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Effect of Tranexamic Acid on Platelet ADP During Extracorporeal Circulation

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Seventeen adults received the antifibrinolytic drug tranexamic acid during cardiac surgery utilizing extracorporeal circulation (ECC). In 8 patients, drug administration began prior to skin incision (pre-ECC); infusions commenced after ECC and protamine administration in another 9 patients (post-ECC). Compared with the post-ECC group, the pre-ECC group exhibited less bleeding via mediastinal drains (420 vs. 655 mL/12 h median, $P = 0.024$), decreased frequency of the presence ($\geq$10 μg/mL) of fibrin split products ($P < 0.05$), and greater platelet dense granule content of adenosine diphosphate after surgery (15.47 vs. 4.05 nmoles/mg protein median, $P = 0.021$).

Follow-up in vitro study of tranexamic acid inhibition of plasmin-induced platelet activation utilizing normal human platelet rich plasma and porcine plasmin revealed a 13-fold lower concentration of tranexamic acid for 50% inhibition when plasmin was preincubated with the drug (1.2 μg/mL, 95% CI = 1.13 – 1.60 μg/mL) compared to when platelet rich plasma was preincubated with the drug (16 μg/mL, 95% CI = 7.3 – 99. μg/mL). Plasmin inactivated with tranexamic acid retained its ability to inhibit thrombin-induced platelet activation, thus suggesting that tranexamic acid inhibits plasmin's catalytic activity and not its binding to platelets.

Both clot lysis and platelet dysfunction may contribute to bleeding after ECC. Tranexamic acid blocks plasmin-induced partial platelet activation during ECC, thus preserving platelet function and promoting hemostasis after ECC.

Key words: platelet aggregation, antifibrinolytic agents, tranexamic acid, fibrin-fibrinogen degradation products, extracorporeal circulation, hemostasis, surgical

INTRODUCTION

Substantial bleeding following extracorporeal circulation (ECC) complicates post-operative care. Attempts to limit bleeding with drugs have met with varied success [1,2,3,4,5,6]. Desmopressin acetate may [1] or may not [3] limit hemorrhage after valve replacement or repeat sternotomy procedures. No hemostatic salutary effect accompanied desmopressin for elective procedures in children [4] or adult aortocoronary bypass grafting [5,6]. Lysine analog plasmin-plasminogen inhibitors (antifibrinolytics) also have provided varied results [7,8, 9,10,11,12,13]. Several trials of the lysine analog ε-aminocaproic acid were neither randomized nor blinded [7–9,11]. One well-designed study demonstrated decreased bleeding with ε-aminocaproic acid administered after ECC [13]. Recent study of the antifibrinolytic agent tranexamic acid demonstrated that its administration before, during, and after ECC decreases bleeding after operation and limits formation of fibrin split products (FSP) [14].

While FSP may exacerbate bleeding by preventing formation of fibrin polymer or by inhibiting platelet aggregation [15], abnormal hemostasis following ECC occurs mainly from platelet dysfunction [16,17]. A significant reduction in releasable platelet adenosine diphosphate (ADP) content occurs after operation [18]. Direct antibody techniques [19] indicate partial activation of platelets during surgery. Since plasmin acts as a weak platelet agonist [20,21] or antagonist [22] depending upon concentration and conditions, local plasmin concentrations during operation may partially activate platelets, resulting in ADP depletion. Thus, a salutary effect of tranexamic acid may arise from inhibition of plasmin-

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ogen and plasmin at platelets instead of, or perhaps in addition to, preventing clot lysis.

This investigation determined if a platelet preservation mechanism contributes to the hemostatic effect of tranexamic acid during ECC. The investigators hypothesized that tranexamic acid given prior to ECC would preserve platelet ADP content, while tranexamic acid given after ECC would not preserve platelet ADP.

METHODS

Following institutional review board approval, 17 patients gave written, informed consent to receive intravenous tranexamic acid during cardiac surgery. Eligible patients did not receive dipyridamole or non-steroidal anti-inflammatory medications within 3 days, or aspirin or warfarin within 7 days of operation. No patient gave a personal or family history of bleeding disorder.

Patients received intravenous tranexamic acid randomly and in a double-blinded fashion beginning either prior to skin incision or immediately after heparin neutralization with protamine. Drug dosing began with a 10 minute infusion of 7 mg/kg followed by 2 mg/kg/hr for 2 hrs; the maintenance infusion rate was 1 mg/kg/hr. Drug infusion ceased 10 hrs following protamine administration.

Clinical Measurements

Prothrombin time (PT), activated partial thromboplastin time (aPTT), platelet count, plasma fibrinogen concentration, fibrin split products (serum fibrin-fibrinogen related antigen, ThromboWellco Test), plasminogen concentration [23], and platelet function studies (see below) were determined prior to surgery and again 4 hrs after operation during drug infusion. Template bleeding time was obtained prior to surgery only; monitoring catheters prevented access for template bleeding time after operation.

Heparin, 400 U/kg, provided anticoagulation for ECC. Automated activated coagulation time (Hemochron, International Technidyne Corp, Edison, NJ), greater than 480 s, assured continued anticoagulation. ECC utilized non-occlusive roller pumps and membrane. After ECC, protamine neutralized remaining heparin to obtain an activated coagulation time within 15 seconds of baseline. Blood drained into calibrated containers via mediastinal tubes for the first 12 hrs after operation constituted blood loss. The study ignored estimates of irrigation fluid, sponge and suction container losses, and soaking of linens; all of these minor components of operative blood loss are notoriously inaccurate.

Platelet Laboratory Studies

Centrifugation (200 × g for 10 minutes) provided platelet rich plasma (PRP) from whole blood collected in citrated plastic tubes. For determination of platelet granule adenosine triphosphate (ATP) and ADP content, 4 mL of PRP were pelleted at 650 × g for 15 min and processed exactly as previously described [24]. Conversion of ADP to ATP proceeded as described by Holmsen et al. [25].

A Chronolog lumi-aggregometer measured ATP content at 37°C as previously described [24]. A 50 μL aliquot of sample or standard was added to 430 μL of Tris buffer (5.5 mM Tris base plus 44.5mM Tris-HCl, pH 7.38). After 30 sec, 20 μL of chrono-lume luciferin-luciferase was added and luminescence measured.

Serotonin (5HT) uptake was determined as previously reported [26]. A modification of Brodie et al.'s procedure [27] determined serotonin content. Approximately 4 mL of PRP was pelleted at 650 × g for 15 min. and processed exactly as previously reported [24]. Serotonin was measured fluorometrically at excitation 295 mμ and transmission 550 mμ in a Perkin-Elmer LS-5 fluorescence spectrophotometer.

Experiments With Normal Human Platelets

In vitro experiments measured tranexamic acid interference with plasmin-induced platelet activation. These studies substituted normal human PRP and porcine plasmin for the usual gel filtered platelets and human plasmin [20–22]. To study the effects of human plasmin, platelets must be washed free of the antiplasmin present in plasma. Human antiplasmin does not, however, completely inhibit the effects of porcine plasmin. Use of porcine plasmin permits substitution of PRP for gel filtered platelets, thus more closely modeling in vivo conditions. We first verified the suitability of these substitutions. Porcine plasmin induced the same pattern of immediate shape change followed by an aggregation-like phase with PRP as with gel filtered (Sepharose CL-2B) platelets. Human plasmin added to gel filtered platelets induced similar responses, although the platelet shape change was smaller and the aggregation response greater.

In the first in vitro experiment, PRP was pre-incubated for 10 min with several concentrations of tranexamic acid, chosen from pilot experiments to yield percent inhibitions of aggregation below and above 50 percent; porcine plasmin (3 U/mL) was added, and platelet shape change and aggregation-like phase measured at 37°C with a Chronolog lumi-aggregometer. Adjustment of the recorder baseline following immediate plasmin-induced shape change permitted monitoring of the aggregation-like phase over the subsequent 5 min. In the second experiment, porcine plasmin was pre-incubated for 5–10 min with tranexamic acid in appropriate concentrations. Then PRP was added, and the platelet shape change and aggregation-like phase recorded. Repetition of these experiments in more limited fashion with gel

filtered (Sepharose CL-2B) platelets substituting for PRP, once using porcine plasmin and again using human plasmin, documented the suitability of using porcine plasmin and PRP. Experiments with PRP were repeated using collagen (84 μg/mL), thrombin (5 U/mL), or ADP (10–25 μM) substituting for porcine plasmin.

The third experiment determined the effect of tranexamic acid on plasmin-inhibition of thrombin-induced platelet aggregation. At low concentrations, plasmin inhibits thrombin- or collagen-induced platelet aggregation [22]. Aggregation was measured after addition of thrombin, 0.2 U/mL, to a mixture of PRP and either porcine plasmin, 3 U/mL, or the same concentration of porcine plasmin preincubated with 2 μg/mL tranexamic acid.

Statitistical Analysis

Paired or unpaired Student's t-test compared normally distributed data, which are reported as mean ± standard deviation. Two-tailed Fisher exact test provided analysis of frequency data. Non-parametric data are reported as median and range, with Wilcoxon signed-rank test comparing paired data, and the Mann-Whitney U test comparing unpaired data. For the in vitro aggregation inhibition experiment, transformation of dose-response data to probit vs. log dose [28] permitted linear regression analysis. Linear calibration for a 50 percent inhibition response (EC_{50}) yielded the 95% confidence intervals (CI) for the EC_{50} for each pre-incubation condition [29]. For all data comparisons, significance required $P < 0.05$.

RESULTS

One patient in each group demonstrated FSP ≥ 10 μg/mL prior to surgery. Analyses did not include data from these patients. The 7 patients who received tranexamic acid prior to ECC (pre-ECC group) did not differ from the 8 who received the drug beginning after ECC (post-ECC group) with respect to age (overall 57 ± 9 years), weight (81 ± 15 kg), ECC duration (81 ± 23 minutes), and initial laboratory measurements (PT 11.3 ± 0.60 s; aPTT 44 ± 15 s; fibrinogen 241 ± 59 mg/dL; plasminogen 84 ± 26 units; platelet count 269 ± 93 K/μL; bleeding time 5 ± 1 min). Post-operative laboratory measurements (see Table I) did not differ between the groups, except for the presence of FSP, which were present more frequently in the post-ECC group ($P < 0.05$, two-tailed Fisher exact test). The groups did not differ in type of operation performed, there being 6 aortocoronary graftings (ACB) and 1 combined valve replacement with ACB in the pre-ECC group and 6 ACB, 1 valve replacement, and 1 atrial myxoma resection in the post-ECC group. Two patients, both for ACB in the pre-ECC group, had undergone a previous ACB in the distant past.

TABLE I. Post-Operative Laboratory Data

	Pre-ECC Group	Post-ECC Group
N	7	8
PT (s)	14 ± 1	14 ± 1
aPTT (s)	38 ± 4	41 ± 5
Fibrinogen (mg/dL)	163 ± 30	192 ± 35
Platelet Count (K/μL)	274 ± 172	296 ± 130
Plasminogen (units)	41 ± 10	42 ± 18
FSP (No. pts ≥10μg/mL)	0 of 7*	5 of 8*

*$P < 0.05$, two-tailed Fisher exact test. All other pre-ECC vs. post-ECC comparisons are not significant by unpaired Student's t-test.
PT = prothrombin time; aPTT = activated partial thromboplastin time; FSP = fibrin split products.
The pre-ECC group received tranexamic acid beginning before surgical incision. The post-ECC group received tranexamic acid beginning after administration of protamine.

TABLE II. Platelet Dense Granule Serotonin and ATP Content*

Group	Sample	Serotonin[a]	ATP[a]
Pre-ECC group	Pre-op	0.464 ± 0.509 (7)	10.43 ± 3.90 (7)
	Post-op	0.417 ± 0.241 (7)	10.21 ± 4.76 (7)
Post-ECC group	Pre-op	0.340 ± 0.153 (6[b])	7.66 ± 3.56 (8)
	Post-op	0.385 ± 0.256 (6[b])	7.37 ± 2.70 (8)
Normal values		0.53 ± 0.35 (21)	9.8 ± 4.4 (21)

*Entries are mean ± SD (N).
[a]Measured in nmoles per mg protein, where 1 mg of protein = $8 \cdot 10^8$ platelets (Soslau G, Giles J: The loss of sialic acid and its prevention in stored human platelets. Throm Res 26:443-455, 1982)
[b]Data from two patients not obtainable.
ATP = adenosine triphosphate; pre-ECC and post-ECC groups as defined in text.

Neither platelet serotonin content nor ATP content differed significantly between the groups pre-operatively or post-operatively (Table II). Table III displays platelet granule ADP data. Pre-operative ADP content did not differ between groups (Mann Whitney U test). For the pre-ECC group, platelet granule ADP content increased post-operatively from pre-operative values (9.43 to 15.47 nmoles/mg protein, Wilcoxon signed rank test, $P = 0.028$). For the post-ECC group, platelet granule ADP content decreased from pre-operative values (9.61 to 4.05 nmoles/mg protein, Wilcoxon signed rank test, $P = 0.017$). Post-operative platelet granules from pre-ECC patients contained more ADP than those from post-ECC patients (15.47 vs. 4.05 nmoles/mg protein, Mann Whitney U test, $P = 0.021$). Patients who received tranexamic acid prior to ECC bled less in the 12 hr study period (median 420 vs. 655 mL; Mann Whitney U test, $P = 0.024$) than those who received the drug following ECC. Least squares linear regression disclosed a significant relationship between blood loss and the ratio

TABLE III. Dense Granule ADP Content, Blood Loss, Fibrin Split Products, and Plasminogen

Pre-ECC	ADP (nmoles/mg protein) Pre-op	Post-op	Blood loss	FSP	Plasminogen
Patient H	14.88	19.75	615 mL/12 hr	(−)	40 units
Patient K	13.00	16.28	305	(−)	42
Patient C	9.43	8.56	420	(−)	50
Patient F	3.71	5.81	490	(−)	35
Patient M	11.10	12.94	310	(−)	60
Patient N	9.27	15.47	490	(−)	31
Patient O	8.79	20.48	375	(−)	32
Median	9.43*	15.47*,+	420‡		40
Post-ECC					
Patient P	9.02	6.47	640	(−)	70
Patient E	10.20	4.15	780	(−)	33
Patient D	7.03	3.23	1005	(+)	29
Patient B	4.29	3.95	480	(+)	38
Patient A	5.95	1.89	541	(−)	28
Patient L	11.35	11.66	375	(+)	59
Patient I	20.80	18.69	670	(+)	19
Patient G	18.78	2.11	705	(+)	60
Median	9.61**	4.05**,+	655‡		35.5

*P = .028, pre-op ADP vs. post-op ADP of pre-ECC group; **P = .017, pre-op ADP vs. post-op ADP of post-ECC group; +P = .021 pre-ECC vs. post-ECC for post-op ADP; ‡P = .024, pre-ECC vs. post-ECC for blood loss; (+), ≥10 μg/mL; (−), <10 μg/mL.

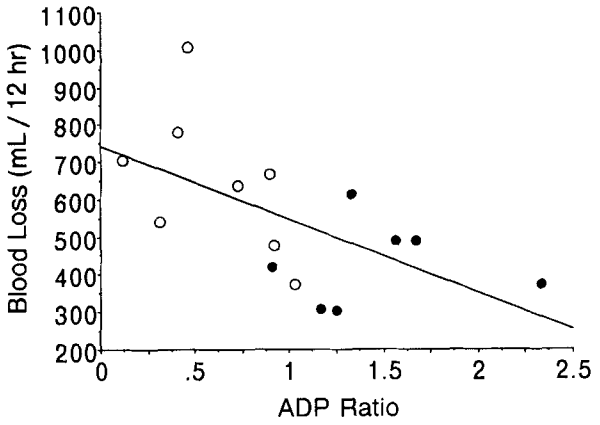

Fig. 1. Blood loss as a function of the ratio of platelet ADP after ECC to ADP before ECC. Solid circles: pre-ECC group; open circles: post-ECC group. Least squares regression line: Blood loss (mL) = 743 − 195 · ADP ratio (N = 15, P = .02, r = .59).

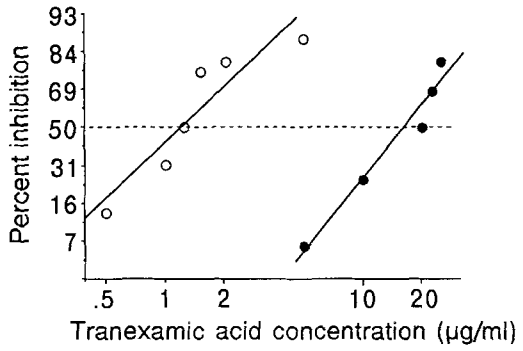

Fig. 2. Dose response relationship for tranexamic inhibition of platelet aggregation by plasmin, as probit of % inhibition vs. log dose. Open circles represent plasmin preincubated with tranexamic acid to which normal human platelet rich plasma (PRP) was added. Least squares regression line is probit of % inhibition = 4.80 + 2.47 · log dose (N = 6, P = .0093, r = .92). Closed circles denote PRP preincubated with tranexamic acid to which plasmin was added. Least squares regression line is probit of % inhibition = 1.16 + 3.19 · log dose (N = 5, P = .0041, r = .98). The dotted line facilitates identification of each EC_{50}.

of ADP in post-operative platelets to that in pre-operative platelets (Fig. 1, N = 15, P = 0.02, r = 0.59).

In normal human PRP, porcine plasmin induced an initial platelet shape change followed by an aggregation-like phase (activation). Light microscopy revealed no platelet aggregates in these preparations. However, gel filtered platelets treated with porcine plasmin formed aggregates with leucocytes or erythrocytes. Any remaining individual platelets appeared enlarged by light microscopy. Tranexamic acid inhibited plasmin-induced platelet activation. Inhibition of platelet activation by tranexamic acid occurred at significantly lower concentrations when porcine plasmin was pre-incubated with tranexamic acid compared to pre-incubation of platelets with the drug (Fig. 2). The concentration of tranexamic

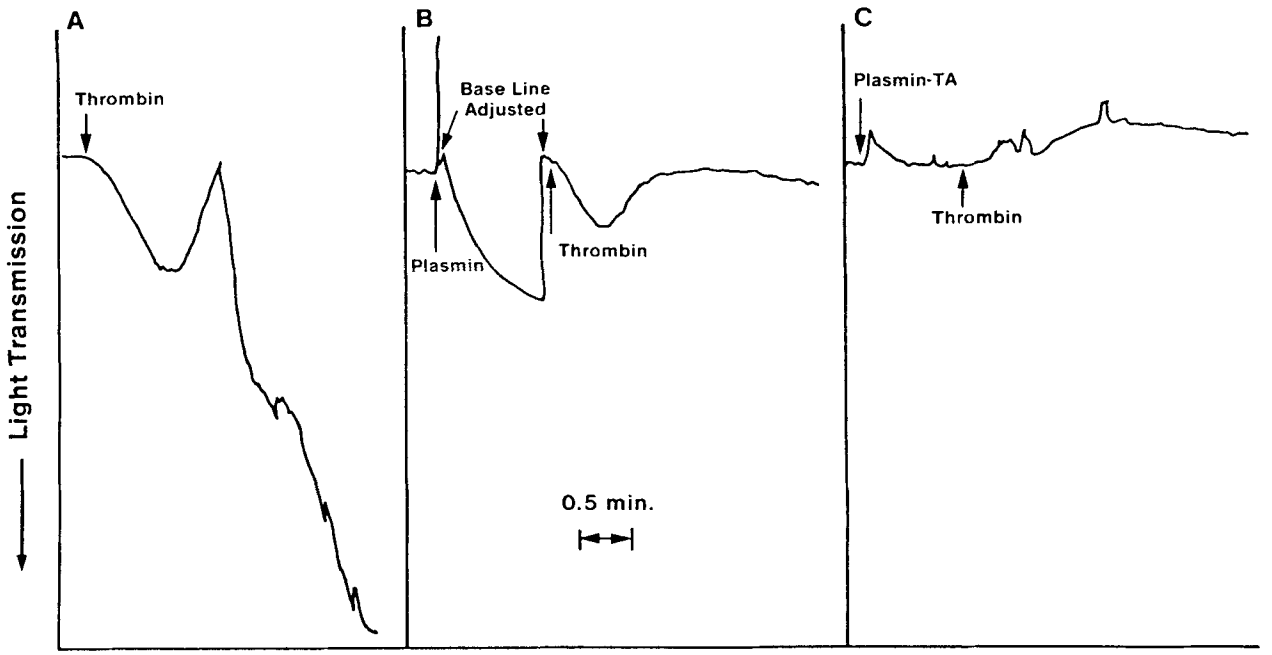

Fig. 3. Thrombin-induced platelet aggregation and its inhibition by plasmin with and without tranexamic acid. Panel A shows platelet aggregation in platelet rich plasma (PRP) monitored with a Chronolog lumi-aggregometer in the presence of 0.2 U/ml human thrombin. The vertical axis indicates light transmission, with the arrow denoting increasing transmission, and the horizontal axis time. Panel B demonstrates light transmission for PRP plus 3 U/ml of porcine plasma. Light transmission baseline adjustment occurred immediately after addition of PRP and again 1 min later, just prior to addition of 0.2 U/ml of human thrombin. Panel C depicts light transmission when PRP is added to a pre-incubated (10 min) mixture of 3 U/ml plasmin and 2 μg/ml tranexamic acid. Addition of 0.2 U/ml human thrombin occurred 1 minute later, without baseline adjustment.

acid providing 50% inhibition of activation (EC_{50}) was 16 μg/mL with platelet-tranexamic acid pre-incubation (95% CI = 7.3–99.) and 1.21 μg/mL with plasmin-tranexamic acid pre-incubation (95% CI 1.13–1.60). Recommended clinical intravenous doses of tranexamic acid produce plasma levels of 5–10 μg/mL [30]. This concentration corresponds to over 85 percent inhibition of platelet activation in the plasmin pre-incubated in vitro model. Tranexamic acid had no effect on collagen-, ADP-, or thrombin-induced platelet aggregation with either preincubation condition. Both porcine plasmin alone and porcine plasmin inactivated with tranexamic acid inhibited thrombin-induced platelet aggregation (Fig. 3). All of the above experiments were conducted with porcine plasmin since human plasmin was inactive in PRP. However, similar results are obtained with human plasmin and gel filtered platelets.

DISCUSSION

Multiple factors contribute to hemorrhage following ECC, among them transient fibrinolysis. Prophylactic (pre-ECC) tranexamic acid decreased bleeding after ECC in a diverse adult cardiac surgery population [14]. In that study, post-operative fibrin split products occurred less frequently in the group that received prophylactic tranexamic acid compared with a placebo control group. This association implies that inhibition of fibrinolysis provides a beneficial hemostatic effect. However, platelet dysfunction, not fibrinolysis, is considered the most common mechanism of post-ECC bleeding [16]. Blood loss in the current 8 post-ECC treated patients, 655 mL median over 12 hrs, parallels the 695 mL median blood loss of the 20 placebo-treated patients in the previous study [14], suggesting that tranexamic acid administration after ECC is equivalent to placebo. The data in table III imply that a platelet-preserving effect contributes significantly, perhaps entirely, to the hemostatic outcome from tranexamic acid. In patients receiving tranexamic acid prior to incision, platelet granules contained more ADP after surgery compared to before surgery (Table II, pre-ECC group). This unexpected finding might arise from entry of young platelets into the circulation from the spleen [31] or other vascular pools coupled with decreased platelet utilization.

By what mechanism might tranexamic acid preserve platelet function? Perhaps a weak plasmin-platelet interaction in vivo is sufficient to release small amounts of granule ADP without affecting the other dense granule components. Platelets can differentially release selected

components from their dense granules [24,32]. Dense granule ATP and serotonin were not altered significantly in platelets derived from either patient group. Tranexamic acid at clinically achieved plasma concentrations completely abolishes plasmin-induced platelet activation in vitro. This inhibition arises from specific binding of tranexamic acid to plasmin and not to the plasmin receptor on platelets. This is evident from the ≈13 fold lower EC_{50} of tranexamic acid when plasmin was preincubated with drug (EC_{50} = 1.21 μg/ml) compared with preincubation of platelets with drug (EC_{50} = 16 μg/ml). Furthermore, in order for plasmin to activate platelets in vivo without being neutralized by antiplasmin, the precurson plasminogen must be tightly associated with the platelet membrane and/or the vessel wall which the platelet contacts. High concentrations of plasmin may be generated on or near platelets in vivo [32]. Adelman et al. demonstrated [33] that the antifibrinolytic agent ϵ-aminocaproic acid also inhibited plasmin proteolysis of platelet glycoprotein Ib. Aminocaproic acid inhibited the plasmin-mediated reduction of ristocetin-induced platelet agglutination in the same concentration range (10^{-4} to 10^{-2} M) as observed in our studies.

Plasmin-induced platelet activation is similar to thrombin-induced activation [21]. However, the mechanism by which plasmin associates with platelets and inhibits thrombin and collagen-induced platelet aggregation has not been resolved. Plasmin inactivated by tranexamic acid has little effect on platelets. Subsequent addition of thrombin yields no change, indicating that tranexamic acid-inactivated plasmin binds to platelets and blocks the thrombin receptor. Similar results with gel filtered platelets indicate that plasma factors are not required for tranexamic acid-inactivated plasmin to inhibit thrombin-induced platelet aggregation.

Tranexamic acid may protect the patient's hemostatic system during surgery in two ways. First, tranexamic acid inhibits plasmin-induced platelet activation. The tranexamic acid-plasmin complex might also prevent any thrombin not inactivated via heparin from activating platelets. Platelets protected during ECC can participate fully in hemostasis after ECC. Second, after ECC, tranexamic acid can inhibit plasmin-induced fibrinolysis. Why doesn't tranexamic acid paralyze platelets after ECC? One explanation is that high levels of thrombin formed upon protamine administration could displace the tranexamic acid-plasmin complex from platelets.

In many previous trials of antifibrinolytic drugs as hemostatic agents during cardiac surgery, the drugs were administered following ECC [7,11–13]. The platelet preincubation experiment models this circumstance: tranexamic acid and plasmin have insufficient time to interact. In that situation, clinical levels of tranexamic acid (5–10 μg/mL) produced minimal inhibition of platelet activation. The plasmin preincubation experiment, which models prophylactic administration of tranexamic acid, revealed near total inhibition of activation at clinical tranexamic acid concentrations. Thus realization of the full hemostatic effect of tranexamic acid requires appropriate timing and plasma concentrations.

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