Heparin immobilization reduces thrombogenicity of small-caliber expanded polytetrafluoroethylene grafts

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Objective: **The patency of small-diameter expanded polytetrafluoroethylene (ePTFE) grafts for vascular reconstruction is impaired by acute thrombotic occlusion. Prosthetic materials are thrombogenic and cause platelet adhesion and activation of the coagulation cascade. Heparin is a potent anticoagulant drug widely used to prevent and treat thrombosis. A new ePTFE graft with long-term bonding of heparin is now commercially available in several European countries, but a basic analysis of its mechanism of action in humans has never been performed. This study was performed to evaluate the thrombogenicity of heparin-bonded ePTFE grafts compared with standard ePTFE in a newly developed human ex vivo model.**
Methods: **Nonanticoagulated blood was drawn from antecubital veins of 10 healthy donors with a 19-gauge needle. The proximal end of a 60-cm ePTFE vascular graft with a diameter of 3 mm was connected to the needle while the distal end was connected to a syringe, which was placed in a syringe pump. Every volunteer served as his or her own control by using a heparin-bonded ePTFE graft on one arm and a standard ePTFE graft on the other arm. The perfusions were performed over 6 minutes with a flow rate of 20 mL/min, corresponding to a shear rate of 74/s. Serial samples were taken at the distal end of the graft for determination of prothrombin fragment 1 + 2, fibrinopeptide A, and P-selectin expression on perfused platelets. Fibrin deposition and platelet deposition were studied by using scanning electronic microscopy.**
Results: **Fibrinopeptide A production over time was significantly reduced on the heparin-bonded ePTFE grafts compared with standard ePTFE grafts ($P < .05$). There was no increase in the production of prothrombin fragment 1 + 2 or P selectin over time on either type of graft. Scanning electronic microscopy scanning showed platelet deposition and fibrin formation on standard ePTFE grafts, whereas no platelets or fibrin were observed on heparin-bonded ePTFE grafts.**
Conclusions: **Heparin immobilization substantially reduces the thrombogenicity of small-diameter ePTFE in a newly developed human ex vivo model. In this study, we provide evidence that the mechanism of action of the heparin bonding is due not only to anticoagulant but also to antiplatelet effects. Heparin bonding may be an important improvement of ePTFE, resulting in better patency rates for vascular reconstructions. (J Vasc Surg 2006;43:587-91.)**

Clinical Relevance: **Heparin immobilization reduces the thrombogenicity of small-caliber expanded polytetrafluoroethylene (ePTFE) grafts. The patency of small-diameter ePTFE grafts for vascular reconstruction is impaired by acute thrombotic occlusion. Prosthetic materials are thrombogenic and cause platelet adhesion and activation of the coagulation cascade. Heparin is a potent anticoagulant drug widely used to prevent and treat thrombosis. A new ePTFE graft with long-term bonding of heparin is now commercially available, but a basic analysis of its mechanism of action in humans has never been performed. This study was performed to evaluate the thrombogenicity of heparin-bonded ePTFE grafts compared with standard ePTFE in a newly developed human ex vivo model. We demonstrated that heparin immobilization reduces thrombogenicity on small-caliber ePTFE grafts. Heparin-bonded ePTFE grafts might therefore result in better patency rates for vascular reconstructions with vascular grafts.**

It is well known that an autologous vein graft is the surgeon's first choice in peripheral arterial bypass procedures because of superior patency rates when compared with prosthetic grafts. In a recent review on venous and polytetrafluoroethylene (PTFE) above-knee femoropopliteal bypasses, 5-year primary patency rates of 74% and 39%, respectively, were reported.[1] However, almost a third of patients who undergo peripheral arterial reconstructive operations do not have suitable autologous veins available for grafting.[2] Therefore, prosthetic grafts, such as PTFE grafts, are frequently used in arterial bypass procedures.

Apart from this clinical evidence, laboratory models also have shown that PTFE grafts are substantially more thrombogenic compared with autologous veins. Prosthetic materials cause platelet adhesion and activation of the coagulation cascade on the graft.[3] One potential strategy for reducing the thrombogenicity of prosthetic material is to bind heparin to the endoluminal surface. Heparin is a polysaccharide anticoagulant with potent inhibitory effects on coagulation and a long history of clinical use in the prevention and treatment of thrombosis.[4] Heparin-bonded (or heparinized) grafts have shown favorable results in

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animal models and humans compared with untreated vascular grafts.[5-7] A new expanded PTFE (ePTFE) graft (GORE-TEX PROPATEN® Vascular graft, W.L. Gore and Associates, Flagstaff, AZ) with long-term bonding of heparin accomplished by covalent linkage of the anticoagulant is now commercially available in those European countries (except France) that accept CE certification. An in vivo canine study showed measurable heparin activity on these heparinized ePTFE grafts throughout a 12-week observation period, with only a marginal drop in activity after 12 weeks.[5] These results indicate that this particular heparin immobilisation method yields grafts that express anticoagulant properties for prolonged periods after implantation. Although the anticoagulant mechanism of action of intravenous heparin is well established, little is known about the precise mechanisms by which immobilization of heparin on vascular grafts reduces the thrombogenicity of ePTFE in humans. In this study we evaluated the potential mechanism of action by analysis of the thrombogenicity of standard ePTFE and heparinized ePTFE grafts by a comprehensive investigation of clotting parameters and deposition of platelets and fibrin in a newly developed human ex vivo model.

METHODS

Ex vivo model. In a newly developed ex vivo model, nonanticoagulated blood was drawn from the antecubital veins of 10 healthy donors with a 19-gauge needle. A 60-cm vascular graft with a diameter of 3 mm was directly connected to the needle (Fig 1). During 6 minutes, the blood was aspirated with a constant flow rate of 20 mL/min by using a syringe pump (Harvard Apparatus, South Natick, Mass). This flow rate and graft diameter result in a shear rate of 74/s, which reflects venous flow conditions and favors fibrin-rich clot formation. A cuff was wrapped around the upper arm to ensure a constant pressure of 45 mm Hg, resulting in a continuous blood flow through the graft during the experiment. Volunteers denied taking any medication 2 weeks before the experiment and gave informed consent.

Every volunteer served as his or her own control. In the first run, a standard Gore-Tex (W. L. Gore & Associates, Inc, Flagstaff, Ariz) ePTFE graft was perfused by using blood drawn from one arm. In the second run, which was performed within half an hour of the first, a Gore-Tex ePTFE graft treated with Carmeda BioActive Surface (CBAS) technology was perfused with blood drawn from the other arm. CBAS is a clinically used heparin-binding technology. This technology is based on covalent end-point attachment of heparin to a biomaterial surface, thus enabling maintenance of functional heparin bioactivity.

Heparin-bonded ePTFE grafts. Standard ePTFE Gore-Tex vascular grafts (internal diameter, 3 mm; length, 60 cm) were used in our study. The luminal microstructure of the heparinized grafts was treated with the CBAS, in which heparin molecules are covalently bound via end-point aldehyde linkages to free amino groups in an underlying polyethyleneimine sublayer. End-point attachment permits the pentasaccharide active sites on the heparin to remain available for binding antithrombin III. The procedure to modify heparin with free aldehyde moieties has been described previously.[5,6] Standard nonheparinized ePTFE grafts were steam-sterilized; heparinized ePTFE grafts were sterilized with ethylene oxide.

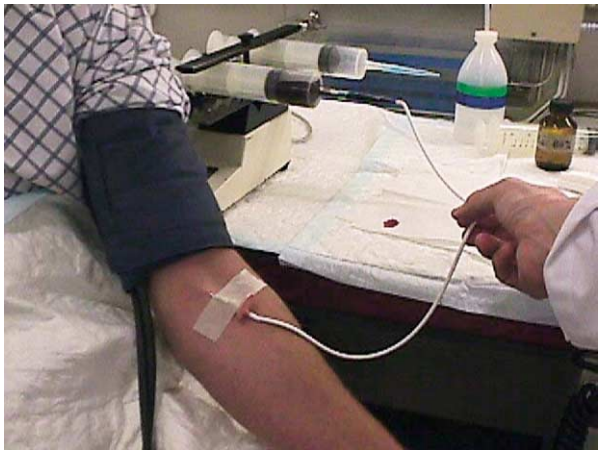

Fig 1. The newly developed ex vivo perfusion system using a cuff at a constant pressure of 45 mm Hg to ensure blood flow. Blood is aspirated through the graft with a constant flow of 20 mL/min, resulting in a shear rate of 74/s.

Blood samples and assays. Blood samples (900 μL) were collected at the end of the graft, starting directly after connection to the vein and thereafter every minute until 4 minutes. After 4 minutes, samples were collected every 30 seconds until the end of the perfusion (total perfusion time, 6 minutes per arm). The samples were mixed immediately with 100 μL of ethylenediaminetetraacetic acid (500 mmol/L) and centrifuged at 3500 rpm for 5 minutes, and aliquots of plasma were stored at −20°C until assayed.

A commercially available enzyme-linked immunosorbent assay was used for the fibrinopeptide A (FPA) measurements as an indicator of fibrin formation (Zymutest FPA; Hyphen Biomed, Andresy, France). Prothrombin fragment 1 + 2 (F_{1+2}), an indicator of thrombin formation, was also measured by using an enzyme-linked immunosorbent assay (Enzygnost–F_{1+2}; Dade Behring, Marburg, Germany). P-selectin expression on perfused platelets, as an indicator of activation of platelets, was determined in whole blood (nonanticoagulated blood). Blood was collected in paraformaldehyde (4%) by flow cytometry analysis by using an R-phycoerythrin–conjugated monoclonal antibody according to the instructions of the manufacturer (Dako, Glostrup, Denmark; code R7200).

Scanning electron microscopy. The deposition of platelets and fibrin onto the grafts was visualized by scanning electron microscopy. The distal end of the graft was cut into small pieces (5 × 5 mm), fixed in 2% glutaraldehyde, and then dehydrated through increasing concentrations of ethanol (80%-100%). The samples were dried with the use of hexamethyldisilazane. Next, ePTFE pieces were sputter-coated with a thin layer of platinum/palladium and

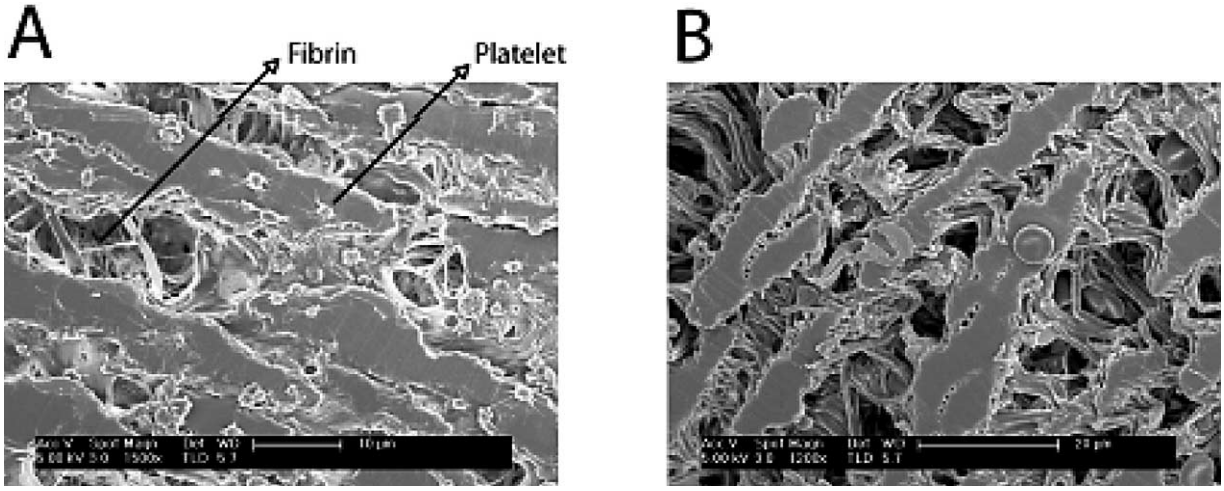

Fig 2. Scanning electron microscopy showing platelet adhesion and fibrin deposition (arrows) on the untreated Gore-Tex expanded polytetrafluoroethylene vascular graft perfused for 6 minutes with nonanticoagulated whole blood **(A)** and no such adhesion or deposition on the Gore-Tex graft treated with Carmeda BioActive Surface technology **(B).** Representative pictures of a single volunteer are shown.

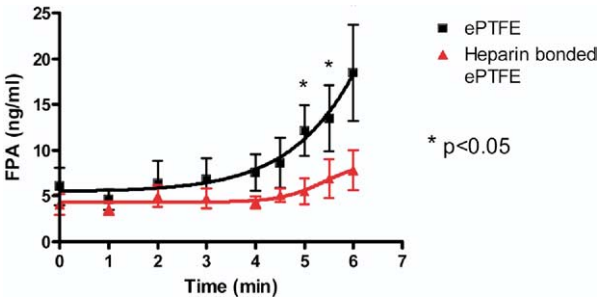

Fig 3. Heparin immobilization reduces fibrin formation during ex vivo perfusions. Perfusions were performed in 10 volunteers by using both a noncoated and heparin-bonded graft in each volunteer. Serial samples were taken during perfusion, in which levels of fibrinopeptide A *(FPA)* were measured by enzyme-linked immunosorbent assay. Error bars indicate SEM. *ePTFE,* Expanded polytetrafluoroethylene.

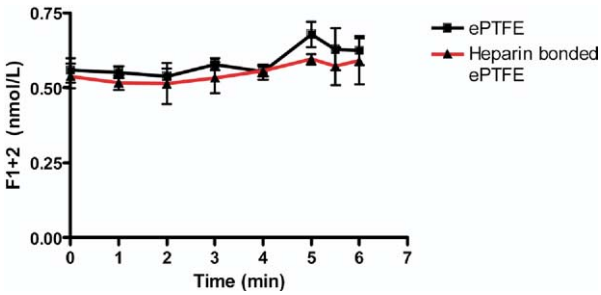

Fig 4. Progression of prothrombin fragment 1 + 2 *(F_{1+2})* levels in the perfusate over time. There was no increase in F_{1+2} in the perfusate over time on either type of vascular graft, thus indicating that no measurable amount of thrombin was generated. Error bars indicate SEM. *ePTFE,* Expanded polytetrafluoroethylene.

analyzed with scanning electron microscopy (Philips XL30; Eindhoven, The Netherlands).

Statistical analysis. Results are expressed as the mean ± SEM. A paired *t* test was used to determine the significance of differences between groups. A *P* value of $<.05$ was considered significant.

RESULTS

In our newly developed ex vivo model, nonanticoagulated blood was drawn directly from the antecubital veins over either standard Gore-Tex ePTFE vascular grafts or grafts to which heparin was covalently bonded by means of CBAS technology. All 10 perfusions were performed without any complications, concerning the volunteers as well as the setup itself.

On standard grafts, platelet deposition and fibrin formation were observed by scanning electron microscopy, whereas on heparinized grafts, no platelets or fibrin deposits were observed (Fig 2). On standard grafts, a progressive increase in the production of FPA was observed in time. FPA production on the heparinized grafts was substantially depressed compared with the standard grafts. A significant difference in FPA values between standard and heparinized grafts was observed at 5 minutes and later (Fig 3).

No increase in F_{1+2} levels in time was observed on either graft (Fig 4) nor was an increase of P-selectin expression in time noted on platelets in the perfusate of either graft (Fig 5).

DISCUSSION

In this study, we evaluated in a novel ex vivo setup the thrombogenicity of small-diameter (3-mm) ePTFE grafts treated with CBAS technology compared with standard

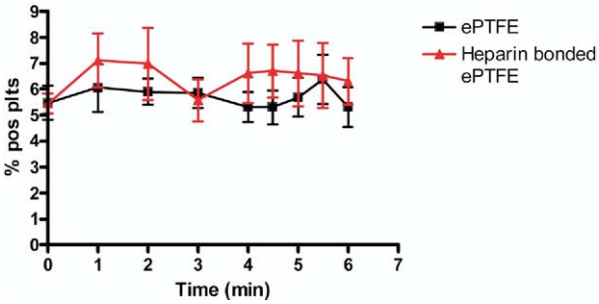

Fig 5. Progression of the percentage of P-selectin–positive platelets *(pos plts)* in the perfusate over time. P-selectin expression on the perfused platelets did not increase over time on either graft surface, thus indicating that no measurable number of platelets was activated. Error bars indicate SEM. *ePTFE,* Expanded polytetrafluoroethylene.

Gore-Tex ePTFE vascular grafts. The advantage of this model, in contrast with other ex vivo models, is that the blood of the volunteer does not come into contact with surfaces other than the materials investigated. In this setup, every volunteer was his or her own control.

We assessed the standard untreated ePTFE always in the first run. We hypothesized that if there was any in vivo activation of the coagulation cascade as a consequence of the procedure after the first run, the outcome of the second run might be influenced. Therefore the condition in which inhibition of thrombogenicity was anticipated was always performed last. However, we did not observe any differences in baseline F_{1+2} and FPA levels in the first and second run, indicating that systemic coagulation activation by the procedure is unlikely. Moreover, we did observe a substantially decreased production of FPA by heparinized grafts in the second run, indeed indicating that thrombogenicity of heparinized grafts is reduced. If an unmeasurable activation of hemostasis was present after the first run, the results of this study might even be underestimated.

We found a substantial reduction in thrombogenicity of the ePTFE grafts as a result of the heparin bonding. Platelet adhesion and fibrin deposition were observed only on the surface of the standard grafts. Moreover, a significant reduction in FPA production was observed on heparinized grafts when compared with standard grafts. There was no increase in F_{1+2} in the perfusate over time on both types of vascular grafts, thus indicating that no measurable amount of thrombin was generated. P-selectin expression on the perfused platelets did not increase over time on either graft surface, thus indicating that no measurable amount of platelets was activated.

The discrepancy between the increase in FPA levels and the absence of significant F_{1+2} production can be explained by the fact that one single thrombin molecule cleaves many fibrinogen molecules into fibrin. Apparently, the amount of thrombin generated is too small to be detected by the F_{1+2} assay used.

In a recently published study by Keuren et al.,[8] comparing heparinized and nonheparinized collagen surfaces, it was noted that thrombin activity became detectable after 12 minutes, and peaked after 20 minutes.[8] In contrast with our study, these experiments were performed with recalcified anticoagulated blood. The time course of coagulation in this more or less artificial system will be different from the time course in vivo or in our ex vivo experiments. Although detectable thrombin generation might have occurred with longer perfusion times, this was not feasible due to the substantial amounts of blood that were used in this experiment (2 times 120 mL of blood was drawn). The clinical extrapolation of our results might therfore be limited to the mechanism of action by which the heparinized graft reduces acute graft thrombosis, although the mechanism by which delayed thrombosis is accomplished is presumably highly similar.

The fact that P-selectin expression did not increase over time on either graft surface may be explained by the limited perfusion time in this model. During the 6 minutes of perfusion, only a small percentage of platelets became activated as a result of the platelet-activating properties of ePTFE. These activated platelets are able to adhere to the graft surface, as shown by scanning electron microscopy. It is conceivable that the platelets that are activated by ePTFE also adhere to the graft; this would explain why no activated platelets were found in the blood collected at the distal end of the graft.

Two potential mechanisms may be responsible for the reduced thrombogenicity of the heparinized grafts. First, the bonded heparin reduces the formation of thrombin because of the inhibition of coagulation. As a result, both fibrin formation and thrombin-mediated platelet activation are diminished. Second, the negatively charged surface directly prevents platelet interaction with the graft because of electrostatic repulsion, because the net charge of the platelet is also negative.[9] The phenomenon of reduction of platelet adhesion by using covalent heparin immobilization has not been shown in humans before.

Because the patency of small-diameter ePTFE grafts for vascular surgery is impaired by graft occlusion due to acute thrombosis, the application of the CBAS technology to vascular grafts may reduce this acute problem. This study is the first to prove that thrombogenicity is reduced in a human ex vivo model using the CBAS technology on Gore-Tex ePTFE vascular grafts. In concordance with our results, a recent study on small-caliber CBAS heparin-coated ePTFE grafts showed significantly reduced platelet deposition in arterial grafts in baboons.[5] In addition, a reduction of anastomotic neointimal hyperplasia and cell proliferation, without measurable side effects, was observed. A recently published prospective randomized trial described patients with femoropopliteal bypass by using a different kind of heparin-bonding technique on vascular grafts. The authors showed significantly increased patency rates after 3 years of follow-up and a reduced incidence of major limb amputation compared with non–heparin-bonded vascular grafts.[7]

In conclusion, heparin bonding reduces the thrombogenicity of ePTFE grafts. Here we provide evidence that the mechanism of action of the heparin bonding is due not only to anticoagulant but also to antiplatelet effects. Heparin bonding

may be an important improvement of ePTFE, resulting in better patency rates for vascular reconstructions.

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Overall responsibility: J.M.M.H

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