Heparin-Resistant Thrombus Formation by Endovascular Stents in Baboons

 Interruption by a Synthetic Antithrombin

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Intravascular mechanical support has been proposed as a solution to the frequent occurrence of vascular narrowing and occlusion after transluminal balloon angioplasty or surgical endarterectomy. Although several endovascular stents are currently in clinical use for angioplasty of larger vessels, acute thrombosis is a troublesome complication of their use with coronary angioplasty. To study thrombus formation associated with metallic mesh endoprostheses, we have evaluated stents placed inside 3-mm expanded polytetrafluoroethylene (ePTFE) grafts incorporated into chronic exteriorized arteriovenous rubber shunts in baboons. We have also compared the antithrombotic capacities of heparin and the synthetic antithrombin D-phenylalanyl-L-prolyl-L-arginyl-chloromethylketone (D-FPRCH2Cl) to interrupt this platelet-dependent process for two different endovascular stents. Acute platelet deposition was continuously measured during 1 hour using gamma camera imaging of platelets labeled with indium-111 oxine. On untreated control ePTFE grafts (n=11), 0.87±0.15x10⁹ platelets/cm² were deposited during 60 minutes. In contrast, balloon-expandable endovascular stents within ePTFE (n=6) accumulated 4.37±0.68x10⁹ platelets/cm² (p=0.003 compared with controls), and self-expandable stents (n=6) accumulated 3.91±0.42x10⁹ platelets/cm² (p=0.006 compared with controls); no difference between stents was detected in this test system (p>0.5). Systemic heparin treatment did not reduce platelet deposition (4.20±0.41x10⁹ platelets/cm² at 60 minutes; p>0.5). Continuous infusion of D-FPRCH2Cl (100 nmol/kg·min) began immediately before placing stented grafts in the shunts maintained plasma levels of D-FPRCH2Cl at 4.30±0.50 μg/ml and significantly reduced platelet deposition (0.45±0.41x10⁹ platelets/cm² for balloon-expandable stents; p=0.0008 compared with untreated stents, and 0.01±0.01x10⁹ platelets/cm² for self-expandable stents; p=0.01 compared with untreated stents). The accumulation of abundant thrombotic material, as demonstrated by electron microscopy in stented grafts, was interrupted by D-FPRCH2Cl treatment. We conclude that stainless-steel endovascular stents within ePTFE grafts induce platelet-dependent, heparin-resistant thrombosis under high-flow conditions and that systemic infusion of D-FPRCH2Cl abolishes thrombus formation. (Circulation 1990;81:570-577)

The frequency of restenosis after coronary or angioplasty ranges from 25% to 70%1-6 and averages 20% after carotid endarterectomy.7-9 To date, thermal,10-12 pharmacological,13,14 and mechanical15,16 approaches to the prevention of early occlusion and restenosis have not been successful. In 1969, Dotter17 reported successful percutaneous insertion of nonexpandable stainless-steel coils into canine popliteal arteries. Two decades later, a variety of endovascular stent designs are being evaluated18-24 for their capacity to prevent dissection and vascular narrowing after balloon angioplasty or endarterectomy.

Because placement is irreversible, stent biocompatibility, biomechanics, and thrombogenicity are important considerations. Whereas biocompatibility and biomechanics have been studied extensively,21,25-27 thrombus formation remains problematic. Indeed, antithrombotic regimens consisting of combinations of heparin, aspirin, and dipyridamole have not prevented this platelet-mediated, high-flow, arterial thrombotic process.28,29
In this investigation, we measured the thrombi formed on two different types of stainless-steel mesh endoprostheses placed inside 3-mm expanded polytetrafluorethylene (ePTFE) grafts by incorporating the stent-containing segments into chronic exteriorized arteriovenous silicone rubber shunts in baboons. The extent of acute thrombus formation was measured in real time by scintillation camera imaging of autologous 111In platelet deposition. Because the synthetic antithrombin D-FPRCH$_2$Cl has been shown to prevent platelet-dependent high-shear thrombus formation,$^{30}$ we have also determined the capacity of D-FPRCH$_2$Cl to inhibit stent thrombosis.

The baboon was selected as the experimental animal because it closely resembles humans with respect to its hemostatic mechanisms$^{29,31-33}$ and its responses to antithrombotic agents in high-flow arterial systems.$^{30,34,35}$ ePTFE grafts were used for stent placement because these grafts are clinically relevant and have less thrombogenicity than other commonly used clinical graft materials.$^{36}$

**Methods**

**Animals**

Fifteen normal male baboons (*Papio anubis*) were used in these studies. The animals weighed 8–12 kg and had been dewormed and observed to be disease-free for at least 6 weeks before use. All procedures were approved by the institutional animal care and use committee and were in accordance with federal guidelines. Chronic arteriovenous shunts were surgically implanted between the femoral artery and vein using the method described previously.$^{38}$ Earlier investigations have established that these permanent Teflon–silicone rubber shunts do not produce measurable activation of platelets or coagulation factors.$^{29,34,39}$ All experiments were performed in awake animals.

ePTFE grafts (with and without stents) were interposed between the segments of the permanent arteriovenous shunt. Mean blood flow rates through the arteriovenous shunt were continuously measured using a Doppler ultrasonic flowmeter (model T101, Transonic Systems Inc., Ithaca, N.Y.) and a cuff-type transducer that fitted appropriately around the silicone rubber tubing immersed in water.

**Vascular Grafts**

 Reinforced ePTFE grafts (30-µm internodal distance) were a gift of the W.L. Gore Co., Flagstaff, Ariz. All grafts were 7 cm in length and 3.0 mm i.d. The grafts were constructed using the method reported by Hanson et al.$^{38}$ for Dacron grafts (Figure 1). The grafts were wrapped with Parafilm (American Can Co., New York) and encased with heat-shrunk Teflon tubing (Small Parts Inc., Miami, Fla.) to ensure that the apparatus was rigidly constrained to a linear geometry with an inner diameter of precise dimensions (3.0 mm). Care was taken to maintain a smooth transition between the silicone rubber tubing and the graft without imperfection due to the coupling procedure because reproducible and uniform platelet deposition required that there be no flow disturbance at the graft junction. The 3-mm graft–silicone rubber tubing apparatus was then connected to additional lengths of 3-mm silicone rubber tubing comprising the arteriovenous shunt system with 2-cm-long tapered Teflon connectors (Small Parts).

**Stents**

We investigated two types of stents, both measuring 3 mm i.d. and 15 mm in length after placement. The first device studied was a balloon-expandable (BE) stent made from tempered stainless steel and was a gift from Johnson & Johnson, New Brunswick, N.J. The endoprosthesis consisted of continuous tubular wire mesh that measured 1.67 mm in collapsed diameter (Figure 2). Wire thickness was 150 µm. The BE stents were compressed to fit snugly around collapsed coaxial angioplasty balloons. They were expanded in a controlled fashion to reach the diameter imposed by inflating the coaxial balloon to expand the steel fibers to the limits of the graft. Placement directly within the center of the ePTFE grafts was determined by direct vision. The six double rows of staggered slots assumed a diamond shape after expansion. The mesh configuration of the stent dictated the degree of shortening that occurred when the stent reached its ultimate diameter within the lumen. This design determined the proportion of open space to metal surface and the ratio of expanded to expanded diameter (which limits delivery catheter size). The open area of the stent ranged from 69% in the collapsed state to a maximum of 88% when fully expanded.

The second type of stent evaluated was a self-expandable (SE), spring-coil device provided as a gift from Medinvent SA, Lausanne, Switzerland. It was fashioned as a cylinder woven from surgical-grade stainless-steel monofilaments (100-µm diameter). Each monofilament was free to pivot at lattice crossing points and had sufficient flexibility to become elastically extended longitudinally with a consequent decrease in diameter. It was enclosed within an invaginated, rolling plastic membrane at the end of a

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**Figure 1.** Drawing of configuration of expanded polytetrafluorethylene (ePTFE) vascular graft connection to silicone rubber tubing. A smooth transition of graft material to tubing is essential to prevent flow disturbance. Stent is deployed exactly at the midpoint of the graft.
specially designed catheter delivery system (Figure 2). When the constraining membrane was progressively removed, the stent self-expanded. The length after expansion was 15 mm.

**Laboratory Procedures**

Autologous baboon blood platelets were labeled with 800–1,000 μCi 111In oxine as previously described35,38 and were re injected at least 2 hours before grafts were placed and imaged. Labeling efficiencies averaged 90%. Platelet counts were performed before each study on whole blood collected in Na2-EDTA (2 mg/ml) with a whole blood analyzer (model 810, J.T. Baker Instruments, Piscataway, N.J.). In selected animals, repeated platelet counts were performed during and after the study to observe potential depletion. Template bleeding times were also performed by the technique described previously.31

Activity levels of d-FPRCH2Cl were measured as antithrombin activity in plasma prepared from blood collected in acid–citrate dextrose (ACD; National Institutes of Health formula A) on samples obtained before and at the end of infusion. Plasma antithrombin activity levels were assayed immediately or plasma was flash-frozen at −70°C for subsequent assay, using a standard curve for d-FPRCH2Cl in the range of 0–150 μg/ml prepared in autologous pre-treatment plasma. The unknown plasma samples were diluted in control autologous plasma to obtain a thrombin time that could be read on a standard curve. Thrombin times were also performed on samples obtained 30 minutes after stopping d-FPRCH2Cl infusion.30

To assess heparin activity, activated partial thromboplastin times were measured before, 30 minutes after, and 60 minutes after injection.

**Treatment Regimens**

Eleven untreated ePTFE grafts served as controls. Six BE stents and five SE stents were deployed in grafts and placed in animals without pharmacological treatment. Five BE stents and five SE stents were deployed in grafts and placed in animals treated with a continuous infusion of d-FPRCH2Cl (100 nmol/kg/min) using a syringe pump (model 22, Harvard Apparatus, Cambridge, Mass.) begun 10 minutes before insertion of the stented graft into the arteriovenous cannula and maintained throughout the 60 minutes of stent imaging. d-FPRCH2Cl was obtained from Calbiochem, La Jolla, Calif., as a purified...
Morphology

At the conclusion of the study, grafts (control and stented) were gently irrigated with normal saline, removed, and inspected for gross thrombus. They were perfused with 2.5% gluteraldehyde in 0.1 M phosphate buffer. Grafts were then dehydrated in increasing concentrations of ethanol, critical-point dried with Freon, and sputter-coated with approximately 35 nm of carbon for scanning electron microscopy using a Hitachi Model S520 scanning electron microscope.

Data Analysis

Data were analyzed using the CLINFO Plus computer program provided by the Division of Research Resources, the Heart, Lung, and Blood Institute, National Institutes of Health. Comparisons between groups were made using Student’s t test (two-tailed) for paired and unpaired data. Variance about the mean is reported as ±1 SEM.

Results

Blood flow rates in the arteriovenous shunt ranged from 100 to 165 ml/min (130±15 ml/min) at the beginning of the study and 80 to 125 ml/min (99±11 ml/min) at the end of the study. In no instance was there a significant difference in mean flow rates between study groups (p>0.3 by paired t test). Moreover, in previous work using the vascular graft model, platelet deposition was not significantly altered over the range of flow rates of 200 to 70 ml/min (p>0.4), that is, those values spanning the variations in shunt flow rate observed in this study (S.R. Hanson, unpublished observations). Circulating platelet counts averaged 280±51x10^3/µl, and there was no significant between-group difference in platelet counts before and after studies were performed (p>0.5; paired t test).

Platelet deposition on control ePTFE grafts (n=11) was detected and reached a plateau value by 60 minutes of 0.87±0.15×10^9 platelets/cm (Figure 3). Images showed uniform distribution of radioactivity over the entire length of the graft. No control graft occluded.

In contrast, endovascular BE stents within ePTFE (n=6) produced substantially greater platelet deposition (Figure 3). By 60 minutes 4.37±0.68×10^9 platelets/cm were accumulated (p=0.003 compared with controls; Figure 3). SE stents accumulated comparable numbers of platelets (3.91±0.42×10^9 platelets/cm, p=0.006 compared with controls [Figure 4]; p>0.2 compared with BE stents). Maximum radioactivity was located at the midpoint of the graft over the stent. Abundant macroscopic thrombus was deposited along the length of the stents and two (one of five BE and one of five SL) stents occluded in untreated animals.

Conventional anticoagulating doses of heparin (100 units/kg given as a bolus prior to inserting the graft) did not reduce platelet deposition on stented grafts (4.20±0.41×10^9 platelets/cm; p>0.5 compared with untreated stents). Activated partial thromboplastin times (aPTTs) averaged 32.3±4.6 seconds before injection of heparin, 194±26 seconds 30 minutes after injection, and 188±32 seconds at 60 minutes after injection, when the study was completed. Indeed, three of five stented grafts occluded by 60 minutes in the heparin-treated group.

In animals (n=10) treated with continuous infusion of d-FPRCH_2Cl (100 nmol/kg/min) begun (99.5%) stable compound. Immediately before its intravenous administration, d-FPRCH_2Cl was dissolved in 0.15 M NaCl and sterilized by filtration.

In an additional five animals, heparin (100 units/kg) was given as a bolus 10 minutes before insertion of stented grafts. This regimen was used to simulate the clinical administration of heparin for short-term coverage associated with the use of cardiovascular devices, in which anticoagulation is produced for several hours by the injection of a large initial bolus of heparin. Porcine heparin (1,000 units/ml) was obtained from Elkins-Sinn, Inc., Cherry Hill, N.J.

Platelet Imaging

Dynamic images of the grafts and accompanying silicone rubber tubing were taken with a Searle PHO/Gamma V scintillation camera (Siemens Medical Systems, Iselin, N.J.) with acquisition at 5-minute intervals for 60 minutes. Images (128×128 byte mode) were analyzed directly with a Medical Data Systems A³ computer (Medtronic, Ann Arbor, Mich.) without image smoothing or other manipulations. The 172-keV energy peak of ^11^In was acquired with a high-sensitivity collimator and a 15% energy window. Silicone rubber tubing and graft were fixed in a groove precisely machined into Plexiglas to maintain a linear geometry and ensure a standard 1-cm distance from the surface of the collimator. Care was taken to position the camera directly over the midpoint of the graft in which the stent was located. Images of the regions of interest 1.7×2.2 cm in area (10×13 pixels, as determined by direct calibration of point sources) of the stented graft segment and of the silicone rubber tubing proximal to the stented region were acquired. Subtracting the blood background counts per minute from the stent region emissions for each image corrected for circulating (nondeposited) radioactivity, yielding deposited radioactivity only. After 60 minutes, 1 ml of whole blood was drawn, placed inside 3-mm-i.d. silicone rubber tubing, and imaged for 5 minutes as described above (region of interest, 100×13 pixels). The activity of this blood standard was also corrected for the small fraction of circulating nonplatelet (i.e., plasma) isotope to give platelet-associated ^11^In radioactivity in counts per minute per milliliter of whole blood. Total platelet deposition, which included both labeled and unlabeled cells, was calculated by dividing stent-deposited counts per minute by circulating platelet counts per minute (blood standard) and multiplying by the circulating platelet count (platelets per milliliter of whole blood) as measured in the blood standard sample. 30,38
immediately before placing the stented graft in the shunt, platelet deposition at 60 minutes was markedly decreased (0.23±0.20×10⁹ platelets/cm for all D-FPRCH₂Cl-treated stents versus 4.21±0.46×10⁹ platelets/cm for all untreated stents; p=0.0001). Platelet deposition on D-FPRCH₂Cl-treated BE stents was 0.45±0.41×10⁹ platelets/cm (p=0.0008 compared with untreated BE stents; Figure 4); platelet deposition on D-FPRCH₂Cl-treated SE stents was 0.01±0.01×10⁹ (p=0.01 compared with untreated SE stents; Figure 5). Stented grafts were free of any visible thrombus, and none occluded.

The thrombin times were prolonged to more than 300 seconds, the aPTTs were prolonged to 243±48 seconds, and the template bleeding times were more than 30 minutes during the infusion of this dose of D-FPRCH₂Cl. Thirty minutes after discontinuing the infusion of D-FPRCH₂Cl, the bleeding time had normalized (5.0±1.6 minutes; p>0.5), the aPTTs had returned to baseline (p>0.3), and the thrombin times averaged 20 seconds compared with the control times of 10 seconds. Plasma levels of D-FPRCH₂Cl were not detectable before infusion, 4.30±0.5 µg/ml at the end of infusion, 0.1±0.04 µg/ml at 30 minutes after stopping the infusion, and undetectable the next day.

There was no clinical evidence of spontaneous bleeding episodes in any of the animals studied.
Scanning electron microscopy of the untreated ePTFE grafts showed detectable platelet deposition (Figure 6A), which became abundant on intraluminal stents in both untreated and heparin-treated animals (Figure 6B). Platelet deposition on stents in animals treated with D-FPRCH₂Cl was abolished (Figure 6C).

**Discussion**

This study demonstrates that platelet deposition is substantial on two different endovascular stents placed within ePTFE grafts under high-flow conditions in nonhuman primates and that thrombus formation is abolished by infusion of the synthetic antithrombin D-FPRCH₂Cl but resistant to comparably anticoagulating levels of heparin. Resistance of platelet-dependent thrombotic processes to heparin has been reported previously. Although the design and delivery systems of BE and SE stents are quite different, both stents accumulated significant and equivalent amounts of thrombus, a process known to complicate their use in vivo. Because the stents were deployed in vitro onto an artificial surface, conclusions regarding the absolute amount of thrombus accumulation and the relative thrombogenicity of the two stents in vivo may not be warranted without additional study. However, the test strategy is well designed to assess the effects of therapies.

Thrombosis has complicated the use of intravascular stents in humans. Sigwart and coworkers implanted 34 stents into iliofemoral (10) and coronary (24) arteries in 25 patients; two stents thrombosed, and one may have indirectly caused a patient's death by inducing coronary vasospasm. In another study, Sigwart et al reported 1-year follow-up of 48 coronary artery stents with an overall complication rate of 14%, including three deaths, five abrupt closures, and two restenoses. Puel et al implanted stents in the coronary arteries of 23 patients; four had acute thrombotic occlusions and five thrombosed within 3–5 days, for a 40% thrombosis rate. Partial or complete thrombosis occurred in 33% of stents placed in peripheral vessels in sheep.

Stainless-steel alloys are thrombogenic prosthetic materials. For example, fibrinogen and platelets adhere to stainless steel upon contact with blood. In large arteries with high blood flow and high ratios of metal to vessel diameter, thrombotic occlusion of the stent is unlikely. However, in smaller vessels such as coronary or superficial femoral arteries, in which stents may have the most clinical potential, the thrombogenicity of stents appears to contribute to the high failure rate. Palmaz et al reported inconsistent results when heparin was combined with antiplatelet agents to prevent stent-induced thrombosis.

Although the precise clinical relevance of stent thrombosis in graft segments in baboon arteriovenous shunts has not been fully defined, this model demonstrates reproducible thrombus formation on
various prosthetic materials suitable for assessing the effects of antithrombotic agents. Graft material, chemical properties, and topography are major factors influencing platelet deposition on vascular prostheses.\textsuperscript{47-50} In this regard, ePTFE, in contrast to knitted Dacron, produces little platelet deposition.\textsuperscript{36,51,52} By constraining grafts to their intended cylindrical geometry, with smooth transitions between tubing and graft material, stents were evaluated in a manner free of confounding hemodynamic variables.

\( \text{D-FPRCH}_2\text{Cl} \) but not heparin profoundly decreases platelet deposition on stents. These data suggest that antithrombins not dependent on antithrombin III may be potentially useful therapeutic agents in this setting. \( \text{D-FPRCH}_2\text{Cl} \), a synthetic antithrombin, produces direct, potent, specific, and irreversible inactivation of thrombin that is independent of antithrombin III. Its mechanism of action involves a two-stage process: the reversible formation of a complex with the active site of thrombin followed by rapid, irreversible alkylation of active-center histidine by chloromethyl ketone.\textsuperscript{53}

There are at least three possible explanations for the disparity in effectiveness of the antithrombins \( \text{D-FPRCH}_2\text{Cl} \) and heparin with respect to stent thrombosis. First, activated platelets release platelet-specific heparin-binding proteins including platelet factor IV and \( \beta \)-thromboglobulin. Thus, platelet-rich thrombi may locally neutralize heparin but not \( \text{D-FPRCH}_2\text{Cl} \) because it does not bind with these platelet-specific proteins. Second, \( \text{D-FPRCH}_2\text{Cl} \) but not heparin may inactivate platelet-bound meizothrombin during thrombin generation.\textsuperscript{54} Meizothrombin is an intermediate product of prothrombin that remains bound to, and may activate, platelets. Meizothrombin is poorly inhibited by the antithrombin III–heparin complex\textsuperscript{55} but highly susceptible to \( \text{D-FPRCH}_2\text{Cl} \). Third, antithrombin III–heparin may be sterically or ionically hindered from interacting with thrombin formed within thrombus, whereas the smaller molecular weight \( \text{D-FPRCH}_2\text{Cl} \) is not.

The early durability of stented vessels appears to be related to the potential for thrombus formation and occlusion, and the late outcome is dependent on the completeness of endothelialization.\textsuperscript{25-27,56} Until endothelialization is realized, initial antithrombotic therapy with a synthetic antithrombin may be useful because of its potent and immediate inhibition of arterial thrombosis after stent deployment. The required duration of treatment and the safety of such agents remain to be defined.

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