Importance of Factor Xa in Determining the Procoagulant Activity of Whole-blood Clots

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Abstract

The binding of thrombin to fibrin is thought to be an important mechanism by which thrombi exhibit procoagulant activity; however, the extent to which other procoagulants are associated with thrombi has not been previously defined. This study was designed to determine whether clotting factors other than thrombin are bound to whole-blood clots and can thereby contribute to significant procoagulant activity. Clots formed in vitro from human blood exhibited minimal thrombin activity when incubated in plasma depleted of vitamin K-dependent factors by barium-citrate adsorption, as indicated by increases in the concentration of fibrinopeptide A (FPA), a marker of fibrin formation, to 72 nM after 30 min. Incubation of clots in barium-absorbed plasma repleted with 0.9 μM human prothrombin under the same conditions resulted in marked increases in the concentration of FPA (> 1,000 nM) and clotting by 30 min. The increases in FPA were attributable to activation of the added prothrombin by clot-associated Factor Xa, judging from concomitant increases in the concentration of prothrombin fragment 1.2. Similar results were obtained with thrombi induced in the axillary arteries of dogs by vascular injury and incubated with plasma in vitro. Activation of prothrombin was inhibited in a dose-dependent manner by tick anticoagulant peptide, a direct inhibitor of Factor Xa, at concentrations of 0.5–5.0 μM. Clot-associated Factor Xa activity was resistant to inhibition by anti-thrombin III, judging from the lack of inhibition of prothrombin activation during incubation of clots in plasma containing heparin pentasaccharide, an anti-thrombin III–mediated inhibitor of Factor Xa. Thus, the activity of Factor Xa appears to be an important determinant of the procoagulant activity of whole-blood clots and arterial thrombi, and is resistant to inhibition by anti-thrombin III–dependent inhibitors. (*J. Clin. Invest.* 1993. 91:1877–1883.) Key words: thrombosis • thrombin • coagulation factors • Factor Xa • anticoagulants

Introduction

It has been suggested that the propensity of thrombi to induce activation of the coagulation system plays an important role in the recurrence of thrombosis after thrombolysis and in the propagation of thrombi. One potential contributor to the procoagulant activity of thrombi is the persistence of activity of thrombin bound to fibrin (1–3). Thrombin appears to bind to thrombi in vivo, judging from the thrombin activity associated with thrombi recovered from patients at the time of vascular surgery (1). Thrombin bound to fibrin has been shown to induce continued formation of fibrin and to be relatively protected from inhibition by anti-thrombin III or anti-thrombin III–heparin complex (2–4). Because thrombin activates Factors V and VIII, even minimal thrombin activity associated with thrombi may play an important role in potentiating activation of the coagulation system. However, the extent to which procoagulants other than thrombin are associated with thrombin and may induce procoagulant activity has not been defined.

In addition to fibrin, thrombi formed in vivo contain varying amounts of platelets, other circulating blood cells, and other adhesive proteins, including fibronectin. It is likely that platelets contribute to the procoagulant properties of thrombi in an important way by providing binding sites for the Factor Xa/Va complex on their membrane surface (5). The Xa/Va complex activates prothrombin, and when bound to phospholipid is relatively protected from inhibition by anti-thrombin III (6). In addition, the Factor IXa/VIIIa complex may bind to platelets. Thus, the procoagulant activity of thrombi formed in vivo may be attributable to the activity of Factor Xa or IXa associated with the thrombus, as well as to thrombin bound to fibrin. This study was designed to determine whether clotting factors other than thrombin are bound to whole-blood clots formed in vitro and thrombi formed in vivo, and to define the extent to which the activity of other clotting factors contribute to the procoagulant activity of thrombi.

Methods

Material. Fibrinogen was purchased from Kabi Vitrum (Kabi L fibrinogen; Kabi Vitrum, Fredrickstown, OH) or prepared as described by Blomback (7). Fibrinogen was depleted of plasminogen by lysine-sepharose affinity chromatography. Human Factor X and prothrombin were isolated and purified from pooled citrated plasma, as previously described (8). Thrombin was prepared from purified human prothrombin by activation with Taipan snake venom, and isolated by cation exchange chromatography on an Amberlite CG-50 column. The thrombin preparation contained > 90% α-thrombin, as determined by migration on reduced and nonreduced SDS-PAGE and staining by Coomassie blue.

Activated Factor X (Xa)[1] was prepared by incubation of human Factor X with purified Factor X coagulant protein from Russell viper venom at a 1:1,000 ratio by weight in 0.02 M Hepes, 0.15 M NaCl, 0.002 M $CaCl_2$, pH 7.4, at 37°C for 30 min. Complete activation of Factor X prepared under these conditions was confirmed by absence of

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1. *Abbreviations used in this paper:* FPA, fibrinopeptide A; PPACK, D-Phe-L-Pro-L-Arg-Chloromethylketone, S-2222, *N*-benzoyl-L-isoleucyl-L-glutamyl-glycl-L-arginine-p-nitroalanine HCl; TAP, tick anticoagulant; TBS, Tris-buffered saline; Xa, activated Factor X.

zymogen Factor X, as determined by migration on SDS-PAGE and staining with Coomassie blue. Fresh-frozen plasma was purchased from the American Red Cross (St. Louis, MO). Whole-blood, as well as platelet-rich and platelet-poor plasma, were from blood samples obtained by venipuncture from healthy, nonhospitalized volunteers who had not taken aspirin or any aspirin-containing medications within the previous 72 h. Plasma was prepared from blood collected into 3.8% sodium citrate (vol ratio 9:1) and subjected to centrifugation at 300 *g* for 15 min for platelet-rich plasma, or 1,500 *g* for 30 min for platelet-poor plasma.

Aprotinin and recombinant hirudin (RHV_2 variant) were obtained from Sigma Chemical Co. (St. Louis, MO). Heparin sodium was purchased from LyphoMed (Rosemont, IL). D-Phe-L-Pro-L-Arg-chloromethylketone (PPACK) was purchased from Calbiochem (La Jolla, CA) (9). Recombinant tick anticoagulant peptide (TAP), a specific Factor Xa inhibitor, was provided by Merck, Sharp, & Dohme (West Point, PA) (10, 11). Heparin pentasaccharide, an anti-thrombin III–dependent specific inhibitor of Factor Xa, was provided by Choay (Paris, France) (12). The chromogenic substrate for Factor Xa activity, *N*-benzoyl-L-isoleucyl-L-glutamyl-glycl-L-arginine-*p*-nitroanaline HCl (S-2222), was purchased from Chromogenix (Franklin, OH).

Preparation of plasma and whole-blood clots. Whole-blood clots were prepared by modification of the method of Chandler (13). Blood was drawn through a 19-gauge needle and then transferred in 1-ml aliquots to lengths of tygon tubing. The tygon tubing was connected to form a closed circle and rotated at 37°C for 1 h. The clot was then removed and washed extensively with saline.

Plasma clots were prepared from citrated platelet-rich or platelet-poor plasma by addition of $CaCl_2$ and thrombin at final concentrations of 25 mM and 2 nM, respectively, followed by incubation at 37°C for ≤ 30 min. The clots were formed on stainless steel wires, retracted, and washed three times in 1-ml aliquots of 0.02 M Na_2PO_4, and 0.15 NaCl, pH 7.4. Complete removal of adherent thrombin associated with plasma and whole-blood clots was confirmed by lack of amidolytic activity of the final wash buffer toward the chromogenic substrate S-2238 (Kabi Vitrum, Fredrickstown, OH), a substrate for thrombin.

Preparation of barium citrate–adsorbed plasma. Plasma depleted of vitamin K–dependent factors was prepared by addition of 100 mM $BaCl_2$ to pooled citrated plasma at 4°C for 60 min, followed by centrifugation to separate the precipitate. The supernatant was recovered and additional barium chloride precipitate was allowed to form. The supernatant plasma was then collected and dialyzed exhaustively against 0.15 M NaCl and 0.012 M sodium citrate, pH 6.0. The barium citrate–adsorbed plasma (barium-adsorbed plasma) was stored as 1.0-ml aliquots at −70°C and thawed at 37°C immediately before use.

Thrombi formed in vivo. Thrombi were recovered from dogs with induced arterial thrombosis. All procedures were conducted according to the guiding principles of the American Physiologic Society and were approved by the Animal Studies Committee at Washington University (St. Louis, MO). Mongrel dogs weighing 22–25 kg were anesthetized intravenously with sodium pentobarbital (30 mg/kg), intubated, and ventilated with oxygen-enriched room air. Body temperature was maintained with a heating pad. The axillary artery was exposed, side branches were ligated, and a 20-MHz Doppler-flow probe was placed proximal to the intended site of thrombosis. A platelet-rich thrombus was formed by applying direct anodal current (300 μA) to an indwelling transluminal electrode for 90 min or until occlusion occurred. The current was then stopped, the vessel ligated, and the thrombus carefully removed from the vessel and placed in saline. The thrombus was extensively washed, as described above, before incubation with plasma.

Characterization of thrombin-dependent clot-associated procoagulant activity. Clot-associated thrombin activity was characterized by incubation at 37°C in barium-adsorbed plasma repleted with $CaCl_2$ to a final concentration of 25 mM. Aliquots of the plasma were obtained at selected intervals for measurement of the concentration of fibrinopeptide A (FPA), a peptide released when fibrin I is formed (14). The plasma aliquot was added to an anticoagulant solution containing EDTA, aprotinin, and PPACK (FPA anticoagulant solution; Byck Sangtek, Dietzenbach, Germany). In some experiments, clots were incubated with recombinant hirudin to confirm that increases in the concentration of FPA were attributable to thrombin activity.

Characterization of clot-associated Factor Xa activity. The activity of Factor Xa associated with clots was characterized by measuring the extent of prothrombin activation induced by clots when incubated in barium-adsorbed plasma at 37°C that was repleted with $CaCl_2$, as noted above, and either 0.9 or 1.3 μM purified human prothrombin. Activation of prothrombin was confirmed both by measurement of changes in the concentration of prothrombin fragment 1.2, a peptide released when thrombin is activated by Factor Xa, and by changes in the concentration of FPA, a marker of thrombin activity. In some experiments, clots were incubated with TAP or heparin pentasaccharide, specific inhibitors of Factor Xa, to confirm that prothrombin activation was dependent on Factor Xa.

Characterization of clot-induced activation of Factor X. To determine whether whole-blood clots induced activation of Factor X, clots were incubated with 0.18 μM purified human Factor X either in recalcified barium-adsorbed plasma repleted with prothrombin, or 0.05 M Tris-HCl, 0.15 M NaCl, 0.002 M $CaCl_2$, pH 7.4, at 37°C for 30 min, as described above. A 30-μl aliquot of the plasma or buffer was removed and added to 0.05 M Tris-HCl and 0.15 M NaCl, pH 7.4, containing 0.75 μM S-2222 and 5 U/ml recombinant hirudin added to inhibit thrombin activity. After incubation at 37°C for 60 min, 15 μl of 20% acetic acid was added and the hydrolysis of the substrate was characterized by measurement of the absorbance at 405 nM with an automated kinetic plate reader (ThermoMax; Molecular Devices, Inc., Palo Alto, CA).

Factor Xa binding to fibrin clots. To characterize the binding of catalytically active Factor Xa to fibrin clots, purified human Factor Xa was added in selected concentrations to a solution of 0.25 mg/ml plasminogen-depleted fibrinogen in 0.01 M Hepes, 0.1 M NaCl, and 0.002 M $CaCl_2$, pH 6.8, and clotted by addition of 2 nM α-thrombin. The clots were incubated for 1 h at 37°C, recovered on a steel wire, washed extensively in the Hepes saline buffer, blotted, and incubated with barium-adsorbed plasma to which 0.9 μM prothrombin was added, as described above. Concentrations of FPA were measured to characterize prothrombin activation by Factor Xa bound to the fibrin clot.

Inhibition of Factor Xa by TAP in the presence of fibrin II monomer. Inhibition of Factor Xa by TAP in the presence or absence of fibrin II monomer was characterized as described by Jordan et al. (15). Fibrin II monomer was prepared by incubating fibrinogen in 0.015 M Tris-HCL and 0.15 NaCl, pH 7.4 (TBS), with 0.5 U/ml thrombin for 60 min. The polymerized fibrin was resuspended in 0.02 M acetic acid and diluted 1:50 in TBS. TAP (0.063–10 nM) was incubated with 0.125 nM Factor Xa in TBS in the presence or absence of 34 nM fibrin II monomer for 30 min. Residual Factor Xa activity was measured by adding a 100-μl aliquot of the Factor Xa/TAP mixture to 0.2 M S-2222 in 0.05 M Tris-HCl and 0.15 mM NaCl, pH 7.4, containing 5 U/ml recombinant hirudin and measuring the absorbance at 405 nM in an automated kinetic plate reader. The initial rate of hydrolysis of the substrate was linear and was measured over 5 min.

Radioimmunoassay of FPA. Plasma was adsorbed with bentonite before assay for FPA, as previously described (16, 17). The concentration of FPA was determined with a polyclonal antisera-based commercial radioimmunoassay (Byck Sangtek, Dietzenbach, Germany).

ELISA for prothrombin fragment 1.2. The ELISA for prothrombin fragment 1.2 was provided by Dr. Juan Ruiz, Baxter Diagnostics, Inc. (Miami, FL). This assay is based on a monoclonal antibody that is specific for fragment 1.2 and does not recognize native prothrombin (18). The specific monoclonal antibody was coated on a microtiter plate and incubated with the plasma sample or purified human prothrombin fragment 1.2 for 30 min. The plate was then washed and a second monoclonal antibody conjugated to horseradish peroxidase that recognizes an independent epitope on fragment 1.2 was added. After a 10-min incubation, the plate was washed, and 3,3′,5,5′ tetramethylbenzadine, a peroxidase substrate, was added. The color reaction was stopped with 1 M sulfuric acid and the absorbance was mea-

sured with an automated microtiter plate reader. Concentrations of fragment 1.2 were determined by comparison with a standard curve obtained from purified fragment 1.2.

Data analysis. The results are expressed as mean±SE.

Results

Thrombin compared with Xa activity of whole-blood clots. To determine the extent of thrombin activity associated with whole-blood clots, washed clots were incubated in recalcified barium-adsorbed plasma, and the rate of thrombin-catalyzed conversion of fibrinogen to fibrin I was characterized by serial measurement of plasma concentrations of FPA. Increases in FPA in this system reflected thrombin activity associated with the clot, since other vitamin K–dependent factors had been depleted by barium adsorption. As shown in Fig. 1, whole-blood clots induced modest increases in the concentration of FPA to 72 nM after a 30-min incubation (n = 2), consistent with minimal clot-associated thrombin activity.

To characterize the extent of Factor Xa activity associated with whole-blood clots, washed clots were incubated in barium-adsorbed plasma to which 0.9 μM purified human prothrombin was added. FPA did not increase in recalcified barium-adsorbed plasma containing prothrombin incubated with purified Factor Xa coagulant protein. This result is consistent with complete depletion of Factor X in the plasma; thus, activation of prothrombin reflected the activity of Factor Xa associated with the whole-blood clot. As shown in Fig. 1, rapid and marked increases in the concentration of FPA occurred when a whole-blood clot was incubated in recalcified barium-adsorbed plasma repleted with prothrombin, resulting in clotting of the plasma by 30 min. To confirm that the generation of FPA reflected activation of the added prothrombin, the change in concentration of prothrombin fragment 1.2 was measured. As shown in Fig. 2, the concentration of fragment 1.2 increased markedly, indicating that whole-blood clots induced activation of prothrombin. The increases in fragment 1.2 were not attributable to contamination of the added human prothrombin preparation with activated Factor X, judging from the minimal increases in the concentration of FPA and prothrombin fragment 1.2 (208 and 1.8 nM, respectively) observed when recalcified prothrombin-repleted, barium-adsorbed plasma was incubated at 37°C in the absence of a clot.

To further confirm the role of Factor Xa activity in inducing the marked activation of prothrombin that was observed, whole-blood clots were incubated in recalcified barium-adsorbed plasma repleted with human prothrombin in the presence of recombinant TAP, a specific inhibitor of Factor Xa. As shown in Figs. 1 and 2, incubation of whole-blood clots in plasma containing TAP markedly attenuated in a dose-dependent manner the increases in FPA and prothrombin fragment 1.2 induced, compared with those observed in the absence of inhibitor. However, activation of prothrombin was not abolished, even at a concentration of 5 μM TAP, as indicated by minimal increases in the concentration of prothrombin fragment 1.2 and FPA.

To determine whether both thrombin and Factor Xa activity were necessary for clot-bound Factor Xa to induce prothrombin activation, whole-blood clots were initially incubated in a PBS with 1 U/ml of recombinant hirudin. The clots were washed to remove unbound hirudin and then incubated in recalcified barium-adsorbed plasma. Preincubation of the clots with hirudin abolished thrombin activity, judging from

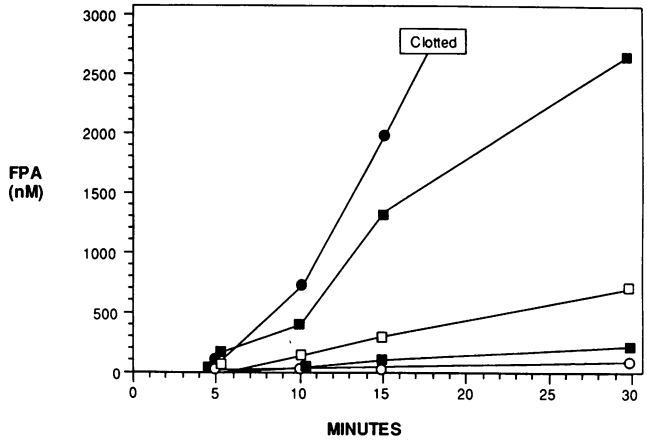

Figure 1. Changes in the concentration of FPA induced by incubation of whole-blood clots in recalcified barium-adsorbed plasma, either with or without addition of 0.9 μM human prothrombin and the specific inhibitor of Factor Xa, TAP. Concentrations of FPA in the baseline plasma (0 min) were < 10 nM. The open circles indicate the modest increases in the concentration of FPA induced by whole-blood clots incubated with barium-adsorbed plasma. In contrast, marked increases in the concentration of FPA occurred when whole-blood clots were incubated with recalcified barium-adsorbed plasma to which 0.9 μM human prothrombin was added (*closed circles*). The increases in the concentration of FPA induced by whole-blood clots in plasma repleted with prothrombin were attenuated in a dose-dependent fashion by TAP at concentrations of 0.5 μM (*closed squares*), 1.0 μM (*open squares*), and 5.0 μM (*shaded squares*).

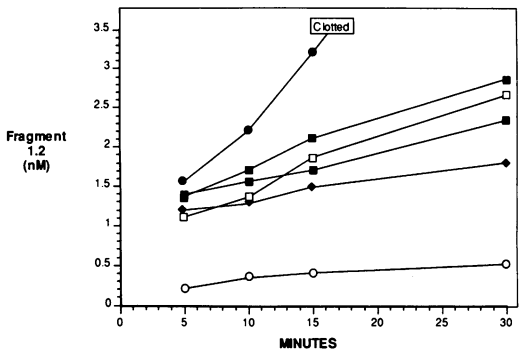

Figure 2. To characterize the extent of prothrombin activation, the changes in the concentration of the prothrombin fragment 1.2 were measured. The open circles indicate the concentrations of fragment 1.2 in barium-adsorbed plasma incubated with whole-blood clots for 30 min. Essentially no increase in fragment 1.2 occurred under these conditions. In contrast, marked prothrombin activation occurred when whole-blood clots were incubated with recalcified barium-adsorbed plasma repleted with 0.9 μM prothrombin (*closed circles*). The extent of activation of prothrombin was attenuated by TAP at concentrations of 0.5 μM (*closed squares*), 1.0 μM (*open squares*), and 5.0 μM (*shaded squares*). The closed diamonds indicate the concentrations of fragment 1.2 measured in barium-adsorbed plasma repleted with prothrombin, but not incubated with clots. Although the preparation of prothrombin appeared to contain modest concentrations of fragment 1.2 (1.2 nM compared with 0.2 nM in barium-adsorbed plasma alone), there was evidence of only minimal prothrombin activation during 30 min of incubation of this plasma after recalcification in the absence of a clot.

the lack of an increase in FPA after 30 min (22 nM [n = 2]). However, marked increases in the concentration of FPA to 2,366 nM (n = 2) at 15 min and clotting by 30 min occurred when whole-blood clots preincubated with hirudin were incubated in the recalcified barium-adsorbed plasma repleted with prothrombin. Thus, clot-bound thrombin activity is not necessary for subsequent prothrombin activation during incubation with whole-blood clots.

Whole-blood clots were also incubated with recalcified barium-adsorbed plasma repleted with 0.18 nM Factor X, under identical conditions described above for incubation with prothrombin-repleted plasma, to determine whether activation of Factor X was induced. An aliquot of the clot-incubated plasma failed to demonstrate any Factor Xa activity, as judged by lack of hydrolysis of the synthetic chromogenic substrate S-2222. Identical results were observed when whole-blood clots were incubated with 0.18 nM Factor X in 0.05 M Tris-HCl, 0.15 M NaCl, 0.002 M $CaCl_2$, pH 7.4, at 37°C for 30 min. The lack of activation of Factor X suggests that clot-associated procoagulant activity is not caused by clot-bound Factor IXa/VIIIa or VIIa/tissue factor complexes.

Thrombin and factor Xa activity of thrombi formed in vivo. To determine whether thrombi formed in vivo exhibit Factor Xa activity similar to that of whole-blood clots formed in vitro, thrombi were recovered from a preparation of axillary artery injury in dogs. Thrombi were extensively washed and handled in a manner identical to the whole-blood clots prepared from human blood in vitro. As shown in Fig. 3, canine thrombi incubated in barium-adsorbed plasma exhibited only minimal thrombin activity, as characterized by slight increases in the concentration of FPA at 30 min (41.8±12.2 nM [n = 5]). However, marked activation of human prothrombin was induced when thrombi were incubated in recalcified barium-adsorbed plasma repleted with 0.9 μM human prothrombin, as indicated by marked increases in the concentration of FPA at 30 min (2,817±710 nM [n = 5]), and clotting of the plasma by 45 min. These results indicate that thrombi formed in vivo in response to arterial injury exhibit characteristics with regard to thrombin- and Factor Xa–associated procoagulant activity that are very similar to those of clots formed from human whole blood in vitro.

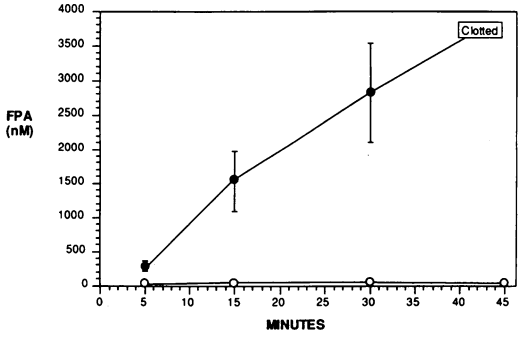

Figure 3. Concentrations of FPA were measured in recalcified barium-citrated plasma incubated with clots recovered from an in vivo preparation of axillary artery injury in dogs. Similar to the results shown in Fig. 1, in the absence of prothrombin (*open circles*) the canine clots induced only minimal increases in the concentration of FPA. However, in recalcified barium-adsorbed plasma repleted with 0.9 μM prothrombin, marked increases in fibrin formation occurred.

Response of clot-bound Xa activity to specific inhibitors. To define the response of clot-associated Xa activity to inhibition, TAP and heparin pentasaccharide were incubated with whole-blood clots in the recalcified barium-adsorbed plasma prothrombin system. Both inhibitors were added to the plasma just before recalcification and addition of the whole-blood clots. Heparin pentasaccharide, which acts by catalyzing antithrombin III–mediated inhibition of Factor Xa, did not inhibit clot-associated Xa activity even at a relatively high concentration (5 μg/ml), judging from the increases in the concentrations of FPA (Fig. 4) after 10 min of incubation. Concentrations of prothrombin fragment 1.2 were > 3 nM after 10 min of incubation of clots with heparin pentasaccharide, confirming activation of prothrombin. The increases in FPA were attenuated by TAP in a dose-dependent manner, but relatively high concentrations of TAP (5 μm) were required (Fig. 5). To determine whether the limited efficacy of heparin pentasaccharide was attributable to the association of Factor Xa activity with clot, 0.25 or 0.5 nM purified human Factor Xa was added to barium-adsorbed plasma repleted with prothrombin and incubated with 1.0 and 5.0 μg/ml of heparin pentasaccharide in the absence of clot. The concentrations of Factor Xa added were selected to induce increases in the concentration of FPA similar to those observed after 10 min of incubation of whole-blood clots in this plasma system under identical conditions. Heparin pentasaccharide, at the concentrations used, markedly attenuated the increases in FPA induced by free Factor Xa (Fig. 4). Thus, clot-associated Factor Xa activity appears to be relatively protected from inhibition by the anti-thrombin III–dependent inhibitor, heparin pentasaccharide. Similarly, only one tenth of the concentration of TAP shown to inhibit clot-associated Factor Xa activity was required to attenuate the increases in FPA induced by Factor Xa in recalcified barium-adsorbed plasma repleted with prothrombin in the absence of clot (Fig. 5). To determine whether the high concentrations of TAP required to inhibit clot-associated Xa activity reflect the slow-binding characteristics of TAP as opposed to inhibition of TAP-me-

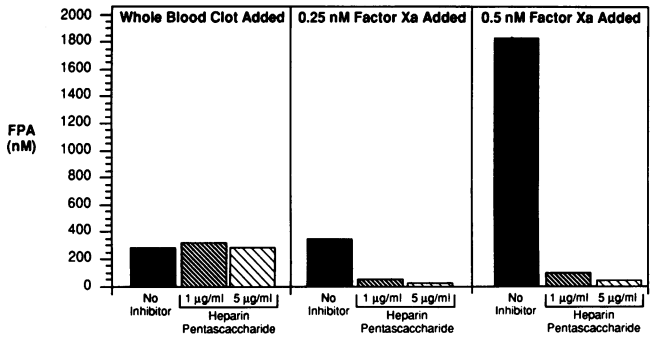

Figure 4. The extent to which heparin pentasaccharide, the Factor Xa–specific inhibitor, attenuated the activation of prothrombin in recalcified barium-adsorbed plasma during incubation with whole blood clots is shown in the first panel. Heparin pentasaccharide at concentrations of 1.0 and 5.0 μg/ml did not inhibit the increases in FPA induced by whole-blood clots after incubation for 10 min in recalcified barium-adsorbed plasma containing 0.9 μM prothrombin. In contrast, heparin pentasaccharide at these concentrations inhibited the increases in FPA induced by addition of both 0.25 nM (*middle panel*) and 0.5 nM (*right panel*) free Factor Xa to the recalcified barium-adsorbed plasma containing prothrombin under identical conditions.

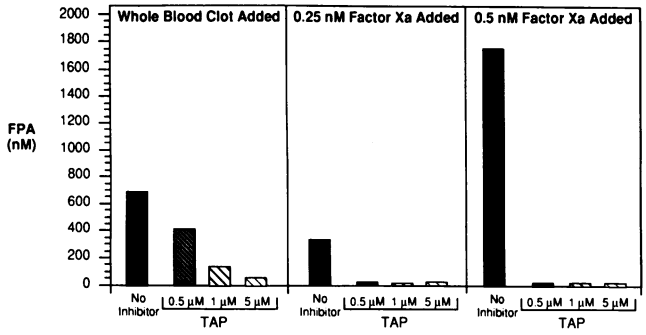

Figure 5. The extent of inhibition of clot-associated Factor Xa activity by TAP. By 10 min and in a dose-dependent manner, TAP attenuated the increases in FPA induced by whole-blood clots during incubation in recalcified barium-adsorbed plasma containing 0.9 μM human prothrombin at 10 min (*left panel*). However, the extent of inhibition was greater when Factor Xa, at concentrations of 0.25 and 0.5 nM (*middle and right panels*), was added to the recalcified barium-adsorbed plasma and prothrombin in the absence of clot. Thus, TAP appeared to more effective in inhibiting free compared with clot-bound Xa activity.

diated inhibition of Xa by fibrin, Factor Xa was incubated with 0.6–10 nM TAP in 0.15 M Tris-HCL, 0.10 M NaCl, pH 7.4, in the presence or absence of fibrin II monomer for 30 min, and residual Xa activity was measured with the chromogenic substrate, S-2222. As shown in Fig. 6, there was no significant difference in the inhibition of Factor Xa by TAP when fibrin II monomer was present.

Factor Xa activity of platelet-rich versus platelet-poor clots. To characterize the extent to which Factor Xa activity was attributable to platelets, clots were prepared from platelet-rich and platelet-poor plasma and incubated in recalcified barium-adsorbed plasma with or without repleted prothrombin. Concentrations of FPA increased only minimally during incubation of these clots in recalcified barium-adsorbed plasma alone

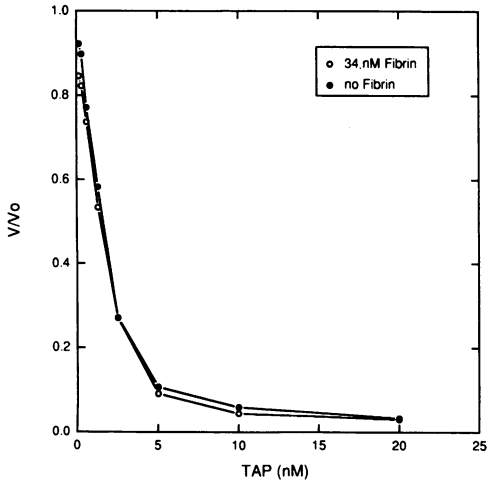

Figure 6. Inhibition of Factor Xa by TAP in the presence or absence of 34 nM fibrin II monomer is shown. Inhibition of Factor Xa is characterized as the ratio of the initial rate of hydrolysis of the chromogenic substrate S-2222 by 0.125 nM Factor Xa after a 30-min incubation with increasing concentrations of TAP compared with the reaction rate in the absence of TAP. Fibrin did not significantly attenuate inhibition of Factor Xa by TAP under these conditions.

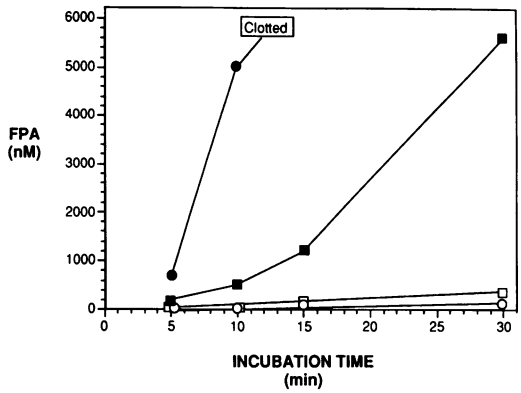

Figure 7. Comparison of procoagulant activity induced by platelet-rich and platelet-poor clots in recalcified barium-adsorbed plasma, with or without 0.9 μM human prothrombin, is shown. Incubation of platelet-rich (*open circles*) and platelet-poor (*open squares*) clots in recalcified barium-adsorbed plasma induced modest increases in the concentration of FPA. In contrast, incubation of platelet-rich (*closed circles*) and platelet-poor clots (*closed squares*) in recalcified barium-adsorbed plasma containing prothrombin resulted in marked increases in the concentration of FPA, consistent with the activation of prothrombin. Platelet-rich clots promoted more rapid formation of fibrin compared with platelet-poor clots, resulting in clotting of the plasma by 10 min.

(Fig. 7), although slightly greater increases were observed with platelet-poor compared with platelet-rich clots. In contrast, incubation of clots with recalcified barium-adsorbed plasma after repletion with 0.9 μM prothrombin resulted in marked increases in the concentration of FPA, consistent with extensive activation of prothrombin. As shown in Fig. 7, platelet-rich clots induced more marked increases in the concentration of FPA than did platelet-poor clots.

Association of Factor Xa with fibrin clots. To determine whether factor Xa activity associated with platelet-poor clots was attributable to binding of factor Xa to fibrin, increasing

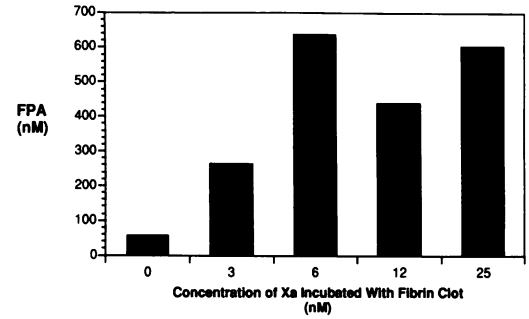

Figure 8. Increases in the concentration of FPA as characterized in recalcified barium-adsorbed plasma containing 0.9 μM prothrombin when incubated with fibrin clots formed in the presence of increasing concentrations of Factor Xa. Fibrin clots formed in the absence of Factor Xa induced modest increases in the concentration of FPA when incubated in the recalcified barium-citrated plasma containing prothrombin. In contrast, clots formed in the presence of increasing concentrations of Factor Xa induced greater increases in the concentration of FPA when subsequently incubated in recalcified barium-adsorbed plasma containing prothrombin. The extent of increase in the concentration of FPA was related to the concentration of Xa present at the time the fibrin clot was formed.

concentrations of Factor Xa were incubated with 0.25 mg/ml of plasminogen-depleted purified human fibrinogen in 0.01 M Hepes, 0.1 M NaCl, and 0.002 M $CaCl_2$, pH 6.8, and clotted by addition of 2 nM α-thrombin. The clots were allowed to form for 1 h at 37°C, extensively washed in the Hepes saline buffer, and incubated with recalcified barium-adsorbed plasma repleted with 0.9 μM human prothrombin for 30 min. As shown in Fig. 8, the increases in the concentration of FPA induced by fibrin clots were related in a dose-dependent manner to the concentrations of Factor Xa (3–25 nM) present when the fibrin clot was formed. The magnitude of increase of FPA induced by incubation of 0.25-mg fibrin clots prepared in the presence of 25 nM Xa was similar to that induced by incubation of clots prepared with plasma containing 0.25 mg of fibrinogen under identical conditions (FPA = 860 nM [n = 2]). Thus, Factor Xa associates with fibrin clots and remains catalytically active.

Discussion

The results of this study indicate that the procoagulant properties of thrombi are in part attributable to the activity of Factor Xa bound to the thrombus. Our results confirm that thrombin binds to fibrin and remains enzymatically active. However, when the clot-bound thrombin activity is inhibited by hirudin, marked activation of prothrombin in plasma still occurs. Thus, clot-bound Factor Xa activity is sufficient, even in the absence of thrombin, to exert significant procoagulant activity. The presence of clot-associated Factor Xa suggests that inhibition of clot-associated procoagulant activity by thrombin-specific inhibitors will be ineffective in preventing continued activation of prothrombin. However, inhibition of Factor Xa may attenuate clot-associated procoagulant activity. Recent results demonstrating the efficacy of TAP in preventing reocclusion after thrombolysis are consistent with this hypothesis (19).

An unexpected finding of this study was that Factor Xa appears to bind to fibrin in clots. An interaction of Xa with fibrin is consistent with the observation that fibrin II monomer attenuates anti-thrombin III–mediated inhibition of Factor Xa (4). Because the association of Factor Xa with fibrin clots that we observed may be in part attributable to nonspecific protein trapping, additional studies characterizing the association of Factor Xa with fibrin monomer will be necessary to determine whether a specific binding of Xa to fibrin occurs, as has been observed with thrombin (1–3).

Although clot-bound thrombin activity induces fibrin formation, the increases in the concentration of FPA are relatively modest (1, 3). Thus, it has been assumed that activation of the coagulation cofactors V and VIII by thrombin accounts for the marked procoagulant properties of thrombi. However, the role of other clot-associated factors in the induction of procoagulant activity by clots formed in vivo has not previously been defined. In the current study, whole-blood clots formed in vitro as well as thrombi recovered after arterial injury in vivo exhibited, in addition to thrombin activity, Factor Xa activity that was sufficient to induce marked activation of prothrombin. The extent of prothrombin activation induced by Factor Xa is significantly greater than that associated with thrombin, judging from the > 10-fold greater increase in FPA induced by Factor Xa activity compared with clot-bound thrombin activity (Fig. 1). Because thrombin activates Factors V and VIII, it is possible that clot-associated thrombin and Factor Xa could act synergystically by inducing prothrombin activation leading to thrombin-mediated activation of cofactors V and VIII, which would potentiate procoagulant activity. These findings have important therapeutic implications because one of the rationales for the use of thrombin-specific anticoagulants is their greater efficacy, compared with heparin–anti-thrombin III, in inhibiting clot-bound thrombin activity. Our findings suggest that thrombin-specific therapy may be effective only as long as it is continued. The persistence of Factor Xa activity is likely to induce continued activation of prothrombin despite potent inhibition of thrombin. This may also account for the recent observation of recurrent thrombosis after discontinuation of heparin in patients with unstable angina (20).

We were unable to demonstrate significant Factor IXa or VIIa activity associated with the whole-blood clots formed in vitro. Nonetheless, we cannot exclude the possibility that the activity of the IXa/VIIIa or VIIa/tissue factor complexes contributes to clot-induced procoagulant activity in vivo. Activation of Factor X by either of these complexes could increase local Factor Xa concentrations to an even greater extent than that which we observed.

Although maximal activation of prothrombin requires formation of a complex of Factor Xa and Factor Va on phospholipid membranes (6), our findings suggest that Factor Xa associated with fibrin clots exhibits procoagulant activity as well (Fig. 8). Additional studies to define the enzymatic activity of Factor Xa bound to fibrin, relative to free Factor Xa, and that associated with Factor Va in the prothrombinase complex, should further clarify the potential importance of Factor Xa bound to fibrin. The finding that platelet-rich clots induce marked activation of prothrombin is consistent with the importance of the factor Xa/Va complex bound to platelets in the procoagulant activity of whole-blood clots (Fig. 7). This may be particularly relevant to the platelet-rich thrombi formed in the arterial system under conditions of high shear stress (20).

Recent studies with TAP in animals indicate that inhibition of Factor Xa is effective in attenuating thrombosis in response to arterial injury and recurrent thrombosis after coronary thrombolysis (19, 21). Patients treated with tissue-type plasminogen activator for acute myocardial infarction exhibit increases in prothrombin activation consistent with increases in Factor Xa activity as well (22). Our results are consistent with these findings and indicate that Factor Xa–induced activation of prothrombin is likely to play a pivotal role in recurrent thrombosis after thrombolysis. Maximal inhibition of the procoagulant activity of thrombi may require inhibition of both thrombin and Factor Xa. Judging from the results of the current study, antithrombin III–independent inhibitors may be more effective in inhibiting clot-induced procoagulant activity, because both thrombin and Factor Xa bound to clot appear to be relatively protected from inhibition by anti-thrombin III (4). Direct inhibition of Factor Xa with TAP was effective in inhibiting Xa associated with clots; however, the effectiveness of this inhibitor may be limited by its slow-binding kinetics. We found that the concentrations of TAP required to inhibit clot-associated Xa procoagulant activity when TAP was not preincubated with the clot were higher than those necessary to inhibit free Xa under the same conditions. However, fibrin II monomer did not attenuate the efficacy of TAP when the inhibitor was preincubated with Xa before the extent of inhibition was characterized (Fig. 6).

In summary, whole-blood clots, particularly when platelet rich, appear to induce activation of the coagulation system by at least two phenomena: one is ascribable to the activity of thrombin bound to fibrin, and the other to clot-associated Xa activity. Both clot-associated thrombin and Factor Xa appear to be relatively protected from inhibition by anti-thrombin III or heparin-anti-thrombin III. These results are consistent with the relative attenuation by fibrin monomer of anti-thrombin III-dependent inhibition of Factor Xa, as demonstrated by Hogg and Jackson (4). Factor Xa activity associated with whole-blood clots induces thrombin formation de novo, even when clot-bound thrombin is inhibited. Clot-associated Factor Xa activity can be inhibited by specific inhibitors such as TAP, and its inhibition may provide an effective means of preventing recurrent thrombosis induced by residual thrombi in the arterial circulation.

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References

1. Francis, C. W., R. E. J. Markham, G. H. Barlow, T. M. Florack, D. M. Dobrzynski, and V. J. Marder. 1983. Thrombin activity of fibrin thrombi and soluble plasmic derivatives. *J. Lab. Clin. Med.* 102:220–230.

2. Wilner, G. D., M. P. Danitz, M. S. Mudd, K. Hsieh, and J. W. Fenton. 1981. Selective immobilization of alpha-thrombin by surface-bound fibrin. *J. Lab. Clin. Med.* 97:403–411.

3. Weitz, J. I., M. Hudoba, D. Massel, J. Maraganore, and J. Hirsh. 1990. Clot-bound thrombin is protected from inhibition by heparin–antithrombin III but is susceptible to inactivation by antithrombin III–independent inhibitors. *J. Clin. Invest.* 86:385–391.

4. Hogg, P. J., and C. M. Jackson. 1989. Fibrin monomer protects thrombin from inactivation by heparin-antithrombin III: implications for heparin efficacy. *Proc. Natl. Acad. Sci. USA.* 86:3619–3623.

5. Miletich, J. P., C. M. Jackson, and P. W. Majerus. 1978. Properties of the factor Xa binding site on platelets. *J. Biol. Chem.* 253:6908–6916.

6. Mann, K. G., M. E. Nesheim, W. R. Church, P. Haley, and S. Krishnaswamy. 1990. Surface-dependent reactions of the vitamin K-dependent enzyme complexes. *Blood.* 76:1–16.

7. Blomback, B., and M. Blomback. 1956. Purification of human and bovine fibrinogen. *Ark. Kemi.* 10:415.

8. Miletich, J. P., G. H. Broze, and P. W. Majerus. 1980. The synthesis of sulfated dextran beads for isolation of human plasma coagulation factors II, IX, X. *Anal. Biochem.* 105:304–310.

9. Kettner, C., and E. Shaw. 1979. D-Phe-Pro-ArgCH2Cl: a selective affinity label for thrombin. *Thromb. Res.* 14:969–73.

10. Waxman, L., D. E. Smith, K. E. Arcuri, G. P. Vlasuk. 1990. Tick anticoagulant peptide (TAP) is a novel inhibitor of blood coagulation factor Xa. *Science (Wash. DC).* 248:593–596.

11. Neeper, M. P., L. Waxman, D. E. Smith, C. A. Schulman, M. Sardana, R. W. Ellis, L. W. Schaffer, P. K. Siegl, and G. P. Vlasuk. 1990. Characterization of recombinant tick anticoagulant peptide. A highly selective inhibitor of blood coagulation factor Xa. *J. Biol. Chem.* 265:17746–52.

12. Choay, J., J. C. Lormeau, M. Petitou, P. Sinay, J. Fareed. 1981. Structural studies on a biologically active hexasaccharide obtained from heparin. *Ann. NY Acad. Sci.* 370:640–649.

13. Chandler, A. B. 1958. In vitro thrombotic coagulation of the blood. *Lab. Invest.* 7:110–114.

14. Nossel, H. L., R. E. Yudelman, V. P. Canfield, V. P. Butler, S. K., G. D. Wilner, G. D. Qureshi. 1974. Measurement of fibrinopeptide A in human blood. *J. Clin. Invest.* 54:43–53.

15. Jordan, S. P., L. Waxman, D. E. Smith, G. P. Vlasuk. 1990. Tick anticoagulant peptide: kinetic analysis of the recombinant inhibitor with blood coagulation factor Xa. *Biochemistry.* 29:11095–11100.

16. Kochum, C., S. Frebelius. 1980. Rapid radioimmunoasay of human fibrinopeptide A-removal of cross-reacting fibrinogen with bentonite. *Thromb. Res.* 19:589–598.

17. Eisenberg, P. R., L. A. Sherman, K. Schectman, J. Perez, B. E. Sobel, and A. S. Jaffe. 1985. Fibrinopeptide A: a marker of acute coronary thrombosis. *Circulation.* 71:912–918.

18. Shi, O., R. Sio, S. Lin, K. Yu, K. Arbuthnutt, J. Ruiz, and P. Gaur. 1991. Performance characteristics of an enzyme-linked immunosorbent assay (ELISA) for prothrombin fragment F1.2. *Thromb. Haemost.* 65:1118*a*.

19. Sitko, G. R., D. R. Ramjit, I. I. Stabilito, D. Lehman, J. J. Lynch, G. P. Vlasuk. 1992. Conjunctive enhancement of enzymatic thrombolysis and prevention of thrombotic reocclusion with the selective factor Xa inhibitor, tick anticoagulant peptide. *Circulation.* 85:805–815.

20. Theroux, P., D. Waters, J. Lam, M. Juneau, J. McCans. Reactivation of unstable angina after the discontinuation of heparin. *N. Engl. J. Med.*

21. Schaffer, L. W., J. T. Davidson, G. P. Vlasuk, P. K. S. Siegl. 1991. Antithrombotic efficacy of recombinant tick anticoagulant peptide. A potent inhibitor of coagulation factor Xa in a primate model of arterial thrombosis. *Circulation.* 84:1741–1748.

22. Eisenberg, P. R., B. E. Sobel, and A. S. Jaffe. 1992. Activation of prothrombin accompanying thrombolysis with rt-PA. *J. Am. Coll. Cardiol.* 19:1065–1069.