

Characterization of Prothrombin Activation during Cardiac Surgery by Hemostatic Molecular Markers

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Background: Prothrombin activation represents the key regulatory step in the hemostatic process. Once formed, thrombin contributes to the generation of fibrin as well as the activation of platelets and fibrinolysis. Failure to suppress thrombin formation during cardiac surgery could result in disorders of hemostasis and thrombosis in the perioperative period. The aim of this study was to determine the time course for prothrombin activation during the perioperative period associated with cardiac surgery.

Methods: We measured prothrombin activation during the perioperative period in 19 adult patients undergoing primary cardiac surgery using enzyme-linked immunosorbent assays for the detection of thrombin formation (prothrombin fragment 1.2 and thrombin-antithrombin III complex) and thrombin activity (fibrinopeptide A and fibrin monomer). Blood samples were obtained preoperatively; at 30-min intervals during cardiopulmonary bypass (CPB); and 1, 3, and 20 h after completion of CPB.

Results: Despite anticoagulation with heparin, plasma concentrations of prothrombin fragment 1.2, thrombin-antithrombin III complex, and fibrin monomer increased throughout CPB. Peak concentrations for all hemostatic markers occurred in the samples obtained 3 h after completion of CPB. By the morning after surgery, plasma prothrombin fragment 1.2 returned to preoperative concentrations; however, fibrinopeptide A and fibrin monomer concentrations remained significantly increased ($P < 0.05$) compared to preoperative values.

Conclusions: These data clearly demonstrate the occurrence of prothrombin activation and thrombin activity during CPB

despite heparin concentrations adequate to maintain the activated clotting time greater than 400 s. Hemostatic markers for the activation of prothrombin demonstrated peak concentrations 3 h after completion of CPB with a return to baseline concentrations by the morning after surgery. Markers for thrombin activity, however, suggest the presence of active thrombin through the morning after surgery. Further investigations will be necessary to determine the role of hemostatic activation in thrombotic complications after cardiac surgery. (Key words: Arteries, coronary: thrombosis. Blood, coagulation: prothrombin fragments; thrombin. Surgery, cardiac: cardiopulmonary bypass; coronary artery bypass graft.)

DESPITE recent advances improving the safety of cardiac surgery, disorders of hemostasis and thrombosis in the perioperative period continue to account for significant surgical morbidity. Historically, hemostatic dysfunction associated with cardiac surgery has received much greater attention than the risk of thrombosis^{1,2}; however, recent evidence demonstrating the essential role of thrombosis in the etiology of myocardial ischemia³ and data demonstrating that myocardial ischemia occurs with high frequency after successful coronary artery bypass graft (CABG) surgery⁴ suggest the need to understand in greater detail the time course of hemostatic activation during the period surrounding cardiac surgery.

Prior investigations of the hemostatic system during cardiac surgery have described alterations of both the plasma protein and cellular mediated components of hemostasis, presumably initiated in large part by exposure to the artificial membranes of the cardiopulmonary bypass (CPB) circuit. Hemodilution, occurring with the initiation of CPB, results in decreased concentrations of the plasma coagulation factors.⁵ Both quantitative and qualitative platelet defects are associated with the exposure of blood to the artificial surface of the CPB circuit.^{6,7} Recent investigations have demonstrated the activation of platelets as well as the formation of procoagulant platelet microparticles during CPB.⁸⁻¹⁰ In addition, plasma concentrations of tissue plasminogen activator increase during CPB, re-

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sulting in a fibrinolytic state that continues into the early postoperative period.¹¹ Although many investigations support the role of platelet dysfunction and fibrinolysis in the etiology of post-CPB coagulopathy, the initiating pathophysiologic mechanism for these abnormalities has not been determined.

Thrombin formation is the key regulatory step in hemostasis and thrombosis. Trace plasma proteins, activated upon exposure to tissue factor or foreign surfaces, initiate a series of reactions culminating in the conversion of prothrombin to thrombin.¹² In addition to its role in converting fibrinogen to insoluble fibrin, thrombin is a potent activator of platelets. Also, thrombin and the fibrin generated by its activity regulate *in vivo* fibrinolysis.¹³

Before the initiation of CPB, large doses of heparin are administered with the expectation that prothrombin activation will be inhibited and that thrombin, if formed, will not propagate blood clotting during CPB. Failure to inhibit prothrombin activation during CPB would lead to depletion of plasma coagulation factors, activation of platelets, and fibrinolysis, all of which have been observed during cardiac surgery. Several investigations have suggested that thrombin formation occurs during CPB.¹⁴⁻¹⁶ These investigations have been limited by the number of sample intervals analyzed and by the failure to assess both the formation of thrombin as well as the subsequent activity of thrombin. The short half-life of active thrombin, primarily due to the inhibitory action of antithrombin III,¹⁷ hinders the direct measurement of plasma thrombin concentration; however, the activation peptide, prothrombin fragment 1.2 (F1.2), cleaved from prothrombin upon conversion to thrombin, provides a direct immunochemical measurement of prothrombin activation and thrombin formation *in vivo*.

Using an enzyme-linked immunosorbent assay (ELISA) for F1.2 in conjunction with assays for fibrinopeptide A (FpA), thrombin-antithrombin III complex (TAT), and fibrin monomer, we examined the time course for thrombin formation and activity during the perioperative period associated with cardiac surgery.

Materials and Methods

This investigation was approved by the institutional review board of Duke University Medical Center. After informed consent had been obtained, 20 adult patients scheduled for elective CABG surgery were enrolled in the study. Exclusion criteria included requirement for

repeat or emergency cardiac surgery, requirement for preoperative heparin infusions, and preoperative use of warfarin.

Blood samples were drawn from a radial artery catheter (Insyte-W; Becton-Dickinson, Sandy, UT) at predetermined intervals: 5 min after insertion of the radial artery catheter; 5 min after sternotomy; 5 min after anticoagulation with heparin; 5, 30, 60, 90, and 120 min after institution of CPB; 1 h after completion of CPB; 3 h after completion of CPB; and 20 h after completion of CPB. A two-syringe technique was used for blood collection. Eight milliliters of blood were withdrawn from the radial artery catheter into the first syringe before sample retrieval in the second syringe. No heparin was present in flush solutions. Samples for determination of F1.2 were collected into Vacutainer tubes containing lithium heparin ($14.3 \text{ U} \cdot \text{mL}^{-1}$ blood [United States Pharmacopeia units]). Blood for FpA determination was collected into tubes containing D-Phe-Pro-Arg-chloromethylketone ($2 \times 10^{-5} \text{ M}$), aprotinin ($150 \text{ kIU} \cdot \text{mL}^{-1}$), and ethylenediamine tetraacetate (4.5 mM) (Haematologic Technologies, Essex Junction, VT). Blood for measurement of TAT complex and fibrin monomer was collected into Vacutainer tubes containing sodium citrate 3.8% (9:1 vol/vol). All Vacutainer tubes, with the exception of the FpA tubes, were purchased from Becton Dickinson (Rutherford, NJ). Blood samples were immediately centrifuged at $1,500 \text{ g}$ for 15 min at 4°C . Plasma supernatant was retrieved and placed into a polypropylene tube for a second centrifugation at $3,000 \text{ g}$ for 15 min at 4°C . Plasma samples from Vacutainers containing heparin were mixed with Sample Treatment Reagent (Organon Teknica, Durham, NC) immediately after centrifugation and before storage at -70°C .

Anesthesia consisted of fentanyl and midazolam infusions with vecuronium for muscle relaxation. Heparin $300 \text{ U} \cdot \text{kg}^{-1}$ was administered into the internal jugular vein *via* the side port of the introducer sheath before cannulation of the vena cava. Additional heparin was administered as necessary to maintain the activated clotting time (Hemochron, International Technidyne, Edison, NJ) greater than 400 s. CPB was performed using a membrane oxygenator at a flow rate of approximately $2.4 \text{ l} \cdot \text{min}^{-1} \cdot \text{m}^{-2}$ using α -stat pH management and moderate hypothermia ($28\text{--}32^\circ\text{C}$). The bypass circuit was primed with Ringer's lactate solution, 20% mannitol 200 ml, hydroxyethyl starch 500 ml (Heta-starch, DuPont, Wilmington, DE), and porcine heparin 5,000 U. After the completion of CPB, plasma heparin

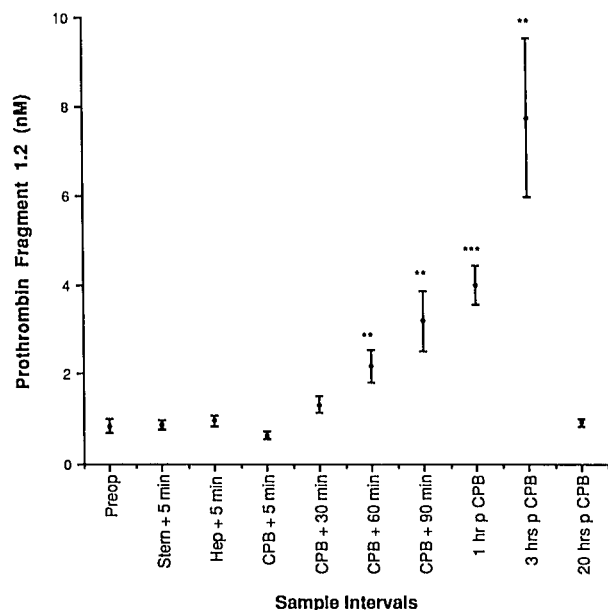


Fig. 1. Perioperative plasma concentrations of prothrombin fragment 1.2. Statistically significant changes from the preoperative value: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

was neutralized by the administration of protamine. Protamine was initially infused to a dose of 200 mg; additional protamine was administered as necessary to restore the activated clotting time to the preoperative value.

F1.2 was determined from plasma samples collected in heparin-containing Vacutainer tubes by the Thrombonostika F1.2 ELISA (Organon Teknika; normal range 1.28 ± 0.7 nM). FpA was measured from plasma samples collected into FpA tubes using the Asserachrom FpA ELISA (Diagnostica Stago, Asnieres-sur-Seine, France; normal range 1.83 ± 0.61 ng/ml). TAT complex concentration was determined from citrated plasma samples using the Enzygnost TAT ELISA (Behringwerke AG; Marburg, Germany; normal range 2.6 ± 0.75 μ g/l). Fibrin monomer was measured in citrated plasma samples by the Coaset Fibrin Monomer chromogenic assay (Kabi Pharmacia Hepar, Franklin, OH; normal range 9.2 ± 1.9 nM). All samples were assayed in duplicate and performed in parallel with standard curves generated from pooled normal plasma.

All data are expressed as the mean value \pm standard error of the mean. A paired t test with $(n - 1)$ degrees of freedom was used to evaluate the statistical significance of changes from baseline values. A P value of $<$

0.05 was selected to designate statistical significance. All data points were further assessed for statistical significance using a Bonferroni adjustment for multiple comparisons.

Results

Of the 20 patients enrolled in the investigation, 1 did not complete the study because of rescheduling. The study population consisted of 15 male and 4 female patients with a mean age of 63 ± 2.3 years. All 19 patients underwent CABG surgery; 2 patients required combined CABG–valve replacement surgery. The mean duration of CPB was 105 ± 5.4 min. The perioperative outcomes were uncomplicated, and no patients required return to the operating room for bleeding.

The mean preoperative plasma concentration of F1.2, 0.9 ± 0.2 nM, falls within the reported normal range for this peptide.¹⁸ Concentrations of F1.2 increased throughout CPB to 3.2 ± 0.7 nM after 90 min of CPB (fig. 1). After the administration of protamine, F1.2 concentrations continued to increase, achieving a peak plasma concentration of 7.8 ± 1.8 nM at 3 h after completion of CPB. By the morning after surgery, plasma concentrations of F1.2 returned to baseline, with a value of 0.9 ± 0.1 nM.

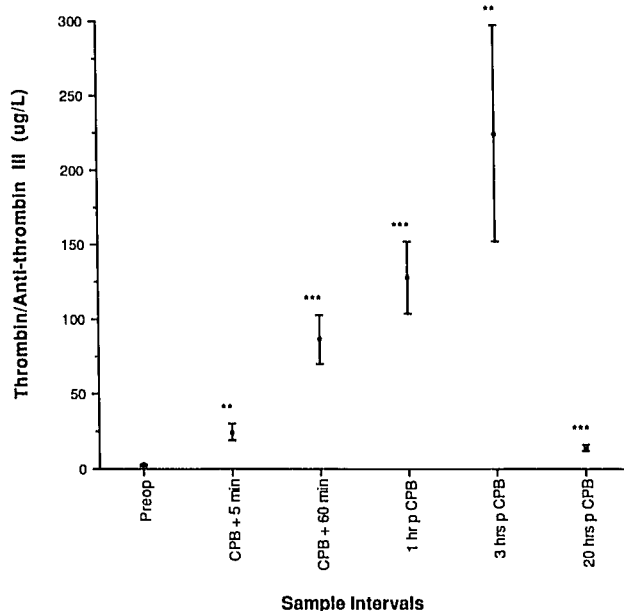


Fig. 2. Perioperative plasma concentrations of thrombin-anti-thrombin III complex. Statistically significant changes from the preoperative value: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

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As depicted in figure 2, plasma concentrations of TAT complex demonstrated a pattern nearly identical to that of F1.2. TAT complex increased from a preoperative value of 2.9 ± 0.5 to $86.6 \pm 16.7 \mu\text{g/l}$ after 1 h of CPB. As observed with F1.2, the peak concentration of TAT complex, $224.4 \pm 72.5 \mu\text{g/l}$, was observed in samples obtained 3 h after completion of CPB. Although TAT complex decreased to $14.4 \pm 2.1 \mu\text{g/l}$ the morning after surgery, this value remained slightly greater than preoperative concentrations in our patient population ($P < 0.001$).

Because FpA concentrations during cardiac surgery have been reported, we examined this marker at four intervals during the perioperative period as a control for our measurements (fig. 3). FpA concentration increased from a preoperative value of $9.2 \pm 3.2 \text{ ng/ml}$ to a peak of $41.5 \pm 3.9 \text{ ng/ml}$ 1 h after completion of CPB. As opposed to the measurements of F1.2 and TAT complex, FpA concentrations the morning after surgery were nearly 2.5-fold greater than preoperative concentrations ($P < 0.05$).

Plasma concentrations of fibrin monomer were also examined as a measure of thrombin activity in the perioperative period. As observed with both F1.2 and TAT complex, fibrin monomer concentration increased throughout CPB, reaching $33.8 \pm 3.0 \text{ nM}$ after 60 min of CPB (fig. 4). Again, peak concentrations of fibrin monomer were measured in the samples obtained at 3 h after CPB. As noted with the measurements for FpA,

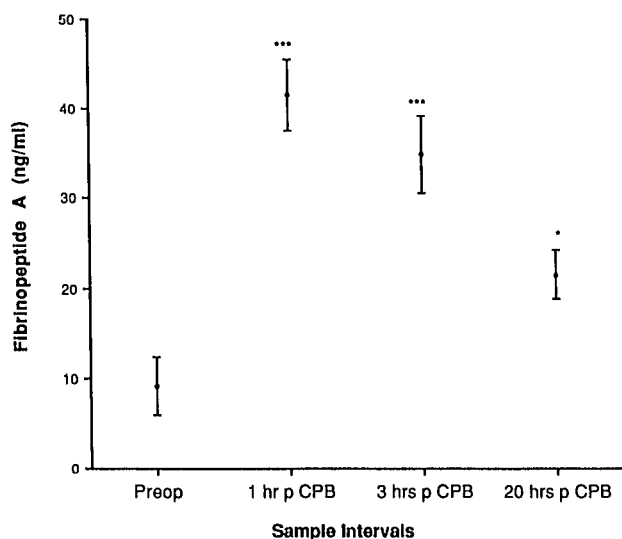


Fig. 3. Perioperative plasma concentrations of fibrinopeptide A. Statistically significant changes from the preoperative value: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

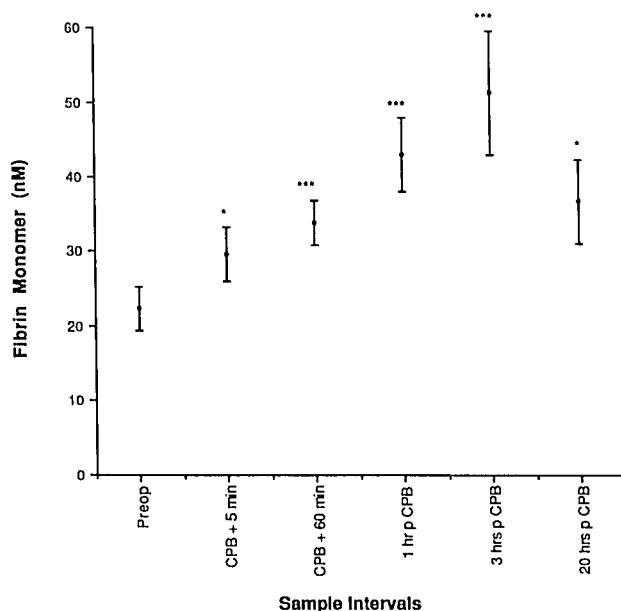


Fig. 4. Perioperative plasma concentrations of fibrin monomer. Statistically significant changes from the preoperative value: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

fibrin monomer concentrations the morning after surgery, at $36.7 \pm 5.7 \text{ nM}$, remained significantly greater than preoperative concentrations ($P < 0.05$).

Discussion

Thrombin executes the key regulatory step in the intravascular processes of hemostasis, thrombosis, and fibrinolysis. In addition to its familiar role of proteolytically cleaving fibrinogen to soluble fibrin monomer, thrombin further carries out the hemostatic process by activating factors V, VIII, and XI.^{19,20} Thrombin-mediated activation of factor XIII contributes to the stabilization of clots against mechanical and fibrinolytic degradation.²¹ Stimulation of thrombin receptors on the platelet surface results in platelet activation and aggregation.²² In addition to its prothrombotic role, thrombin is capable of modifying the thrombotic response by triggering the release of tissue plasminogen activator from the endothelial surface.²³ Failure to suppress thrombin formation during CPB could account for platelet dysfunction and fibrinolysis after cardiac surgery.

The regulation of thrombin formation is a complex process controlled in part by a membrane-bound activation complex comprising factor Va, factor Xa,

phospholipid, and calcium.²⁴ The function of the prothrombinase complex is to accelerate the rate of thrombin formation. In the absence of the prothrombinase complex or any of its components, thrombin formation is essentially inhibited. Only small amounts of thrombin need be formed to supply the prothrombinase complex with activated factors Va and Xa, which are essential to the amplification of thrombin formation.

The optimal dose of heparin required to suppress thrombin formation during CPB remains largely empirical. Based on the measurement of plasma FpA concentration in monkeys, Young *et al.* have suggested that heparin concentrations resulting in an activated clotting time greater than 400 s provide adequate anticoagulation during CPB.²⁵ The value of 400 s has been widely accepted, based on the apparent lack of clot formation with this degree of anticoagulation. However, it should be apparent that the successful prevention of grossly visible clotting during CPB does not ensure inhibition of coagulation at the molecular level, with subsequent activation of platelets, depletion of coagulation factors, and fibrinolysis.

Despite maintaining what is generally considered to be adequate anticoagulation for cardiac surgery, our patient population demonstrated increasing plasma concentrations of F1.2 with increasing duration of CPB. Plasma F1.2 concentration provides a direct measure of prothrombin cleavage by the prothrombinase complex and therefore of thrombin formation.²⁶ Measurements of TAT complex were in agreement with the F1.2 data, providing further evidence for thrombin formation during CPB. Increased plasma concentrations of TAT complex imply that thrombin is being generated and subsequently inactivated by the heparin-antithrombin III complex. The apparent failure of heparin to inhibit prothrombin activation during CPB may be explained by the protective effect provided by fibrin.²⁷ Thrombin bound to fibrin clot remains enzymatically active; however, the thrombin is protected from inhibition by the heparin-antithrombin III complex.²⁸ Fibrin monomers were formed throughout CPB, providing a site for continuous thrombin activity in the presence of heparin. The prothrombinase complex acts in a similar manner to shield its constituents from inhibition.²⁹ The surface of activated platelets and of platelet microparticles provides prothrombinase complex assembly sites³⁰ resistant to the inhibitory effects of the heparin-antithrombin III complex and so may contribute to the propagation of active thrombin during CPB.

Evidence for thrombin formation does not necessarily imply that the thrombin remained active to generate fibrin. Under normal conditions, plasma serine protease inhibitors rapidly inactivate and eliminate thrombin from the circulation, thereby down-regulating the formation of fibrin. To determine whether the thrombin generated was active during CPB, we measured the plasma concentrations of both FpA and fibrin monomer. As active thrombin converts fibrinogen to fibrin, two molecules of FpA are cleaved from each molecule of fibrinogen. Increased concentrations of FpA and fibrin monomer indicate the presence of thrombin activity in plasma. In agreement with prior investigations,^{14,15,31} we found increasing concentrations of FpA throughout CPB. Increased concentrations of fibrin monomer further demonstrate that proteolytically active thrombin was present during CPB despite the presence of heparin.

The peaks of thrombin formation (F1.2 and TAT complex) and of thrombin activity (FpA and fibrin monomer) were measured in the samples drawn 3 h after the completion of CPB. We believe that the sudden increases in prothrombin activation and thrombin activity after surgery were due to reversal of the anticoagulated state by the administration of protamine. After the neutralization of heparin, sites of tissue injury would be capable of accelerated rates of prothrombin activation, resulting in the formation of fibrin. The normal concentration of F1.2 in healthy subjects has been determined to be 1.28 ± 0.7 nm. Preoperative values in our patient population were within this range; by 3 h after completion of CPB, however, F1.2 concentrations had increased fivefold over baseline values. The other measures of thrombin formation and activity followed a pattern similar to that of F1.2, again suggesting that a significant amount of active thrombin is present in the early postoperative period after cardiac surgery.

Because prothrombin activation occurred throughout CPB, it is not unexpected that thrombin formation would continue into the postoperative period after heparin neutralization. On the basis of this investigation, we are unable to localize the sites of prothrombin activation; however, both activated platelets and sites of surgical tissue injury could serve as a nidus. If prothrombin is being generated on the surface of activated platelets, this could serve as a mechanism of propagating unwanted thrombin generation to the coronary vascular bed postoperatively. It is of interest that the peaks in thrombin formation and activity in our measurements correlate with the postoperative period,

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during which the highest incidence of myocardial ischemia after CABG surgery has been reported.⁴

The return of plasma concentrations of both F1.2 and TAT complexes to near baseline values by the morning after surgery raises the possibility of using these measurements as diagnostic markers of postoperative thrombotic complications. Boisclair *et al.* recently reported similar findings in a series of five patients undergoing cardiac surgery.³² Failure of plasma F1.2 or TAT complex concentration to return to baseline by the morning after surgery may be predictive of a thrombotic complication. The small number of patients examined, however, precludes any definitive conclusions regarding this important issue.

As opposed to the measures of thrombin formation, both FpA and fibrin monomer remained increased the morning after surgery. Because FpA has a half-life of only 2–3 min in plasma, increased concentrations of this marker suggest the presence of residual thrombin activity.³³ Soluble fibrin circulates with a half-life of several hours and may act as a sanctuary for thrombin.³⁴ Subsequent degradation of soluble and insoluble fibrin may release active thrombin into the circulation, leading to the formation of both FpA and fibrin monomers.

In summary, in cases of elective CABG surgery, we have documented the generation of both thrombin and fibrin throughout CPB and postoperatively. Current approaches to anticoagulation with heparin during CPB do not ensure the inhibition of prothrombinase activity. Further investigations will be necessary to define the site of thrombotic activation in the postoperative period and the role of thrombotic activation in postoperative ischemic complications.

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