Heparin Neutralization by Platelet-Rich Thrombi
Role of Platelet Factor 4

Daniel T. Eitzman, MD; Liguo Chi, MD; Leopoldo Saggin, MD; Robert S. Schwartz, MD; Benedict R. Lucchesi, PhD, MD; William P. Fay, MD

**Background** Platelets contain several factors that inhibit heparin. This study was designed to assess the heparin-neutralizing activity present in acute, platelet-rich arterial thrombi formed at sites of arterial injury in animals.

**Methods and Results** Platelet-rich thrombi (n=3) were induced in pig coronary arteries by balloon catheter-mediated arterial injury. Soluble extracts were prepared from each thrombus and assayed for the capacity to inhibit heparin in an in vitro clotting assay (activated partial thromboplastin time). Mean heparin-neutralizing activity was 28 U of heparin neutralized per milliliter of thrombus, indicating that 1 vol of coronary thrombus completely inhibited the heparin present in 140 vols of therapeutically anticoagulated (0.2 U heparin/mL) plasma. In contrast, thrombus extracts had no effect on the anticoagulant activity of hirudin, a direct-acting thrombin inhibitor. The heparin-neutralizing activity present in coronary thrombi bound to heparin-agarose and was eluted from it by 1.4 mol/L NaCl, suggesting that platelet factor 4 mediated the antiheparin effect of thrombi. Consistent with this hypothesis, a murine monoclonal antibody to rabbit platelet factor 4 nearly completely inhibited the heparin-neutralizing activity present in rabbit thrombi (n=3) generated by carotid artery injury.

**Conclusions** Extracts prepared from platelet-rich arterial thrombi significantly inhibited the in vitro anticoagulant potency of heparin but not of hirudin. This antiheparin effect appears to be mediated by platelet factor 4. These results are consistent with the hypothesis that localized inhibition of heparin at sites of platelet activation may reduce its anti-thrombotic efficacy. In addition, they suggest an additional mechanism for the apparent superiority of hirudin over heparin as a thrombin inhibitor at sites of arterial injury. (Circulation. 1994;89:1523-1529.)

**Key Words** • thrombosis • anticoagulation • hirudin • heparin resistance

Acute coronary artery thrombosis, usually triggered by disruption of an atherosclerotic plaque, is common in patients with myocardial infarction and unstable angina pectoris. This process is dependent on the formation of thrombin, a serine protease that promotes thrombus formation by converting fibrinogen to fibrin and by activating platelets. Heparin, a sulfated glycosaminoglycan, is used routinely to inhibit thrombin in the setting of acute coronary heart disease. Heparin functions as an anticoagulant by markedly potentiating the capacity of antithrombin III to inhibit thrombin and factor Xa. Treatment with heparin reduces the incidence of myocardial infarction in patients with unstable angina, lowers mortality in the setting of acute myocardial infarction, and enhances vascular patency in patients receiving tissue-type plasminogen activator.

Despite the clinical utility of heparin in patients with acute ischemic syndromes, its efficacy as an anticoagulant within the coronary circulation is not uniform, as evidenced by formation and/or extension of coronary thrombi in some patients receiving high doses of heparin. Possible explanations for the occasional failure of heparin therapy include the presence of intense stimuli for thrombin formation and platelet activation at sites of plaque rupture and the diminished capacity of heparin–antithrombin III to inhibit thrombin that is bound to fibrin, as opposed to free in the circulation. Another potential explanation for ineffective thrombin inhibition by heparin is the development of heparin resistance in the setting of acute thrombosis. Systemic resistance to anticoagulation with heparin has been described in patients with acute coronary disease. However, it is also possible that a localized state of heparin resistance might exist at sites of coronary thrombosis. Coronary thrombi are frequently platelet rich, and platelets contain several factors that inactivate heparin, such as platelet factor 4, heparitinase, histidine-rich glycoprotein, and vitronectin. These proteins may contribute to the failure of heparin therapy in some patients with acute arterial thrombosis.
To test the hypothesis that platelet-rich clots formed in vivo exhibit heparin-neutralizing activity, arterial thrombi were generated in animals, retrieved, and analyzed in vitro for their capacity to inhibit heparin. The results of these studies suggest that platelet-rich thrombi contain potent heparin-neutralizing activity and that this activity is mediated by platelet factor 4.

Methods

Preparation and Processing of Platelet-Rich Thrombi

Generation of Porcine Thrombi

Platelet-rich coronary thrombi were induced in adult pigs by percutaneous implantation of an oversized tantalum wire stent within the proximal left anterior descending coronary artery, as previously described. In this model, high-pressure (14 atm) inflation of an oversized angioplasty balloon during stent deployment results in deep injury to the arterial media, thereby inducing acute coronary thrombosis. After retrieval from the coronary artery, thrombi were immediately frozen at −70°C. Before freezing, portions of some thrombi were placed in Trump’s fixative and subsequently analyzed by electron microscopy.

Generation of Rabbit Thrombi

New Zealand White rabbits were anesthetized with ketamine (100 mg/kg IM) and 6% sodium pentobarbital (1 mL/kg). Carotid thrombi were induced by electrical injury to the carotid endothelium as previously described in a canine model. This procedure has been demonstrated to elicit the formation of a platelet-rich clot. A segment of the carotid artery that included the site of injury was excised 2 hours after initiation of electrical current. With the aid of a dissecting microscope, the thrombus was retrieved from the arterial segment, weighed, and then processed as described below.

All animal procedures conformed to the guiding principles of the American Physiological Society and were performed in accordance with the institutional guidelines of the Mayo Clinic (porcine studies) or the University of Michigan (rabbit studies).

Preparation of Thrombus Extracts

Soluble extracts were prepared from each thrombus and subsequently assayed for heparin-neutralizing activity, as described below. For porcine thrombi, 9 vol of extraction buffer (0.02 mol/L Tris-HCl, 0.01% Tween 80, pH 8.0) was added to 1 vol of thrombus in a 1.5-mL polypropylene tube. The thrombus then was subjected to 15 applications of a miniature pestle. The suspension was centrifuged (14 000g for 1 minute), and the supernatant was removed and frozen at −70°C. Rabbit thrombus extracts were prepared similarly, except that 20 μL of extraction buffer was added per milligram of thrombus.

Determination and Characterization of Heparin-Neutralizing Capacity of Thrombi

Clotting Assay

The activated partial thromboplastin time (APTT) was used to determine the heparin-neutralizing capacity of porcine thrombus extracts. In this assay, 0.1 mL of human plasma was incubated with 0.1 mL of APTT reagent (Organon Teknika) according to the manufacturer’s instructions, and the time to clot formation (initiated by the addition of 0.1 mL of 25 mmol/L CaCl₂) was recorded. Unfractionated porcine heparin (Elkins-Sinn) was added to plasma to prolong the clotting time. To determine the heparin-neutralizing activity of thrombus extracts, 2.5 μL of each extract was added to 0.1 mL of heparinized plasma (0.12 USP U/mL) and incubated at 37°C for 5 minutes. APTT reagent and CaCl₂ were added, and the clotting times were recorded and compared with control reactions in which 2.5 μL of extraction buffer was substituted for thrombus extract. Heparin-neutralizing capacity of thrombus extracts was determined by comparison to a standard curve of APTT versus heparin concentration. In similarly performed experiments, the capacity of thrombus extracts to inhibit the anticoagulant effect of recombinant hirudin also was determined.

Heparin Affinity Experiments

The capacity of the heparin-inhibitory activity present in thrombi to bind to heparin-agarose was determined. Heparin-agarose (Sigma) was equilibrated with 0.02 mol/L Tris-HCl, 0.15 mol/L NaCl, pH 7.5. Equilibrating buffer was removed, and packed resin (5 mg) was added to 10 μL of thrombus extract, then incubated for 30 minutes at room temperature. The suspension was centrifuged and the supernatant removed. The pelletted heparin-agarose was incubated with 10 μL of 0.02 mol/L Tris-HCl, 0.4 mol/L NaCl, pH 7.5, for 5 minutes at room temperature. The suspension was centrifuged and the 0.4 mol/L NaCl eluate removed. In similar fashion, the heparin-agarose was eluted sequentially with 0.8, 1.4, and 2.0 mol/L NaCl. The heparin-neutralizing activity of heparin-agarose-adsorbed thrombus extract and the 0.4, 0.8, 1.4, and 2.0 mol/L NaCl heparin-agarose eluates (10 μL) were determined in the APTT assay, as previously described. Control experiments revealed that NaCl present in heparin-agarose eluates did not affect the APTT.

Factor Xa Assay

The heparin-neutralizing activity of rabbit thrombus extracts was determined using a factor Xa chromogenic assay, as previously described. In this assay, heparin activity is determined by measuring the hydrolysis of factor Xa chromogenic substrate (S-2222, Kabi Pharmacia) after incubation of factor Xa (0.17 nkat, Kabi Pharmacia) with antithrombin III (5 × 10⁻³ IU, Kabi Pharmacia) and heparin for 1 minute at 37°C. Dilutions (160- to 640-fold) of thrombus extract (10 μL) were incubated with heparin (0.2 μM) in 0.05 mol/L Tris-HCl, 0.15 mol/L NaCl, pH 8.4 (total volume of 50 μL), for 15 minutes at 37°C. Reaction mixtures were added to the factor Xa assay described above, and residual heparin activity was determined by comparison to a standard curve generated by incubation of factor Xa and antithrombin III with known amounts of heparin (0.0 to 0.4 μU). To test the hypothesis that the heparin-neutralizing activity present in thrombus extracts was mediated by platelet factor 4, experiments were performed with 10B2, a murine monoclonal antibody to rabbit platelet factor 4 that neutralizes the anticoagulant activity of this protein. Dilutions of rabbit thrombus extracts were incubated with 10B2 (or phosphate-buffered saline as control) for 2 hours at 37°C. Heparin-neutralizing activity of these extracts then was determined, as described above.

Results

Porcine Coronary Thrombi

Thrombi obtained from three consecutive animals were analyzed. The platelet-rich composition of each
Heparin neutralization by platelet-rich thrombi and effect of 10B2. A, Porcine experiments: As described in "Methods," extracts (2.5 μL) prepared from coronary thrombi were added to 0.1 mL plasma anticoagulated with heparin (0.12 U/mL) or hirudin (0.17 μg/mL). Activated partial thromboplastin times (APTT) were performed and compared with control reactions lacking thrombus extract. To facilitate comparison of heparin and hirudin experiments, APTTs are expressed as a percentage of the APTT obtained for nonanticoagulated plasma (ie, 35±1 second). Values shown are mean±SD of three experiments, each performed with a different thrombus extract. Note significant inhibition of the anticoagulant effect of heparin but not of hirudin by thrombus extracts. B, Rabbit experiments: Neutralization of the antiheparin activity of carotid thrombi by anti-platelet factor 4 monoclonal antibody (10B2) is demonstrated. Dilutions (320-fold) of each thrombus extract were incubated with 10B2 or phosphate-buffered saline for 2 hours at 37°C, then residual antiheparin activity was determined in a modified factor Xa assay, as described in "Methods." Results are expressed as percentage inhibition of heparin activity, with 0% (no inhibition of heparin) as residual factor Xa activity observed after incubation with antithrombin III and 0.2 μL of heparin and 100% (complete heparin inhibition) defined as factor Xa activity observed after incubation with antithrombin III alone (no heparin). Values shown are mean±SD of three experiments, each performed with a different thrombus extract.

Thrombus was evident on gross inspection (Fig 1A), and electron microscopy of a sample from a typical thrombus confirmed that it consisted predominantly of densely packed platelets (Fig 1B). Multiple foci of fibrin and occasional erythrocytes and leukocytes were noted. Mean thrombus volume was 40 μL. To assess the heparin-neutralizing capacity of thrombi, extracts were prepared from each thrombus, as described in "Methods." The capacity of thrombus extracts to inhibit heparin in vitro was determined by assessing their effects on the APTT. The baseline APTT was 35±1 second, and addition of heparin (0.12 U per milliliter of plasma) prolonged the APTT to 70±1 second (200±5% of control). Addition of 2.5 μL of thrombus extract to plasma had no effect on the APTT in the absence of heparin. However, preincubation of heparinized plasma samples with 2.5 μL of thrombus extract resulted in shortening of the APTT by 26, 23, and 10 seconds, respectively, for each extract, compared with control reactions in which heparinized plasma was preincubated with 2.5 μL of extraction buffer. By comparison to a standard curve of APTT versus heparin concentration, the mean heparin-neutralizing capacity of coronary thrombus extracts was determined to be 2.8 U of heparin neutralized per milliliter of thrombus extract (or 28 U heparin per milliliter of thrombus). Hence, extract derived from 1 vol of platelet-rich thrombus contained sufficient activity to completely inhibit the heparin in 140 vol of therapeutically anticoagulated (0.2 U/mL) plasma.

To determine if the capacity of thrombus extracts to shorten the clotting time of anticoagulated plasma was specific to heparin, the above experiments were repeated, except that recombinant hirudin (0.17 μg per milliliter of plasma) was used to prolong the APTT to ≈200% of baseline (from 35 to 79 seconds). In contrast to the experiments with heparin, addition of thrombus extracts to hirudin-anticoagulated plasma samples had no significant effect on the APTT (APTTs were 79, 79, and 80 seconds, respectively, in the presence of each extract). Thus, extracts prepared from coronary thrombi significantly attenuated the in vitro anticoagulant capacity of heparin but not of hirudin (Fig 2A).

To characterize the heparin-neutralizing activity present in porcine coronary thrombi, a sample of thrombus extract was incubated with heparin agarose, as described in "Methods." Heparin-agarose−adsorbed thrombus extract (10 μL) shortened the APTT of heparinized plasma by only 8 seconds (from 71 to 63 seconds), whereas an equal volume of nonadsorbed thrombus extract completely inhibited the heparin present in the assay. Eluates (10 μL) prepared by sequential incubation of adsorbed heparin agarose with 0.4, 0.8, 1.4, and 2.0 mol/L NaCl shortened the APTT of heparinized plasma by 7, 9, 34, and 11 seconds, respectively, indicating that the antiheparin activity that adsorbed to heparin agarose was recovered predominantly in the 1.4 mol/L NaCl eluate. These observations suggested that the antiheparin effect of thrombus extracts might be mediated substantially by...
platelet factor 4, a basic protein that inhibits heparin by binding to it with high affinity. To test this hypothesis, the capacity of 10B2 (a monoclonal antibody to rabbit platelet factor 4 that neutralizes its antiheparin effect) to deplete thrombus extracts of heparin-neutralizing activity was studied. However, 10B2 did not cross-react with purified porcine platelet factor 4 either in the functional assay or by Western blotting (data not shown). Therefore, a rabbit carotid thrombus model was used to determine the contribution of platelet factor 4 to the antiheparin effect of platelet-rich thrombi.

Rabbit Carotid Thrombi

Carotid thrombi from three consecutive rabbits were assayed for heparin-neutralizing activity. Like porcine samples, extracts prepared from rabbit thrombi (mean weight, 2 mg) also exhibited potent heparin-neutralizing activity in the APTT assay. However, because of the potent heparin-neutralizing activity present in thrombus extracts, relatively large volumes of 10B2 (ie, greater than 0.1 mL) were necessary to neutralize their antiheparin effect, which resulted in nonspecific prolongation of the clotting time. Therefore, a modified factor Xa activity assay was used to quantify the heparin-neutralizing activity of rabbit thrombus extracts, since this assay has been used previously to measure platelet factor 4 activity and inhibition of platelet factor 4 by 10B2. Thrombus extracts were diluted to obtain ≈80% inhibition of heparin, then incubated with 10B2 (8.4 μg) or an equivalent volume of phosphate-buffered saline (as control) for 2 hours at 37°C, after which residual heparin-neutralizing activity was determined, as described in “Methods.” Preincubation of thrombus extracts with 10B2 reduced the heparin-neutralizing capacity of each extract to 0% (that is, no residual antiheparin activity), 0%, and 5%, respectively, compared with control reactions (Fig 2B), indicating that 10B2 essentially depleted the heparin-neutralizing activity in thrombus extracts. Control experiments revealed that 10B2 had no effect in the factor Xa assay when performed in the absence of thrombus extract.

Discussion

Although heparin is administered systemically in patients with acute ischemic syndromes, the goal of anticoagulant therapy in this clinical setting is to inhibit activation of the blood coagulation system within a localized region of the coronary vasculature, ie, at the site of blood vessel wall injury. This study was designed to assess whether factors present within platelet-rich thrombi generated at sites of arterial injury inhibit heparin, which potentially could attenuate the clinical efficacy of this anticoagulant. The results of these experiments suggest that platelet-rich thrombi contain potent heparin-neutralizing capacity. This antiheparin activity was present in all six thrombi tested, with 1 vol of coronary thrombus containing sufficient antiheparin activity to completely inhibit the heparin in 140 vol of therapeutically anticoagulated plasma. The tight binding affinity of this activity for heparin suggested that platelet factor 4, which elutes from heparin agarose at 1.4 mol/L NaCl,21 contributed substantially to the antiheparin activity present in platelet-rich clots. This hypothesis was confirmed in rabbit experiments in which a monoclonal antibody to rabbit platelet factor 4 virtually completely neutralized the antiheparin activity present in carotid thrombi. Although platelets contain several other proteins that inactivate heparin (eg, heparitinase, vitronectin, and histidine-rich glycoprotein14,15), the experiments with 10B2 suggest that the magnitude of their antiheparin effect in rabbit platelet-rich thrombi is small compared with that of platelet factor 4. However, 10B2 cross-reacts with human platelet factor 4, and the antiheparin activity present in human platelet releasates is >90% neutralized by this antibody,20 suggesting that the results observed with rabbit thrombi probably are relevant to human platelet-rich thrombi as well.

Platelet factor 4 is a 29-kDa homotetrameric protein that is present in high concentrations in platelet α-granules.14 Although the biological functions of this factor have not been firmly established, its potent capacity to inhibit heparin is well recognized,14 and recombinant platelet factor 4 has been used to efficiently reverse heparin anticoagulation in animals and in humans after cardiac catheterization.22,23 Although our quantification of heparin-neutralizing activity is derived from a static system, it is reasonable to hypothesize that localized heparin reversal could occur at sites of platelet activation, particularly under states of low blood flow, as can occur during coronary thrombosis.24,25 Since 1 mg of purified porcine platelet factor 4 was able to neutralize 29 U of heparin in the factor Xa assay (which is in accordance with previous experiments using human and rabbit platelet factor 4 in this assay20), it was possible to estimate the concentration of platelet factor 4 in rabbit platelet-rich thrombi (=0.9 mg of platelet factor 4 per milliliter of thrombus). This amount is approximately 45 000-fold higher than the concentration of platelet factor 4 present in plasma from patients with acute myocardial infarction (=20 ng/mL, with considerable variation26) and is consistent with the remarkable concentration of this protein in platelets relative to normal plasma ([PF₄platelet]:[PF₄plasma] = 280 000:1).27 However, caution must be exercised when drawing conclusions regarding the functional significance of the antiheparin activity detected in thrombus extracts. Mechanical disruption in vitro was used to release antiheparin activity from platelet-rich clots. This method does not reproduce the processes that regulate the secretion of platelet factor 4 or other platelet factors in vivo. In addition, only factors that could be extracted from thrombi were assessed in
these functional assays. Nevertheless, similar methods have been used to measure the concentrations of other factors in thrombi, such as plasminogen, tissue-type plasminogen activator, and plasminogen activator inhibitor-1,2,8.

Although thrombus extracts inhibited heparin, they had no effect on the in vitro anticoagulant capacity of recombinant hirudin, a direct-acting thrombin inhibitor.29 In animal models, hirudin is more effective than heparin in preventing platelet-rich thrombus formation,13,30 and recent clinical trials in humans suggest that hirudin may be superior to heparin as an anticoagulant within the coronary circulation.31 In addition, hirudin is insensitive to inactivation by platelet factor 4.32 Weitz et al.10 have demonstrated that hirudin is more effective than heparin–antithrombin III in inhibiting thrombin that is bound to fibrin. The experiments with porcine and rabbit thrombi suggest an additional localized mechanism that could contribute to the apparent superiority of hirudin as an anticoagulant at sites of vascular injury, i.e., the inactivation of heparin but not hirudin by platelet factor 4.

In conclusion, these studies have assessed the capacity of platelet-rich thrombi formed at sites of vascular injury to inhibit anticoagulation in vitro. These experiments suggest a possible mechanism for the occasional failure of heparin in preventing arterial thrombosis and for the potential superiority of hirudin over heparin as a thrombin inhibitor at sites of arterial injury. Additional studies using an animal model of arterial injury and anticoagulation, in conjunction with systemically administered 10B2, should further clarify the physiological significance of these findings.

Acknowledgments

This work was supported by a Department of Veterans Affairs Medical Research Service Advisory Group Grant (W.F.) and National Institutes of Health grant HL-02728 (W.F.) and HL-17982-15 (B.L.). We wish to thank Dr. S. Niewiarowski (Temple University) for supplying porcine platelet factor 4, Dr. G. Friederichs (University of Michigan) for assistance in obtaining carotid thrombi, and Drs. K. Cooney and D. Ginsburg (University of Michigan) for reviewing the manuscript.

References


11. Hogg PJ, Jackson CM. Fibrin monomer protects thrombin inactiva-


14. Hogg PJ, Jackson CM. Fibrin monomer protects thrombin inactiva-


17. Hogg PJ, Jackson CM. Fibrin monomer protects thrombin inactiva-


20. Hogg PJ, Jackson CM. Fibrin monomer protects thrombin inactiva-


Heparin neutralization by platelet-rich thrombi. Role of platelet factor 4.
D T Eitzman, L Chi, L Saggin, R S Schwartz, B R Lucchesi and W P Fay

Circulation. 1994;89:1523-1529
doi: 10.1161/01.CIR.89.4.1523

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/89/4/1523

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation is online at:
http://circ.ahajournals.org/subscriptions/