opens a new therapeutic strategy in the prevention of acute coronary thrombosis after plaque disruption. (Circulation. 1999;99:1780-1787.)

Key Words: tissue factor • TFPI • thrombosis • coronary disease • atherosclerosis

There is increasing evidence that acute clinical manifestations of coronary atherosclerotic disease are caused by plaque disruption and subsequent platelet-thrombus formation resulting from exposure of deeper plaque components to flowing blood.1–3 The presence of thrombus on disrupted atherosclerotic plaque has been clearly documented in patients with myocardial infarction, unstable angina, and sudden cardiac death.4–6

The relative thrombogenicity of the different types of human atherosclerotic plaques has been characterized by our group. The atheromatous core is the most thrombogenic component of human atherosclerotic plaques.7 Recently, we showed that tissue factor (TF) content of the plaque seems to predict plaque thrombogenicity.8 Expression of TF antigen by vascular cells was identified in both normal and atherosclerotic human vessels.9–11 TF is particularly abundant in the relatively acellular lipid core and recently has been identified in atherectomy specimens from patients with unstable angina.12 These observations suggest that TF is an important determinant of the thrombogenicity of human atherosclerotic lesions after spontaneous or mechanical plaque disruption.

TF is considered a major regulator of coagulation, homeostasis, and thrombosis.13–15 TF rapidly forms a high-affinity complex with factors VII/VIIa. The TF-VIIa complex activates factors IX and X, which in turn leads to thrombin generation.13–15 TF may be synthesized by almost all the cells present in atherosclerotic lesions. Under normal conditions it is not exposed to flowing blood, but in certain circumstances, such as vascular injury, TF could interact with circulating factor VII, forming the active complex TF VIIa activating the coagulation cascade and thrombosis.
The major physiological inhibitor of TF is tissue factor pathway inhibitor (TFPI), previously known as lipoprotein-associated coagulation inhibitor (LACI) or extrinsic pathway inhibitor. TFPI is present on endothelium and circulates in association with plasma lipoproteins and platelets. The structure of TFPI consists of an acidic N-terminal region, followed by 3 repeated Kunitz-type domains and a highly basic C-terminal region. TFPI acts by initially forming a complex with factor Xa, which then forms a quaternary complex with TF VIIa. The initial binding of factor Xa by the second Kunitz domain potentiates inhibition of the TF-VIIa complex with TF VIIa. The objective of the present study was to redefine the role of TF in human atherosclerotic arterial wall thrombogenicity and its possible inhibition by specific and local blockade of its functional activity with recombinant TFPI (rTFPI).

Methods
Preparation of Substrates for Perfusion Studies
Atherosclerotic and normal (nonatherosclerotic) arteries were collected at autopsy within 24 hours of death from 8 donors. Lesions macroscopically characterized by raised white or yellow-white plaques containing a soft yellow core of toothpaste-like consistency, separated from the lumen by a fibrous cap, were classified as atheromatous, lipid-rich lesions. To mimic the in vivo situation of plaque disruption and directly expose the internal components of the arterial substrates, plaques were mechanically disrupted as previously described. In all instances, the exposed substrates were part of the intimal layer of the diseased vessel (above the internal elastic lamina). All the human specimens were devoid of endothelium and thus lacked any endothelial TFPI activity. The initial classification of the lesions was later confirmed by postperfusion histological analysis. As a model of nonatherosclerotic severe arterial injury, porcine tunica media was obtained from normal animals. Substrates were prepared by peeling off the intimal layer with a thin portion of subjacent media, starting from a corner of the arterial segment as previously described. Special care was taken to avoid gross irregularities on the surface.

Ex Vivo Perfusion Chamber and Experimental Design
The Plexiglas perfusion chamber used in this study has been described elsewhere. All arterial segments were exposed to flowing blood for a period of 5 minutes at a flow rate of 10 mL/min (theoretically calculated shear rate of 1690/s; Reynolds number 60; average blood velocity 21.2 cm/s). Local shear rate conditions developed on the perfused vessels modeled local rheological conditions on mild to moderately stenotic coronary arteries. Our previous work demonstrated that these rheological conditions result in consistent levels of platelet deposition. Yorkshire albino pigs (body weight 30±2 kg) served as blood donors. Twenty-four hours before the perfusion studies, autologous platelets were labeled with 111In-tropolone in plasma as previously described. Labeling efficiency was 63±7%. The mean injected activity was 302±26 μCi with the 3.3±0.4×10⁹/μL 111In-labeled platelets injected in a total of 4 mL of autologous platelet-poor plasma.

To study the contribution of fibrinogen deposition to thrombus formation, perfusions were performed with double-labeled blood (platelets and fibrinogen). Fibrinogen was isolated from the same breed of pigs used for the perfusion and labeled with as described. The amount of radioactivity bound to protein was >95% as estimated by trichloroacetic precipitation. The ability of the isolated fibrinogen to clot was not affected by the labeling procedure.

The day of the study, pigs were sedated with ketamine (20 mg/kg body wt) followed by sodium pentobarbital (25 mg/kg IV). Anesthesia was maintained by infusion of pentobarbital as needed. The perfusion chamber was placed within a carotid artery–jugular vein shunt as described. Blood perfused the exposed substrates at a constant blood flow regulated by a peristaltic pump placed distal to the chamber. After carotid cannulation, baseline blood samples were taken for measurements of hematocrit, red blood cells, platelet count, fibrinogen levels, and activated partial thromboplastin time (aPTT). The animals were given a standard regimen of heparin (50 IU/kg bolus followed by a continuous intravenous infusion of 50 IU/kg per hour) to achieve an aPTT ratio of 1.5±0.03 times greater than baseline values. The specimens were mounted in the chamber and perfused with PBS, (0.01 mol/L, pH 7.4; 37°C) for 60 seconds. At the end of the blood perfusions, PBS was again passed through the chamber for 30 seconds to wash away unattached cells and plasma proteins. All the perfusions were carried out in a 37°C water bath. Hematocrit, platelet count, and fibrinogen levels were constant throughout the experiment. At the end of each perfusion the arterial segments were removed, immersed in 4% paraformaldehyde in 0.1 mol/L PBS, pH 7.4, and individually counted in a gamma-well counter (Packard Auto-Gamma 5650). The gamma-well counter was set at a window of 150 to 300 keV to capture both In photopeaks (171 and 274 keV). Total platelet deposition on the perfused substrates was calculated from the radioactivity of the substrates and blood (counts per minute), and the platelet count in blood and results were normalized by surface area as previously described. Platelet deposition was expressed as platelets/cm².

Local Inhibition of TF
Human TFPI (rTFPI) was donated by Dr Abba Crease from CHIRON Corp (Emeryville, Calif). rTFPI was expressed in Escherichia coli as a nonglycosylated protein with an additional alanine attached to the amino terminus of wild-type TFPI. In a TF-induced clotting assay in human plasma, the rTFPI exhibited 3 to 10 times higher specific activity than rTFPI obtained by transfecting mammalian cell lines and 2 times higher activity, on a molar basis, than that of full-length SK hepatoma-derived TFPI. rTFPI (5 mg/mL) was dissolved in a buffer containing 200 mmol/L arginine, 20 mmol/L sodium citrate buffer, 0.01% polysorbate-80, and 150 mmol/L NaCl (pH 7.2). The same solution buffer without rTFPI served as control. To test the specificity of the treatment, human atherosclerotic plaques (n=4) were incubated with an anti-human TF antibody (Dr Y. Nemerson, Mount Sinai Hospital). The same antibody was previously used to demonstrate the presence of TF activity on subendothelium.

Conventional Histology and Immunohistochemistry
Specimens were either routinely processed for paraffin embedding according to conventional techniques or cryoprotected as described below. Exposed segments, cut parallel to the direction of the flow, were step-sectioned every 100 μm, and 5-μm sections were mounted onto lissamine-coated slides and stained with Mason’s trichrome and oil red O. The Masson’s trichrome stain for connective tissue allows marking of muscle cells, collagen fibers, fibrin, and erythrocytes. The oil red O was used to identify fatty infiltration; unsaturated hydrophobic lipids and mineral oils are stained red. Histological evaluation verified the initial macroscopic classification of lesion type.

Perfused arterial segments were fixed in 4% paraformaldehyde solution, PBS 100 mmol/L, pH 7.4, for ≥6 hours. Then, the vessels were cryoprotected (2.3 mol/L sucrose in PBS) and immediately frozen in dry ice with OCT (Tissue-Tek OCT compound 4583, Miles Inc). Serially cut 4- to 5-μm sections were obtained on a Reichert-Jung 2800 Frigocut cryostat, mounted on lissamine-coated slides and...
Thrombus Formation on Severely Injured Normal Arterial Wall

Platelet-thrombus formation on severely injured nonarteriosclerotic arterial substrate was also evaluated (Figure 1). The specific inhibition of TF activity on the substrates by TFPI was associated with a significant reduction ($P<0.05$) in the number of platelets deposited on the substrates. Platelet recruitment on the rTFPI-treated arterial segments ($n=12$) was $160\pm13$ platelets/$10^6$/cm$^2$ versus $208\pm12$ platelets/$10^6$/cm$^2$ on the buffer-treated segments ($n=9$).
Immunohistochemistry Results

Human advanced and mild atherosclerotic plaques as well as normal (nonatherosclerotic) tissue, after incubation with buffer or TFPI, were perfused in parallel runs with blood from the same donors. Total platelet deposition and axial dependent platelet deposition on the specimens were calculated, and the specimens were processed for immunohistochemical analysis. The thrombogenicity of the advanced plaque (462±196×10^6 platelets/cm^2) was significantly reduced by TFPI (140±30 platelets/cm^2). Likewise, immunohistochemical analysis also indicated that the inhibition of TF reduced platelet deposition in association with a significant inhibition in fibrin deposition as compared with the control segments (Figure 3). The severe atherosclerotic tissue induced significant thrombosis (average platelet deposition on the central segment that was further analyzed by immunohistochemistry was 180×10^6 platelets/cm^2 with fibrin-rich [green] and platelet/fibrin colocalization [orange] areas). When TF in adjacent tissues was inhibited by TFPI, a significant reduction in thrombotic mass was observed. The average platelet deposition in the central segment that was further analyzed by immunohistochemistry (Figure 3, bottom left) at 3 days was 45×10^6 platelets/cm^2. In addition, the inhibition of TF also induced a significant inhibition in fibrin/platelet colocalization areas (orange).

Mild atherosclerotic tissue showed significantly less thrombogenicity (47×10^6 platelets/cm^2) than more advanced lesions, but TFPI was still able to reduce total platelet deposition (30×10^6 platelets/cm^2). TFPI was able to reduce fibrin deposition on mild atherosclerotic lesions, even though these plaques showed less platelet recruitment than the more advanced atherosclerotic lesions (Figure 4). The fatty streak–rich plaque (average platelet deposition on the central segment further analyzed by immunohistochemistry was 15×10^6 platelets/cm^2). It showed little fibrin deposition and platelet accumulation, and TFPI almost abolished deposition (average platelet deposition on the central segment further analyzed by immunohistochemistry was 4×10^6 platelets/cm^2).

Non–lipid-rich human tissue with minimal lesion (Figure 5) showed much less deposition (average 11×10^6 platelets/cm^2) of both platelets and fibrin. Disrupted lipid-rich human atherosclerotic lesions are significantly more thrombogenic that disrupted normal arterial wall as demonstrated by the significantly higher number of platelets deposited on these substrates when exposed to flowing blood at the same shear rate conditions. Figure 6 shows the Masson’s trichrome stain of the human atherosclerotic lipid-rich plaques (A and B) and human fatty streaks (C and D) seen in Figures 4 and 5 when the same human atherosclerotic lesions were stained with oil red O to visualize fatty infiltration (Figure 7). Atheromatous...
lesions (A and B) showed a clearly visible lipid infiltration; fatty streaks (C and D) have much less lipid deposition and only superficial infiltration in fatty streaks.

Discussion

The present study clearly establishes the central role of TF in acute thrombosis after the disruption of atherosclerotic plaques. It also provides evidence for therapeutic possibilities of the inhibition of TF activity on the prevention of arterial thrombosis in the clinical situations associated with disruption of atherosclerotic plaques, whether induced spontaneously or by coronary interventions.

Clinical and experimental studies have established the role of plaque disruption and acute thrombus formation in the onset of the acute coronary events and atherosclerosis progression. The most widely accepted hypothesis is that arterial injury activates circulating platelets by exposing deeper components of the vascular wall to flowing blood. The mechanism responsible for the observed thrombogenicity is not well understood. Collagen exposure on plaque disruption was suggested by some investigators. Our group reported that atheromatous, lipid-rich lesions were the most thrombogenic of the studied substrates, including collagen. We also reported that TF is highly expressed in human lipid-rich lesions and in the close relation between thrombogenicity and TF content. This study further demonstrates the thrombogenic activity of TF by the significant antithrombotic effect of its specific inhibition.

The enzymatic activity of factor VIIa on its substrates, factors IX and X, is enhanced several thousandfold in the presence of its cofactor TF. Immunohistochemical studies have detected TF antigen in human atherosclerotic plaques. More recently it was shown that the TF present in coronary atherectomy specimens is active and capable of activating factor X. All of these observations strongly suggest a major role for TF in the thrombogenicity of disrupted human atherosclerotic plaques.

The principal physiological inhibitor of the TF–factor VIIa complex is TFPI. TFPI develops its regulatory effect in 2 steps. First, it inhibits the proteolytic capacity of factor Xa by binding to the active site of Xa through its second Kunitz domain, and then it inhibits the activity of TF/VIIa complex by forming a quaternary complex with TF–factor VII/VIIa.

Our experimental design investigated whether the specific inhibition of TF activity present in lipid-rich atheromatous lesions would reduce plaque thrombogenicity. Atherosclerotic plaques were treated locally with TFPI or the vehicle before their exposure to flowing blood. The rheological conditions (1690/s) used in this study mimic those typical of
a mildly stenosed coronary artery. These rheological conditions were selected because 68% of the plaques responsible for an acute myocardial infarction have <50% stenosis. Our results demonstrate that inhibition of TF activity by rTFPI was associated with a 66% reduction in platelet thrombus. A similar reduction in plaque thrombogenicity was achieved when the plaques were preincubated with an antihuman TF antibody. When the selected artery was nonatherosclerotic, a 25% reduction in substrate thrombogenicity was achieved by the local inhibition of TF activity by TFPI. Given the lower baseline thrombogenicity (platelet deposition) and lower TF content in nonatherosclerotic arteries than in the atherosclerotic ones, a diminished inhibition of thrombogenicity was attained by pretreatment with TFPI of these substrates.

The origin of the TF found in the lipid-rich core of human atherosclerotic lesions is poorly understood. Several authors have suggested that the monocyte/macrophage-type cells play a crucial role not only in the development and progression of the atherosclerotic lesions but also in their disruption and thrombogenicity. Furthermore, our group also identified macrophage infiltrates in specimens obtained from patients with unstable angina and showed their colocalization with the TF-rich areas in the same specimens. These observations provide a possible link between macrophages, plaque instability, TF content, and plaque thrombogenicity.

Administration of TFPI has been reported to prevent venous thrombosis and to sustain arterial patency after thrombolysis in a canine model of arterial thrombosis. TF activity has been shown in rabbit subendothelium, and an antibody against rabbit TF, AP-1, has shown to inhibit thrombus formation in rabbit carotid. Additionally, inhibition of TF activity may serve as adjunctive therapy to tissue plasminogen activator–induced thrombolysis by shortening the time needed for the lysis of thrombus and preventing reocclusion in the carotid rabbit model. Our study demonstrates that TF pathway inhibition by local administration of TFPI or antibodies to TF is highly effective in reducing arterial thrombosis in human atherosclerotic lesions.
In conclusion, our results clearly establish the causal role of TF activity in thrombus formation after atherosclerotic plaque disruption. Therefore, the specific inhibition of TF activity by TFPI would significantly reduce the thrombogenicity of disrupted atherosclerotic plaques, whether induced spontaneously or by coronary interventions.

**Acknowledgments**

This work was partially supported by a grant from the National Institutes of Health, P50 HL-54469 (J.J.B.), CHIRON (Emeryville, Calif), a grant from PNS SAF 712/94, and Fundacion de Investigacion Cardiovascular-Catalana de Occidente (L.B., Spain).

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Circulation. 1999;99:1780-1787
doi: 10.1161/01.CIR.99.14.1780

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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