In Vivo Dynamic Real-Time Monitoring and Quantification of Platelet-Thrombus Formation

Use of a Local Isotope Detector


Abstract—Current methods for monitoring thrombosis and thrombus growth are invasive and provide only single–time-point data. Animal models rely mainly on flow changes as a surrogate of thrombus formation. Our aim was to validate a unique potentially noninvasive system to detect and quantify dynamic thrombus formation in vivo by using a porcine model of carotid artery injury. Thrombus growth was monitored by deposition of autologous 111In-labeled platelet activity over the injured artery by use of miniaturized gamma detectors and Doppler blood flow. Counts were recorded at 2-minute intervals for 2 hours. The technique was validated by comparing standard antithrombotic agents against controls. Platelet recruitment was detected before significant change in flow. Thrombus formation, calculated as the area under the curve (platelets×minutes×106), was greatest for control animals (11.7±1.28), followed by animals treated with aspirin (6.13±0.91, P<0.05), heparin (2.45±0.34, P<0.05), and hirudin (0.2±0.01, P<0.01 compared with heparin). The rate of platelet deposition was assessed as the slope of the curve in the first 30 minutes (platelets×106 per minute) for the following treatment groups of animals: control, 3.53±0.34; aspirin, 1.67±0.34 (P<0.01); heparin, 1.55±0.3 (P<0.01); and hirudin, 0.25±0.03 (P<0.001). There was no statistical difference between heparin and aspirin treatments. Change in flow was assessed as reduction from baseline: control, >99±0.34%; aspirin, 39±9.1%; heparin, 36±12.5%; and hirudin, 17±5.4%. There was no statistical difference between the aspirin- and heparin-treated groups. Morphometric analysis revealed >99±0.63% occlusion of the luminal area with thrombus for the control group, 43±14.3% for the aspirin-treated group, 30±5.6% for the heparin-treated group, and <10±1.8% for the hirudin-treated group. Assessment of platelet-thrombus formation with this technique was more sensitive than change in flow in determining antithrombotic efficacy, and thrombus formation was detected earlier. This study validates a new quantitative, sensitive, potentially noninvasive, portable, in vivo monitoring of dynamic thrombus growth, which appears applicable to phase II studies in humans. (Arterioscler Thromb Vasc Biol. 2000;20:860-865.)

Key Words: platelets • thrombus • antithrombotics • radioisotopes

Platelet–vessel wall interaction and thrombus formation are ongoing dynamic processes. The extent of thrombus formation at the site of arterial injury (whether spontaneous or induced), in addition to acute complications, has long-term consequences because it promotes neointimal proliferation and restenosis. Current methods for the diagnosis of thrombus formation in the clinical setting include intravascular ultrasound, angiography, and angioscopy. All of these are invasive and qualitative, and the results are limited to a single time point of an ongoing dynamic process. Furthermore, their invasive nature does not permit the continuous and quantitative monitoring of thrombus growth or assess the efficacy of treatment over time. Noninvasive methods such as vascular ultrasound are qualitative, not specific for thrombus, and time consuming, and they require frequent recordings to monitor change in volume as a marker for thrombus growth. Biochemical markers of thrombus formation and vessel occlusion are both insensitive and inadequate for quantifying thrombus formation. Thus, clinical development of new antithrombotic therapies has relied on “all or none” symptoms, with often dire consequences of death, myocardial infarction, or arterial occlusion. These occur with a relatively low incidence and are insensitive to gradations of thrombus growth. Consequently, clinical studies for determining optimal dosage or drug combinations to reduce thrombus formation are expensive, time consuming, and insensitive and require moderate to large numbers of patients, and the end points are potentially dangerous to patients.

Animal models, such as the Folts model, monitor changes in flow and obstruction to flow as a surrogate for in vivo thrombosis. This model is amenable for the study of antiplate-
let drugs in a dose-dependent manner and has significantly increased our knowledge of these agents. However, it has limitations of the inability to monitor or quantify dynamic thrombus formation and inability to mimic the high thrombogenicity and minor initial obstruction associated with initial plaque disruption.

The objective of the present study was to evaluate the sensitivity and feasibility of using the new technique for continuous in vivo isotope monitoring compared with changes in Doppler flow velocity for quantifying thrombus growth. Using a portable miniaturized gamma detector, we report that the continuous monitoring of platelet deposition and its rate of growth better quantifies total thrombus formation than does assessing changes in flow velocity.

We hypothesized that changes in $^{111}$In-labeled platelet deposition would result before changes in arterial blood flow and that the former would more accurately reflect the amount of mural thrombus formed after deep arterial injury.

**Methods**

**Animal Model**

The animal model for the study was the male Yorkshire albino pig (weighing between 25 and 32 kg). The pigs were housed at the Mount Sinai Medical Center for Laboratory Animal Sciences (CLAS) facilities for 1 week before any experiment to eliminate the stress effects of transportation and change in habitat. They were individually caged in a light-controlled, temperature ($22^\circ\text{C}$)-controlled, and humidity ($50 \pm 10\%$)-controlled environment with controlled feeding (normal pig chow, Purina Laboratory) and free access to water. All procedures performed in the present study were approved by the Institutional Review Board and follow the American Heart Association Guidelines for the Use of Animals in Research. The Mount Sinai School of Medicine animal management program is accredited by the American Association for the Accreditation of Laboratory Animal Care.

**Experimental Design**

After an overnight fast, blood was withdrawn on the morning of the experiment, and autologous platelets were labeled with $^{111}$In as previously reported. After complete hemostasis, the autologously labeled platelets were reinjected within 3 hours of withdrawal. All the animals were sedated with 15 mg/kg IM ketamine (Ketaset, Fort Dodge Animal Health). Deep anesthesia was induced with 25 mg/kg IV pentobarbital (Veterinary Laboratories Inc) and maintained by injections of 130 to 195 mg every 30 to 40 minutes to minimize hemodynamic changes. Throughout the 2-hour study period, the flow rate in the contralateral control carotid artery did not fall below 350 mL/min. Animals were intubated and ventilated with a Harvard respirator, and ECG was monitored throughout the experiment.

The carotid artery was exposed through a midline neck incision, and the miniature gamma detector was placed above the artery. A Doppler flowmeter (Transonic Flow Systems) was placed around the artery distal to the injury, and flow rates were recorded at 5-minute intervals. After injury, the vessel was bathed with 1% lidocaine solution to prevent vasospasm. Blood was collected for baseline determination of platelet number and activated partial thromboplastin time.

The gamma spectrometry system was activated, and on stabilization of radioactivity (10 minutes) at baseline, a 1-cm length of the carotid artery was deeply injured by external crush (6 serial hemostat clamps with a hemostatic forceps capable of distributing a homogeneous circumferential pressure on the vessel wall (7-62 Lahey hemostatic forceps curved, Miltex Instrument Co). The crush was always performed with the same hemostat and by 1 operator to minimize variability. Characterization of vascular injury by this methodology has been extensively studied for reproducibility. At the end of the 2-hour monitoring period, the injured segment of carotid artery was removed, after which the animal was euthanized (Slepeaway, Fort Dodge Animal Health). The carotid arteries were rinsed gently in 0.01 mol/L PBS and immediately transferred to 4% paraformaldehyde solution. The carotid arteries were later sectioned at 4-mm intervals and kept in fresh fixative. Specimens were then paraffin-embedded, sectioned at 5-μm sections, and routinely stained with combined Masson and elastin techniques.

**Study Protocol**

Three different antithrombotic regimens (heparin, oral aspirin, and hirudin) were compared against baseline (animals receiving intravenous saline, $n=5$).

For the heparin regimen ($n=5$), an intravenous bolus of 100 IU/kg heparin followed by an intravenous infusion of 100 IU/kg per hour was started 30 minutes before injury.

For the aspirin regimen ($n=5$), oral aspirin (160 mg/d) was given for 2 days before the start of the experiment. On the day of the experiment, aspirin was administered as an intravenous bolus of 1 mg/kg started 30 minutes before injury.

For the hirudin regimen ($n=5$), hirudin was administered 30 minutes before the start of the experiment as an initial bolus of 1 mg/kg followed by a continuous infusion of 0.8 mg/kg per hour.

**Detectors**

The detectors (Eurorad), shown in Figure 1, were of the following specifications: spectrometer grade CdTe detector, 440 mV/meV (10 mm×17 mm×5 mm); FET stage in Densimet housing, Al window; optimum energy range, 20 to 300 keV.

The detectors were connected to a preamplifier (TPR306SF, Eurorad) that was optimized for the CdTe sensor and FET amplifier and that had a 0.5-μs shaping time.

The preamplifier was connected to Canberra NaI/plus boards installed in a personal computer containing a Pentium (Intel) microchip. Gamma spectrometry software specifically written for the detectors (GammaSys 1.0, Lorenz Strahlentechnik) was installed and allowed sequential counting in up to 3 regions of interest for user-specified time intervals.

**Monitoring of Platelet-Thrombus Growth**

Continuous monitoring was performed for 2 hours, and actual total counts over the region of interest were recorded in 2-minute cycles. The kinetics of platelet-thrombus growth was assessed as changes in the radioactivity counts over the same area.

At the end of the experiment, the net counts were corrected for decay for the duration of the counting period. Platelet counts were obtained at half-hour intervals, and blood samples at the same time were assessed for activity in a gamma counter. The same blood samples were counted with the use of the miniature detectors. Adjusted radioactivity counts were subsequently converted to platelets deposited over the injured segment.

**Evaluation of Platelet-Thrombus Growth**

The kinetics of thrombus growth was recorded as platelets deposited every 2 minutes for 2 hours. The rate of thrombus growth at the site...
of injury was assessed by determining the slope of the curve in the first 30 minutes after crush. The extent of thrombus formation and its stability was determined by calculating the area under the curve for the observation period after crush, and this was compared with the thrombus formation determined by morphometric analysis of the histological specimens.

**Morphometric Analysis**

The histopathologic sections were digitized to a Macintosh computer from a camera (3CCD Video Camera, Sony) attached to a Zeiss Axioskop light microscope. Cross-sectional areas of the arterial lumen and luminal thrombus were determined by manual tracing with ImagePro Plus (Media Cybernetics). A separate investigator, blinded to the antithrombotic treatment, performed each analysis. From this analysis, the percentage of thrombus occupying the vessel lumen was determined.

**Data Analysis**

Individual values of platelet counts at the various time points were averaged, and the mean platelet deposition for each study group was plotted over time. The mean flow in the carotid artery at baseline and during the last 30 minutes was recorded, and percentage decrease in flow from baseline to the end of the experiment calculated. All results are presented as mean±SEM. All statistical analyses were performed by the Student t test (unpaired observations). Statistical significance was considered as a 2-tailed probability (P<0.05).

**Results**

**Dynamics of Platelet-Thrombus Growth**

The dynamic characteristics of platelet-thrombus growth after injury is clearly represented in Figure 2, which shows the curve obtained for the untreated control group. The rate of platelet deposition over the injured area reflects blood reactivity to the thrombogenic stimulus and is indicated by the slope of the curve in the first 30 minutes. Platelet deposition reaches a peak ~45 minutes after crush injury. Thereafter, the jagged edge profile of the graph, representing instability and embolization and regrowth of thrombus, is demonstrated after 60 minutes with fluctuations in the platelet count. Total thrombus formation is represented by the area under the curve from the time of crush injury to the end of the 2-hour observation period.

The rate of platelet deposition (slope of curve, platelets×10⁶ per minute) presented in Figure 3 reveals the fastest rate of platelet deposition in control animals, leading ultimately to total occlusion of the vessel. Aspirin-treated (1.67±0.34) and heparin-treated (1.55±0.30) animals demonstrated moderately reduced rates of platelet deposition compared with control animals (3.53±0.34, P<0.01), whereas hirudin-treated (0.25±0.03) animals demonstrated the lowest rate of platelet deposition (P<0.05 compared with the heparin-treated group).

Total platelet-thrombus deposition, estimated as area under the curve (platelets×minutes×10⁶) during the observation period, is presented in Figure 4. Control animals demonstrated the highest platelet deposition over time (11.7±1.28) compared with values in the aspirin-treated animals (6.13±0.91, P<0.05), and hirudin-treated animals demonstrated the least platelet deposition over time (0.2±0.01) compared with values in heparin-treated animals (2.45±0.34, P<0.01).

**Flow**

For all animals, platelet deposition detected by the miniaturized detectors preceded changes in flow. The percentage...
decrease in flow rate from baseline is shown in Figure 5 and was most marked in the control group (99\% 
0.34\%, \( P < 0.01 \) compared with other groups). Flow was absent 1 hour after injury in the control animals. The reductions in flow for both the aspirin- and heparin-treated groups were similar (39\% 
9.1\% and 36\% 
12.5\%, respectively; \( P = \text{NS} \)), and hirudin-treated animals demonstrated only a 17\% 
5.4\% reduction from baseline (\( P = \text{NS} \) compared with aspirin- and heparin-treated animals). Flow during the 2-hour observation period is shown in Figure 6. Flow distal to the injury decreased in all vessels and became undetectable in the control animals at the point of maximal platelet deposition. The flow rates for all the experiments demonstrated an inverse relation to both the rate of platelet deposition and the total platelet-thrombus formation. The control group had a noticeable reduction in flow 40 minutes after injury, whereas platelet deposition at the injury site was evident after 10 minutes. In the aspirin-, heparin-, and hirudin-treated groups, flow was reduced after maximal platelet deposition, but vessel patency was maintained throughout.

**Effect of Treatment**

An immediate increase in platelet deposition (Figure 7) was seen for all experiments after severe injury. In contrast to control animals, animals treated with antiplateletics demonstrated a more gradual increase over this time period, ultimately reaching a much lower plateau. For all animals, increased platelet deposition was associated with decreased flow. The most effective agent in reducing the rate of growth and total platelet-thrombus formation was the direct thrombin inhibitor hirudin. Of importance, none of this group demonstrated fluctuations in platelet deposition, indicating stability of thrombus.

The ratios of activated partial thromboplastin time to baseline that were measured 1 hour (and 2 hours) after injury were as follows: control, 0.95 (1.0); aspirin, 1.15 (1.1); heparin, 3.86 (3.7); and hirudin, 2.61 (3.1).

**Histological Analysis**

Histological analysis revealed the degree of injury to be similar in all experiments as previously shown with use of the above technique to induce arterial injury.\(^{10–13}\) All control vessels were occluded (>99\% 
0.63\% occlusion of vessel lumen, \( P = 0.02 \) compared with aspirin-treated group and \( P < 0.01 \) compared with heparin- and hirudin-treated groups). Aspirin- and heparin-treated animals revealed platelet-thrombus occlusion involving 43\% 
14.3\% and 30\% 
5.6\% of the vessel lumen, respectively (\( P = \text{NS} \)). One of the 5 aspirin-treated animals had total occlusion of the vessel. None of the 5 heparin-treated animals had total occlusion. In spite of similar degrees of injury, hirudin-treated animals had the least amount of thrombus formation (10\% 
1.8\% of vessel lumen area, \( P = 0.02 \) compared with heparin-treated animals and \( P = 0.08 \) compared with aspirin-treated animals), with no animal demonstrating complete occlusion.

**Discussion**

The portable gamma spectrometry system with miniature detectors used in the present study is a simple, reliable, sensitive, and potentially noninvasive method for the continuous (minute-to-minute) monitoring and quantification in vivo of dynamic platelet-thrombus formation. The dynamic nature of thrombus formation and greater sensitivity compared with flow measurements was clearly documented in the control group. This group demonstrated the greatest instability during thrombus growth, most likely due to platelet embolization and much earlier changes in platelet deposition before flow changes were evident. The end-stage fluctuations seen before occlusion mimic the cyclic flow variations seen in the Folts model.\(^{15}\)

The importance of documenting quantitative changes in platelet deposition rather than change in flow is highlighted by the difference between heparin and aspirin. Increased platelet deposition for aspirin was clearly evident on the real-time curve; consequently, the area under the curve was greater. However, the decrease in flow for the 2 groups was similar, and this criterion alone would fail to distinguish the relative antiplatelet potency of the 2 drugs. Thus, it is
apparent that the described method for quantifying platelet deposition increases the sensitivity of assessing efficacy between different antithrombotic treatments.

In addition, our results show that this new model allows quantification of both the rate and amount of platelet deposition at the site of arterial injury and can be used as an assay for antithrombotic drugs. This technique can be applied to other animal species, such as dogs, nonhuman primates, and rabbits.

Currently available models of thrombus monitoring fail to give real-time dynamic quantification of thrombus formation. Certain criteria are prerequisites for any model designed to study platelet deposition and thrombosis.9 First, to maximize thrombus formation, the technique should produce controlled degrees of intimal and medial damage with exposure of subintimal structures. The extent of deep compared with subendothelial injury induced is of critical importance and determines the extent of the resulting thrombus formation. Second, the rate and amount of platelet deposition at the injury site should be detectable. Third, the rate and amount of platelet deposition and ultimate luminal narrowing should correlate with flow measured distal to the occlusion. Fourth, the technique should be specific for thrombus, reliable, and reproducible, thus allowing dose-response curves for different antiplatelet and antithrombotic drugs to be established. Finally, any model designed to study the dose-response relation of drugs in a clinical model should, ideally, be noninvasive.

One of the most widely used models to study platelet aggregation and thrombosis is the Folts coronary thrombosis model of cyclic flow variations.9 This model is based on cyclic flow reductions resulting from periodic acute occlusive platelet thrombus followed by embolization.15 It uses flow as a surrogate for thrombus formation. Its advantages are that it meets most of the above criteria and is reproducible. However, it fails to monitor the rate of change of thrombus formation or dissolution, is not specific for thrombus, and does not quantify platelet deposition. Therefore, it is less sensitive for comparison of different antithrombotic agents.

Morphometric analysis is able to provide detailed information, but the method is time consuming and limited to end-point measurements.16 In animal models, thrombus formation can be quantified by measuring the deposition of autologous radiolabeled platelets continuously in a gamma scintillation counter.17,18 Dynamic measurements have also been performed in nonhuman primates by placing the subject in a gamma scintillation camera.19 We previously reported the use of a gamma camera and computer-assisted nuclear scintigraphy to dynamically monitor platelet deposition.20 However, both methods are cumbersome and, therefore, not readily applicable to a clinical setting.

We validated our method by testing commonly used antithrombotic agents against control animals and correlated the results with change in flow and histological analysis of the injured artery. The effect of these commonly used drugs on platelet deposition is well known.21 Our model confirmed the weak antiplatelet effects of aspirin,22 the modest effect of heparin, and the potent effect of hirudin but, importantly, showed that compared with flow measurements, monitoring continuous isotope-labeled platelet deposition can more sensitively quantify thrombus formation, even before changes in flow or total occlusion occur.

This isotope model of continuous quantification of platelet deposition appears valuable and could be used as an assay to test the dose-response relation of new drugs in a clinical setting. The surface detectors are small and can be applied over superficial arteries. Furthermore, they are able to record 3 isotopes simultaneously and, thus, would also permit the monitoring of radiolabeled fibrinogen and red blood cell response to injury. For dose-response studies, smaller numbers of patients would be needed, expense would be reduced, and patient safety would be improved because death, myo-

Figure 7. Graph of $^{111}$In-labeled platelet deposition with time for all treatment groups. This graph demonstrates a rapid and early rise in platelet deposition occurring 10 to 20 minutes after injury for all treatment groups.
cardiac infarction, stroke, or arterial occlusion would not be necessary for phase II dose-response studies.

We have shown that this new model meets all of the criteria required for sensitive quantification of thrombus growth and dissolution after platelet deposition after arterial injury. The model permits the safe and accurate in vivo evaluation of antithrombotic drugs with a significant reduction in the number of subjects needed. It allows quantification of dynamic thrombus formation, thus reducing the risk of arterial occlusion or end-organ damage in clinical dosing studies. More important, the noninvasive and portable nature of this technique makes it ideal to assess the efficacy of new antithrombotic agents in humans. Ongoing studies are directed toward monitoring acute thrombus growth after peripheral vascular intervention.

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References

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