



Regular Article

Aprotinin counteracts heparin-induced inhibition of platelet contractile force

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Abstract

Background: Aprotinin interferes with heparin binding to platelets and decreases blood loss during cardiopulmonary bypass (CPB). Heparin abolishes platelet force during CPB, and the extent of platelet force recovery after protamine administration appears to correlate with blood loss. This study assessed the effect of aprotinin on heparin suppression of platelet force. **Methods:** Platelet force was measured using the Hemodyne® Hemostasis Analyzer. Clots were formed from platelet-rich plasma (PRP) by the addition of batroxobin and 10 mM CaCl₂. Clotting conditions included pH 7.4, ionic strength 0.15 M, fibrinogen level 1 mg/ml and 75,000 platelets/μl. **Results:** After 1200 s of clotting, force was reduced from 7110 ± 1190 to 450 ± 450 dyn by 0.2 U/ml of heparin. Platelet force in aprotinin [20 μg/ml (140 KIU/ml)] containing PRP was not suppressed by heparin addition (7480 ± 2410 dyn). Aprotinin [40 μg/ml (280 KIU/ml)] addition to previously heparinized plasma counteracted heparin force suppression. Aprotinin (40 μg/ml) increased platelet force from 5630 to 11,138 ± 562 in PRP devoid of heparin. Aprotinin did not affect thrombin activity, fibrin structure, platelet aggregation or secretion. **Conclusions:** Aprotinin counteracts heparin suppression of platelet force and enhances platelet force in the absence of heparin. Aprotinin–heparin–platelet interactions may help explain aprotinin's ability to reduce blood loss during CPB.

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The qualitative platelet disorder acquired during cardiopulmonary bypass (CPB) is thought to significantly contribute to blood loss [1]. Microvascular bleeding is the result of a combination of thrombocytopenia, platelet dysfunction and major surgical trauma [2]. CPB-induced platelet dysfunction is believed to be the result of both intrinsic (α-granule depletion, surface adhesion receptor disruption) and extrinsic (hypothermia, inadequate in vivo agonist) platelet defects [1–4]. Exposure of circulating blood to the bypass circuit results in platelet and kallikrein activation [2–4]. Whereas platelet activation causes partial degranulation, kallikrein activation leads to plasmin-mediated alterations

in platelet adhesion receptors [2,4,5]. Growing evidence further suggests that residual heparin present following CPB may make a significant contribution to postoperative bleeding [3,6]. Indeed, several studies have demonstrated that intravenous heparin prolongs the bleeding time [7], interferes with von Willebrand Factor (vWf) binding [8] and ablates platelet contractile force (PCF) during CPB [9].

Aprotinin, a serine protease inhibitor, has been shown to decrease bleeding and blood product utilization during CPB [4,10]. Aprotinin is thought to preserve platelet function by decreasing kallikrein [10,11], thrombin [5,10] and fibrinolytic activity [4,10]. In patients undergoing CPB, aprotinin inhibits plasmin-mediated cleavage of glycoprotein Ib (Gp Ib) receptors [4] and thrombin-induced platelet activation [2,10,11]. Improved platelet preservation post-CPB has also been documented through electron microscopic studies [12] and improved platelet aggregation on extracellular matrix

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[13]. Aprotinin has also been shown to block or reverse heparin binding to platelet membranes [6].

Actin is the predominant protein in platelets. Contractile proteins are critical to multiple platelet functions including platelet shape change, granule secretion and clot retraction. Direct visual assessment of clot retraction has served as a test of platelet function for greater than 200 years. Development of an instrument and technique capable of directly measuring contractile force now permits quantitative assessment of platelet-mediated clot retraction [14].

In the new assay, platelet contractile force (PCF) is directly measured during clot formation. If no platelets are present, no force is measured and PCF increases in a linear fashion with increasing platelet concentration [15]. Metabolic inhibition of platelets reduces PCF, and blockade of glycoprotein IIb/IIIa produces dose-dependent reductions in PCF [16–18]. PCF is reduced in Glanzmann's thrombasthenia [19] and in uremic patients who bleed [20], but is increased in Buerger's disease [21]. The development of PCF is thrombin-dependent and is sensitive to clotting factor deficiencies [22] and a variety of anticoagulants [23–26]. The development of PCF dramatically alters fibrin structure, resulting in significant increases in clot elastic modulus [27], and PCF is increased in a variety of hypercoagulable states including diabetes [28,29] and coronary artery disease [30].

Platelets obtained from patients undergoing CPB produce no contractile force [9]. Heparin reversal with protamine sulfate returns PCF to varying degrees of its baseline value. The degree to which PCF recovers inversely correlates with the amount of blood loss at 12 and 24 h [9]. An *in vitro* study confirmed that force suppression during CPB was due in part to heparin [24]. This *in vitro* study further demonstrated that at the high dose of heparin (4 U/ml) used during CPB, protamine reversal of the anticoagulant effects of heparin may not ensure recovery of PCF [23]. These findings suggest that the presence of low levels of residual heparin following CPB might contribute to the extrinsic platelet defect in these patients.

The ability of aprotinin to reduce heparin binding to platelets prompted this examination of aprotinin's influence on heparin-mediated suppression of platelet contractile force. We hypothesized that exposure of platelets to therapeutic doses of aprotinin would significantly decrease the inhibitor effect of low levels of heparin on PCF. The effects of aprotinin on fibrin assembly, platelet secretion and platelet aggregation were measured to assess potential changes in these parameters in relation to PCF.

1. Materials and methods

1.1. Materials

Human thrombin, greater than 90% alpha, was purchased as a lyophilized powder from Sigma (St. Louis, MO). The

material, with a specific activity of 4300 NIH U/ml was dissolved in water, diluted with 0.10 M NaCl to a final concentration of 20 U/ml, divided into 1-ml lots and frozen at -90°C . Thrombin was free of plasmin and plasminogen. Batroxobin was purchased as Atroxin[®]. *Bothrops atrox* venom protein (Sigma Diagnostics) was dissolved in deionized water and used without further purification. It was maintained on ice and used within 3 h of being dissolved. Aprotinin was provided as Trasylol[®] by Miles (West Haven, CT). The preparation contained 1.4 mg/ml of protein with an activity of 10,000 KIU/ml. The synthetic chromogenic substrate S2238, purchased from Kabi Diagnostics (Stockholm, Sweden), was used in thrombin activity assays. Nanopure water was used in the preparation of all solutions.

Under a protocol approved by the Institutional Review Board for Human Studies of Virginia Commonwealth University and after informed signed consent, human blood for each experiment was obtained in citrated (3.8%) glass tubes by aseptic venipuncture of normal volunteers ($n=4$). Platelet-rich plasma (PRP) was prepared by centrifuging blood samples at low speed (500g) for 10 min. Platelet-poor plasma (PPP) was prepared by additional centrifugation at 2000g for 20 min. The fibrinogen concentration of plasma samples was determined by the modified method of Clauss [31].

1.2. Measurement of platelet contractile force

The Hemodyne[®] Hemostasis Analyzer (Hemodyne, Richmond, VA, USA) (Fig. 1) measures forces generated by platelets within a clot formed between a thermostated cup and parallel upper plate [14,20]. Before clotting, the upper plate is centered above the cup and lowered into the sample. As the clot forms, it attaches to the inner walls of the cup and the bottom of the upper plate. The entire sample volume is contained between the upper and lower surfaces.

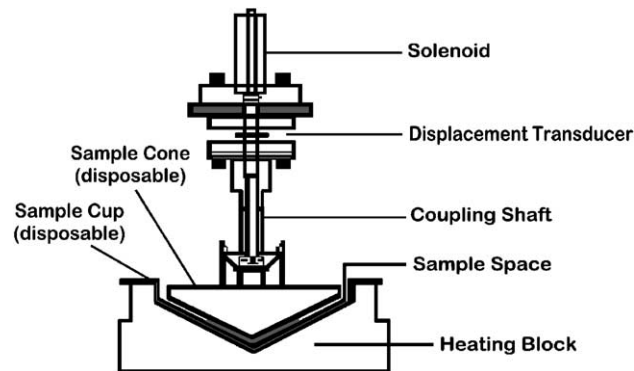


Fig. 1. Schematic diagram of the Hemodyne instrument used to measure force developed by platelets during clot formation. As the clot retracts, a downward force is exerted on the plate attached to the transducer. The resultant downward deflection of the plate generates a voltage, which is proportional to the downward force.

Once clotting is completed, platelets within the network pull fibrin strands inward, transmitting force through the network to the surfaces to which the clot is adherent. Force measurement is accomplished by utilizing a displacement transducer coupled to the upper plate. As platelets contract, the transducer produces an electrical output proportional to the amount of force generated. The transducer output is continuously recorded. Normal values for force development at 1200 s have been established [32].

Plasma clot formation was induced by the addition of batroxobin (0.25 µg/ml). Batroxobin is a serine protease which is not inhibited by antithrombin III and is, thus, insensitive to the effects of heparin. Although batroxobin does not directly activate platelets, clot retraction and platelet force development (PFD) are noted in such clots if calcium or a platelet agonist is added [24].

Clot dissolution experiments were performed using single-chain human recombinant tPA (Activase®, 580,000 IU/mg) purchased from Genentech, South San Francisco, CA. Genentech tPA was diluted with normal saline containing 0.5% albumin (human) USP (Cutter Biological, Miles, IN).

1.3. Measurement of platelet ATP secretion in platelet-rich plasma

ATP release was measured utilizing Chrono-Lume® reagent (Chronolog, Havertown, PA) and a Chrono-Log® (Chronolog) whole blood lumi-aggregometer. For each assay, 900 µl of platelet-rich plasma (platelet count = 200,000/µl) and 100 µl of Chrono-Lume reagent were placed in an aggregometer cuvette equipped with a stirring bar. The amount of ATP secreted was determined by comparing the peak luminescence signal for a test sample to luminescence signals generated by known amounts of a Chrono-Lume ATP standard.

1.4. Measurement of platelet aggregation in platelet-rich plasma

Platelet aggregation was measured utilizing a Chrono-Log® whole blood lumi-aggregometer. Nine hundred microliters of platelet-rich plasma was placed in an aggregometer cuvette equipped with a stirring bar. Platelet concentration was set to 200,000/µl by diluting platelet-rich plasma (PRP) with platelet-poor plasma (PPP). Platelet aggregation was monitored electrically as an increase in impedance (ohms).

1.5. Measurement of clot formation and fibrin mass/length ratios

Plasma gels were formed by adding a varying amount of plasma to 1.2 ml of buffered thrombin to yield a final fibrinogen concentration of 1 mg/ml. All gels were made in triplicate. Effects of aprotinin on the kinetics of fibrin

assembly and structure were studied on clots formed at 37 °C.

Turbidity measurements were made at 633 nm utilizing a thermostated Shimadzu scanning spectrophotometer (Hagerstown, MD). Turbidity was monitored for 30 min after which time gelation was allowed to go to completion unobserved. After 24 h, purified and platelet-poor plasma gels were scanned from 400 to 800 nm and the mass/length ratios of the fibrin fibers were determined according to the following equation [33]:

$$\tau = ((88/15)\pi^3 n(dn/dc)^2 C \mu) / N \lambda^3$$

where τ is the turbidity, n the solution refractive index, dn/dc the refractive index increment, λ the wavelength, C the concentration of fibrinogen in g/ml, N the Avogadro's number and μ the mass/length ratio.

1.6. Kinetics of clot dissolution

Plasma clot dissolution was studied by forming gels in the presence of r-tPA. Thrombin and r-tPA (final concentration 70 IU/ml) were added simultaneously. Gel turbidity was monitored continuously at 633 nm. Clot formation was seen as an increase in turbidity while subsequent digestion was monitored as the gradual decrease in gel turbidity. Turbidimetric lysis time was defined as the time required, after addition of thrombin, for gel turbidity to decrease by 50% from the maximum optical density [34].

The ability of aprotinin to affect heparin suppression of platelet force development was measured directly. The ability of aprotinin to prevent changes in platelet force development was examined by adding aprotinin to platelet-rich plasma prior to the addition of heparin. The ability of aprotinin to “reverse” heparin-induced suppression of platelet force development was measured by addition of aprotinin to previously heparinized plasma. The potential effects of aprotinin on platelet aggregation and secretion were assessed by adding increasing amounts of aprotinin to platelet-rich plasma and subsequently inducing aggregation and secretion by addition of the platelet agonists ADP and collagen. The potential effects of aprotinin on fibrin assembly and structure were monitored by clotting plasma in the presence of increasing aprotinin concentrations. The effect of aprotinin on fibrinolysis was monitored by clotting plasma in the presence of tPA and increasing amounts of aprotinin.

Current dosing regimens result in plasma aprotinin concentrations of 1–8 µmol/l [35]. A single dose of 1–2 million KIU yields 30–60 mg/l of aprotinin, which is equivalent to 210–430 KIU/ml. The concentrations of aprotinin used in this study were 20–80 µg/ml. Given aprotinin's molecular weight of 92,000 and a hematocrit of 40%, this yields final plasma concentrations of 0.362–1.45 µmol/l. This is within the range normally achieved during routine aprotinin administration.

1.7. Statistics

All data are presented as the means \pm S.E.M. of three measurements. Statistical analysis was performed using Student's paired *t*-test. A *p* value <0.05 was considered statistically significant.

2. Results

The effect of aprotinin on heparin-induced suppression of PCF is presented in Fig. 2. Data are presented as the means \pm S.E.M. of three measurements. Heparin at 0.20 U/ml reduced force development from 7110 ± 1190 to 450 ± 450 dyn. The addition of 20 $\mu\text{g/ml}$ aprotinin to PRP just prior to the addition of heparin and batroxobin prevented heparin-induced suppression of platelet force development (PFD = 7480 ± 2410 dyn). Force development began earlier in the aprotinin-containing gels, but the difference in PCF after 1200 s was not statistically significant.

The ability of aprotinin to restore PCF in previously heparinized PRP is presented in Fig. 3. As with the previous experiment, heparin totally inhibited force development. The addition of 40 $\mu\text{g/ml}$ of aprotinin, followed by a 20-min incubation, produced marked recovery of force. The extent of force recovery varied with aprotinin dose, with lower doses being more effective. Heparinized plasma to which 40 $\mu\text{g/ml}$ aprotinin was added produced 9916 ± 984 dyn of force, whereas 60 $\mu\text{g/ml}$ aprotinin produced 5820 ± 1200 dyn of force (data not shown) and 80 $\mu\text{g/ml}$ aprotinin produced 1938 ± 538 dyn of force. At aprotinin concentrations above 80 $\mu\text{g/ml}$, force development was reduced further (data not shown).

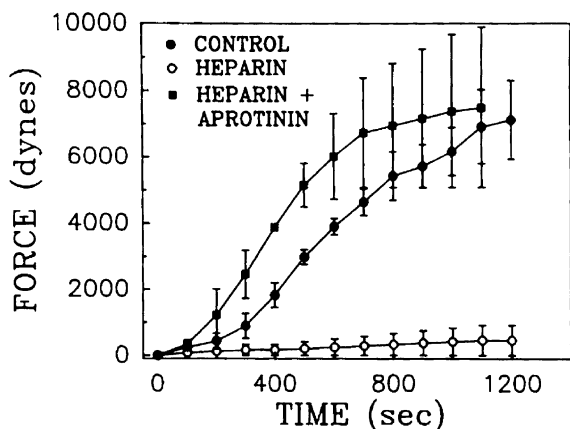


Fig. 2. Effect of aprotinin on heparin suppression of platelet force development in platelet-rich plasma clotted with batroxobin. Aprotinin (20 $\mu\text{g/ml}$) was added 1 min prior to the addition of heparin (0.20 U/ml) and batroxobin (0.25 $\mu\text{g/ml}$) at time 0. Data are presented as the means \pm standard error of the mean (S.E.M.) of three measurements. Other clotting conditions included 10 mM CaCl_2 , pH 7.4, ionic strength 0.15 M, 75,000 platelets/ μl , fibrinogen level 1 mg/ml and temperature 37 $^\circ\text{C}$.

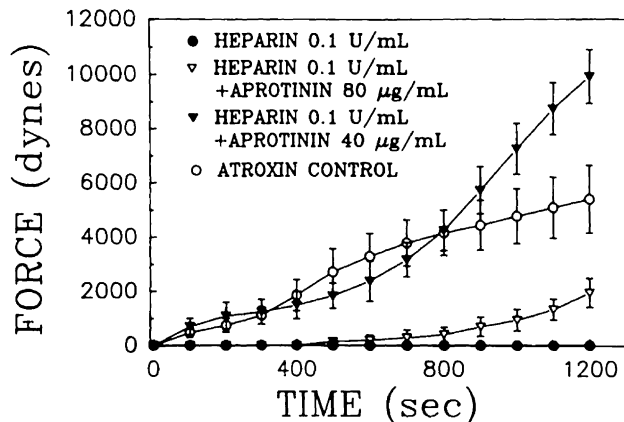


Fig. 3. Ability of aprotinin to reverse heparin-induced suppression of platelet force development in platelet-rich plasma. Heparin was added to plasma 1 min prior to the addition of aprotinin. Plasma was allowed to incubate at 27 $^\circ\text{C}$ for 20 min prior to the addition of batroxobin. Data are presented as the means \pm S.E.M. of three measurements. All other clotting conditions were the same as for Fig. 2.

The effect of aprotinin on PCF, in the absence of heparin, is presented in Fig. 4. In batroxobin-induced clots containing 20 $\mu\text{g/ml}$ aprotinin, force development after 1200 s (7000 ± 1130 dyn) was not statistically greater ($p > 0.50$) than force developed (5630 dyn) in control clots. At 40 $\mu\text{g/ml}$ aprotinin, however, force after 1200 s ($11,138 \pm 562$ dyn) was significantly increased ($p < 0.001$).

The ability of aprotinin to enhance PCF of normal platelets was further investigated by monitoring the effects of aprotinin on platelet aggregation (upper panel, Fig. 5) and platelet secretion (lower panel, Fig. 5). Platelet aggregation, whether induced by collagen or ADP, was unaltered by aprotinin concentrations as high as 80 $\mu\text{g/ml}$. ADP-induced ATP secretion was unaffected by aprotinin. Collagen-

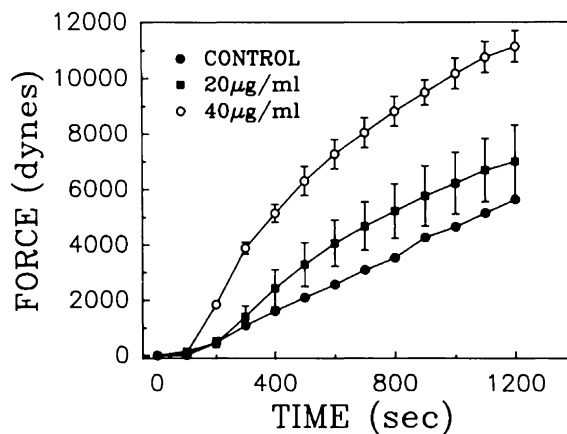


Fig. 4. Effect of aprotinin on platelet force development in platelet-rich plasma clotted with batroxobin (0.25 $\mu\text{g/ml}$) in the absence of heparin. After aprotinin addition, plasma was allowed to incubate at 27 $^\circ\text{C}$ for 20 min prior to the addition of batroxobin at time 0. Control clots did not contain aprotinin. All other conditions were the same as indicated in Fig. 2. Data are presented as the mean \pm S.E.M. of three measurements.

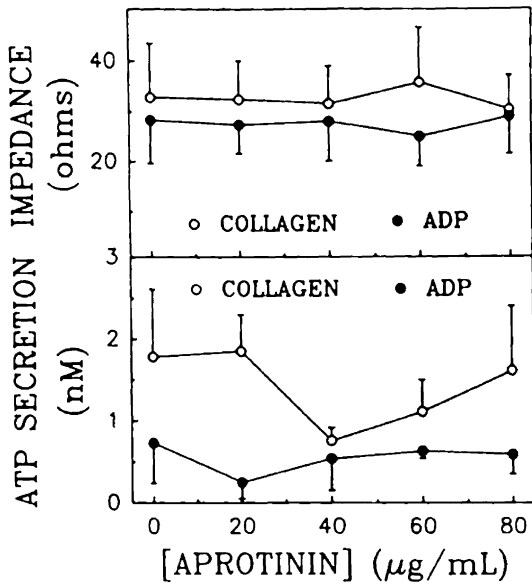


Fig. 5. Effect of aprotinin on platelet aggregation (upper panel) and platelet secretion (lower panel) induced by either collagen (○) or ADP (●). Data are presented as the means \pm S.E.M. of three measurements. Error bars are unidirectional to clarify presentation.

induced ATP secretion, although more variable, was not significantly altered by aprotinin.

Since thrombin is such a profound agonist of platelet function, possible direct effects on thrombin activity were assessed. The ability of thrombin to cleave the chromogenic synthetic substrate S2238 was not altered by aprotinin concentrations as high as 80 $\mu\text{g}/\text{ml}$. Initial slopes of optical density development at 405 nm (OD_{405}) were 1.08, 0.99 and 1.05×10^{-3} OD U/s for aprotinin concentrations of 0, 40 and 80 $\mu\text{g}/\text{ml}$. At 240 $\mu\text{g}/\text{ml}$ aprotinin, cleavage of S2238 was significantly slowed ($p < 0.05$) with the initial slope reduced to 0.68×10^{-3} OD U/s.

Possible effects of aprotinin on thrombin cleavage of fibrinogen on fibrin assembly and on fibrin structure were studied turbidimetrically. Aprotinin at concentrations as high as 80 $\mu\text{g}/\text{ml}$ did not alter either the onset or initial rate of turbidity (OD_{633}) development upon addition of thrombin to plasma (data not shown). Final gel turbidities were slightly decreased at higher aprotinin concentrations. This decreased gel turbidity resulted from a small decline in fibrin fiber size (Fig. 6). The fibrin fiber mass/length ratio declined from 2.9 ± 0.165 to $2.5 \pm 0.17 \times 10^{13}$ Da/cm as the aprotinin concentration rose from 0 to 80 $\mu\text{g}/\text{ml}$ (closed circles). This decline in fiber size was completely accounted for by shifts in ionic strength caused by saline within the aprotinin preparation. As increasing amounts of aprotinin were added to the blood sample, increasing NaCl resulted in an increase in the ionic strength of the sample. Fibrin formation and structure are sensitive to ionic strength. As the ionic strength increases, the size (μm) of the fibrin fibers decreases. A second series of measurements were performed by adding amounts of NaCl identical to that present in the

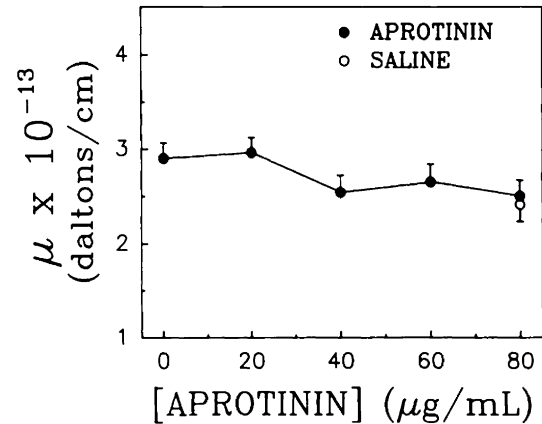


Fig. 6. Effect of aprotinin on fibrin fiber mass/length ratio in plasma clots formed by the addition of thrombin (1 NIH U/ml). Gels were scanned 4 h after thrombin addition. The open circle represents a control gel formed by addition of an equivalent amount of saline contained in the aprotinin preparation. Data are presented as the means \pm S.E.M. of three measurements.

aprotinin samples to blood prior to addition of thrombin. The resulting fibrin structures were identical to those seen in the presence of aprotinin, indicating that the diminished size seen in the presence of aprotinin solutions was due to the NaCl in those solutions. For simplicity, only one saline point (open circle) was included in Fig. 6.

The effects of aprotinin on tPA-mediated fibrinolysis in plasma are demonstrated in Fig. 7. In the absence of aprotinin, 70 IU/ml of tPA produces 50% lysis within 900 s. In the presence of 20 $\mu\text{g}/\text{ml}$ aprotinin, lysis is completely inhibited during the 3000-s period of observation. At 40 $\mu\text{g}/\text{ml}$ aprotinin, lysis is completely inhibited and gel optical density is slightly reduced due to an increase in sample ionic

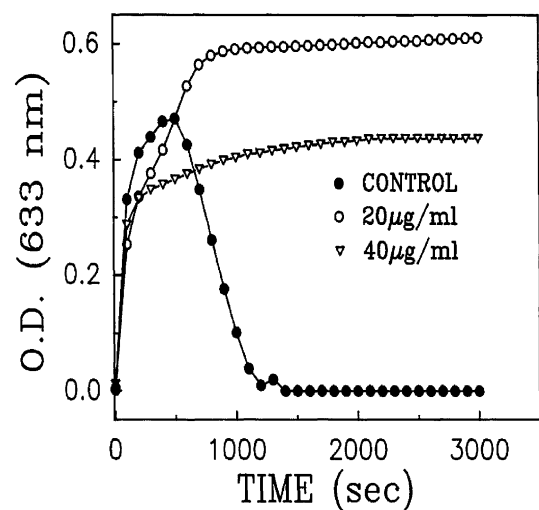


Fig. 7. Effect of aprotinin on tPA-induced clot lysis in plasma clots formed in the presence of increasing amounts of aprotinin. Thrombin (1 NIH U/ml) and tPA (70 IU/ml) were added at time 0. Other assay conditions included 10 mM CaCl_2 , pH 7.4, ionic strength 0.15 M, fibrinogen level 1 mg/ml and temperature 37 °C.

strength due to the presence of saline in the aprotinin preparation.

3. Discussion

The importance of heparin–platelet interactions is being increasingly appreciated. Heparin binds to platelet membranes [36], prolongs the bleeding time [7], interferes with vWf binding [8] and suppresses platelet force development [24]. The latter finding appears to be important for hemostasis during CPB. During CPB, force development is completely suppressed. Upon reversal of heparin with protamine, force development partially recovers and the degree of recovery is inversely correlated with perioperative blood loss [9].

Aprotinin has been shown to decrease heparin binding to platelets [6]. The demonstration by this study that aprotinin can prevent or counteract heparin suppression of PCF is another indication that heparin binding to platelets alters platelet function. We have previously shown that protamine causes recovery of platelet force as anticoagulant effects are reversed [23]. This is clearly not the case with aprotinin. Rather than reversing, aprotinin may actually potentiate anticoagulation via inhibition of procoagulant serine proteases [10]. In addition, since fibrinolysis was not occurring in our system, the recovery of force development cannot be ascribed to suppression of plasmin activity.

At the high heparin doses employed during CPB, protamine reversal of heparin force suppression may be incomplete when anticoagulant effects are totally reversed [23]. Additional protamine may completely restore platelet force, but at high concentrations, protamine becomes an anticoagulant and enhances fibrinolysis [23,37]. If force suppression persists once heparin's anticoagulant effects are reversed, direct restoration of platelet force by aprotinin may be appropriate.

Enhancement of PCF in normal platelets by aprotinin was unexpected. Previous studies have shown no effect of aprotinin on platelet function in the absence of heparin [6], inhibition of aggregation in normal platelets or a combination of inhibition and enhancement [38]. We have reported that a very low dose (0.005 U/ml) of heparin increases force development [24], and other investigators have noted enhanced platelet aggregation at very low heparin concentrations. As demonstrated in this study, higher doses of heparin suppress force development. Enhancement at low and inhibition at high ligand concentration can result from the presence of multiple receptors of varying affinity. Evidence already exists for both high- and low-affinity heparin receptors on the platelet membrane [36]. Aprotinin's potential interaction with these receptors has yet to be defined. The clinical relevance of increased PCF has not been defined, but very high platelet force has been noted in patients with severe coronary artery disease [27], in patients with chest pain [30], in

patients with Buerger's disease [21] and in patients with diabetes [29].

Most of the available information on aprotinin–platelet interactions comes from studies of patients undergoing cardiopulmonary bypass (CPB). Platelet aggregations in response to arachidonic acid, ADP, thrombin and ristocetin are decreased in blood drawn from patients on bypass [39–42]. Altered platelet aggregation is accompanied by altered expression of GP IIb/IIIa [39,40,43,44], GP Ib [39,44,45], GP IV [39] and P selectin [39,42–44]. While some evidence has been found for preservation of thrombin-induced platelet aggregation [41], aprotinin apparently does not protect platelets from CPB-induced reductions in other forms of platelet aggregation [39,40,42]. Interestingly, *in vitro* studies indicate that aprotinin blocks protease-activated receptor 1 (PAR1) but does not affect aggregation induced by other agonists [46,47]. If aprotinin binds at or near the PAR1 receptor during CPB, this finding could offer a potential explanation for the isolated protective effect of aprotinin for thrombin-induced aggregation [41]. The potential importance of platelet–leukocyte interactions and aprotinin have been raised by the finding of diminished responsiveness to thrombin receptor agonist peptide after CPB in whole blood but not in platelet-rich plasma [48]. Aprotinin may aid platelet function by reducing leukocyte activation and thereby reducing platelet–leukocyte interactions.

Since thrombin is both a serine protease and a profound agonist of platelet function, we investigated possible effects of aprotinin on thrombin enzyme activity. Aprotinin neither enhanced nor inhibited thrombin amidolytic or proteolytic activity over the range of interest. In like fashion, aprotinin neither altered the rate of fibrin formation nor the resulting fibrin structure. Although there is significant evidence that heparin-mediated antithrombin activity may be at least partially responsible for heparin-induced force suppression, aprotinin does not appear to directly alter thrombin enzyme activity [24]. Some altered heparins with intrinsically low antithrombin and anti-Xa still possess significant ability to suppress platelet force. Aprotinin may be affecting this non-antithrombin-mediated heparin activity.

The major limitations of this study are that it was completely *in vitro* and a relatively small number of individuals, all of whom were normal control subjects, were utilized for the plasma samples. Whether the same results will be seen in samples from patients who are being administered heparin and aprotinin or whether the results are altered by underlying disease processes such as coronary artery disease awaits additional investigation. It should be noted, however, that the results in the normals appeared to be entirely consistent with respect to the effects of heparin and aprotinin.

In summary, we present evidence that aprotinin can prevent or counteract heparin-induced suppression of PCF. This aprotinin effect is not due to reversal of anticoagulation, suppression of plasmin activity or direct effects on thrombin enzymatic activity. Since force development

appears to be important for hemostasis, this adds to the explanation of how aprotinin may reduce blood loss during CPB.

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