Quantification of platelet retention in aortocoronary femoral vein bypass graft in dogs treated with dipyridamole and aspirin

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ABSTRACT  Autologous femoral vein segments were implanted as aortocoronary bypass grafts in 50 dogs, 25 of which were treated with dipyridamole and aspirin to inhibit platelet deposition and 25 that were not. Autologous platelets labeled with indium-111 were injected into some dogs 48 hr before they were killed on the first day after surgery; other dogs were injected 24 hr before they were killed (3, 7, 30, and 90 days after surgery). Radioactivity on the grafts and on control specimens of contralateral femoral veins was converted to quantification of platelets adhering to the vessel wall (platelets/cm²). The treated group had fewer graft platelets per square centimeter than the untreated group on postoperative days 3, 7 (p < .01), and 30 (p < .05). Graft and control vein platelets per square centimeter were nearly equal by day 90. Comparison of graft and control specimens by scanning electron microscopy revealed deendothelialization at 1 and 7 days after grafting and reendothelialization at 30 and 90 days. The data suggest that indefinite prolongation of therapy to inhibit platelet deposition after bypass grafting may be unnecessary (although other atherosclerotic vessels may benefit from therapy).


A MAJOR LIMITATION of aortocoronary bypass with autologous saphenous vein grafts is the high frequency of graft occlusion by platelet thrombosis in the early postoperative phase and by proliferation of intimal smooth-muscle cells in the late phase. All vein grafts develop some morphologic changes with time,1-11 but the degree and functional significance of the changes may differ because of several factors — loss of endothelial cells during harvesting and implantation of the graft, deposition of platelets or fibrin, graft wall ischemia, intraluminal pressure, vortexting and shear stress at distal anastomoses and vein valves,7 and functional alteration and repair of damaged endothelium.3,4,9 During harvesting and implantation of femoral vein grafts in dogs, the grafts become partially deendothelialized (as shown by scanning electron microscopic studies); consequently graft permeability and cholesterol uptake increase.10,11

We have demonstrated previously, with use of autologous platelets labeled with indium-111 (111In), that treatment with dipyridamole and aspirin in dogs reduces platelet deposition in grafted veins during the short-term phase.6 Now, with a new technique for quantifying platelets on vein implants and their anastomoses, we have evaluated the results of dipyridamole-aspirin therapy during early and late postoperative phases by comparing our observations of implants and control vein segments from treated and untreated animals.

Methods

Aortocoronary bypass grafting. With use of cardiopulmonary bypass, an autologous segment of undistended reversed femoral vein was implanted as a bypass vessel from the aorta to the left anterior descending coronary artery in 50 mongrel dogs weighing 18 to 26 kg.5 The design of the connecting graft is shown in figure 1.

Medication with dipyridamole and aspirin. The dogs were divided into two groups: 25 received no medication other than systemic antibiotics during surgery (and are called “untreated”); the other 25 were given dipyridamole, 55 mg orally, on each of the 2 days preceding surgery and then were given 55 mg of dipyridamole and 325 mg of aspirin at 1 hr after surgery and daily thereafter until they were killed.

Platelet labeling. Autologous platelets of the dogs were labeled with 111In-(tropolone)₃ according to the method of Dewanjee et al.12,13 In brief, platelet-rich plasma was separated from blood anticoagulated with acid citrate dextrose solution

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(ACD) (7:43) by centrifugation for 10 min at 180 gmax. The platelet pellet was obtained by centrifugation of platelet-rich plasma (1600 gmax for 10 min). Next, 400 to 500 μCi of 111In-(tropolone) was added to the platelet pellet suspended in 4 ml of ACD-saline. After incubation at room temperature for 20 min, unbound 111In was removed by washing with 4 ml of ACD-plasma (1600 gmax for 10 min). Residual platelet aggregates and red cells were removed by centrifugation (100 gmax for 5 min) before administration. Efficiency of platelet labeling of 80% to 90% was obtained. An aliquot of labeled platelet preparation was used for checking cell contamination.

From 300 to 400 μCi of 111In-labeled platelets was injected into each dog — at 24 hr before grafting in the dogs to be killed on postoperative day 1 (48 hr after injection) and at 24 hr before death in the dogs to be killed on later days. Five untreated dogs and five treated dogs were killed on each of five different days (1, 3, 7, 30, and 90 days) after surgery. They were heparinized (4 mg/kg) and killed with an overdose of barbiturate.

The dogs were killed in accord with the Guiding Principles in the Care and Use of Animals (American Physiological Society). All animals had been under continuous care of a veterinarian for the first 2 postoperative days, and morphine and acetaminophen had been administered as indicated by the veterinarian. His supervision and care were continued until the animals were killed.

Preparation of specimens. After the death of each animal, a 3 ml sample of its blood was collected into an EDTA tube. The graft, including the two anastomoses, was excised. Each anastomosis, with 2 to 3 mm of vessel on each side of the suture line, was removed, and the remaining control graft was divided into three segments — proximal, middle, and distal. All five segments were opened and rinsed with saline.

A 4 cm segment was taken from the femoral vein contralateral to the graft source; this control vein specimen was carefully cleaned of adventitia and was tied proximally and distally. To avoid adhesion of platelets to the abluminal side, blood in the control vein specimen was removed by a 27-gauge needle before residual blood was flushed out with heparinized saline.

Quantification of platelets in specimens

Blood. The platelet count in the blood samples was determined with a Coulter counter. The samples were weighed in a microbalance, and radioactivity per weight unit of blood was calculated.

Blood samples in duplicate were centrifuged to remove plasma, and the radioactivity (111In-cpm) in plasma was used to determine the percentage of free 111In. With that, the percentage of radioactivity bound to platelets was determined, and next the platelet-bound 111In-cpm/ml blood was determined:

\[
\text{Platelet-bound } 111\text{In-cpm} \left( \frac{\text{ml blood}}{\text{ml blood}} \right) = \frac{\text{Whole-blood } 111\text{In-cpm}}{\text{ml blood}} \times (100 - \% \text{ free } 111\text{In})
\]

Then, from 111In-cpm/ml of blood and the platelet count in EDTA blood, the number of platelets per cpm was calculated:

\[
\text{No. platelets/ml blood} = \frac{\text{No. platelets} \times 111\text{In-cpm}}{\text{111In-cpm/ml blood}}
\]

Graft and saphenous vein. Each graft and anastomotic segment and each control vein specimen was spread on linear graph paper and outlined carefully, and its area was determined by counting the number of squares. With the data then available — the area (cm²) of each segment, radioactivity (cpm) in each, and number of platelets per cpm — the number of platelets adhering to each square centimeter of surface of blood vessel and anastomosis was calculated:

\[
\text{No. platelets} \left( \frac{111\text{In-cpm in specimen}}{\text{area (cm²)}} \right) = \frac{111\text{In-cpm}}{\text{No. platelets}} \times \text{area (cm²)}
\]

The amount of free 111In in the labeled platelet preparation was in the range of 4% to 6%. The amount of free 111In in the 1 day study group was 6% to 8%; in all other study groups the level of free 111In measured at 4% to 6% 24 hr after administration.

Ratios of platelet radioactivity: graft vs control vein. For determination of thrombogenicity of grafted vein with respect to that of control femoral vein (with treatment and without), the mean 111In-cpm/mg from each of the three central graft segments was expressed as a ratio to the mean from the control vein specimens.
Examination by scanning electron microscope. To determine the status of the endothelium, graft and control vein specimens were prepared by pressure-perfusion (120 mm Hg) with HEPES-buffered glucose (4.6% at pH 7.4), HEPES-buffered silver nitrate (0.2%), and 0.1M phosphate-buffered glutaraldehyde (3%). Sections of the fixed veins were coated with gold-palladium alloy. The intimal surface was examined with a scanning electron microscope (ETEC Autoscan), and representative sections were photographed.

Statistical analyses of platelet deposition on normal and grafted vein. For the dogs of each group — treated and control — killed on 1, 3, 7, 30, and 90 days after surgery, the means and standard deviations of platelet deposition per unit area of each of the five graft segments were calculated, as were the means and standard deviations of the radioactivity ratios (each central graft segment vs control vein specimen). The differences between treated and control groups were tested for significance with two-sample t tests.14

Results

Course of platelet deposition

Platelet density. At 24 hr after the grafting (figure 2), the quantity of adherent platelets per area unit (platelets/cm²) of the five graft and anastomotic segments did not differ significantly between the untreated and treated groups (two-sample t test). At day 3, however, significant differences between the untreated and treated groups appeared in the platelets per square centimeter of each of the five segments (p < .01). Except for the distal graft segment, these differences persisted at day 7 (p < .01) and likewise at day 30 (p < .05). But by day 90 the platelets per square centimeter of the two groups were virtually equal in every segment and were lower than at day 1 by almost two orders of magnitude.

Site predilection in graft and anastomoses. At day 1, in treated and untreated groups alike, the proximal and distal anastomoses had more platelets per square centimeter than any of the three central graft segments, but the difference was not significant and it tended to decrease with time. At day 90, again in each group, the platelet density on the five graft and anastomotic segments was nearly identical.

Ratios of platelet radioactivity: graft vs control vein. The ratios of radioactivity (¹¹¹In-cpm/mg) — of central segments of graft (proximal, middle, and distal) vs control vein specimen — are presented by group, untreated in figure 3 and treated in figure 4.

Significant differences between the two groups appeared at 3 days after grafting (p < .01) but not later. Also, at day 3 within the untreated group, the radioactivity ratio of the distal segment was higher than those of the middle and proximal segments (although not significantly so), whereas within the treated group the ratios of all segments were more alike.

Response of endothelium. Examination of graft specimens by scanning electron microscope revealed deen-

FIGURE 2. Course of platelet deposition. Platelets per square centimeter on surface of graft segments and anastomoses: means of data from untreated (control) dogs and dogs treated with dipyridamole and aspirin (ASA + DP) — five of each group killed at 1, 3, 7, 30, and 90 days after grafting. (In each group of five joined points, the points, left to right, represent means from proximal anastomosis, proximal graft segment, mid-graft segment, distal graft segment, and distal anastomosis.)
dothelialized surface at 1 and 7 days after grafting (figure 5) and almost total reendothelialization and absence of platelet adhesion at 30 and 90 days (figure 6).

Discussion

Pathologic and therapeutic mechanisms. Aspirin inactivates platelet cyclooxygenase by acetylation and thus inhibits the formation of the potent aggregating agents prostaglandins G₃ and H₃ and thromboxane A₂ from arachidonic acid. Its inhibition of platelet cyclooxygenase persists about three times longer than the inhibition of endothelial cyclooxygenase. Prostacyclin synthesis by the endothelial cell is also affected by aspirin, although vessel wall cyclooxygenase is less sensitive to it. These effects are better demonstrated at 3 days than at 1 day after bypass grafting.

Furthermore, prostacyclin production in the vascular endothelium is compromised by atherosclerosis and by the partial deendothelialization of the edematous vessel wall. Dipyridamole is an inhibitor of cyclic AMP phosphodiesterase and thus increases cyclic AMP in platelets. Drugs that increase cyclic AMP in platelets also suppress platelet activation. Physiologically, its potentiation of the inhibitory effects of prostacyclin may be the most important action of dipyridamole. In addition, vasodilation by dipyridamole may reduce endothelial sloughing during the harvesting of vein grafts.

Study methods. Although increased platelet deposition at the anastomotic sites had been demonstrated previously by light microscopic and electron microscopic techniques, investigation had been limited by lack of a suitable technique for a calculation of the number of platelets per unit of surface area. We have recently quantified platelet lysis and platelet oxygenator consumption during cardiopulmonary bypass in the dog. Approximately 20% of the platelets are consumed in the bubble oxygenator, and another 10% to 18% are consumed in repair of blood vessels. Therefore, for the 1 day study reported here, the ¹¹¹In-labeled platelets were administered 24 hr before surgery so that they would undergo the same trauma as unlabeled platelets during open heart surgery.

We have reported noninvasive imaging of platelet deposition in the short-term phase. Since the number of adherent platelets decreases severalfold within a short period of grafting, this imaging technique may not be applicable in the long-term phase without appropriate background subtraction. We did not use the subtraction technique in this study.

Our recently developed process has been applied for evaluation of drugs intended to inhibit platelet deposition in bilateral femoral vein and graft (Gore-Tex) implants in dogs. Electron microscopic techniques are useful in demonstrating deendothelialization and the adherence of single and multiple platelets to deendothelialized surface, but pressure-perfusion, fixation, and other manipulations involved in preparing specimens for such examination lead to loss of surface-adherent platelets and underestimation of platelet thrombosis. The radioactivity-measurement technique is useful for studying platelet thrombosis in the arterial system as well as for evaluation of platelet-inhibitor drugs in models of bilateral femoral graft implants.

Because thrombosis is a surface reaction, we think our measurements of platelet thrombosis should be expressed as the number of adherent platelets per unit of surface area. Such expression has been useful in both of our studies of platelet thrombosis in various cardiovascular prostheses and vascular grafts and of platelet inhibitors, which have been carried out in large numbers of animals and 200 human subjects. In this study, it enabled us to compare radioactivity in the

![Figure 4: Serial radioactivity ratios from dogs treated with dipyridamole and aspirin. (GFV and CFV represent grafted and control femoral vein, respectively.)](http://circ.ahajournals.org/content/v1/2/353.full.pdf)
With regard to the amount of platelet deposition we found on graft sections at 1 day after grafting, the insignificant difference between the control and treated groups of dogs might be due to platelet trauma during cardiopulmonary bypass and contamination of the local adventitial surface by blood oozing from the anastomosis.

Clinical implications of the findings. In management of patients who have undergone aortocoronary bypass grafting, it is essential to prevent thrombotic occlusion of the graft without inducing excessive postoperative bleeding. Side effects of, variability of intolerance to, and compliance of patients to drug therapy complicate decisions as to how long the treatment should be continued — drug cost also is a factor. Although the data obtained from our studies suggest that a relatively short period of therapy is necessary, the studies are limited by the use of almost healthy dogs. Their healthy coronary arteries and healthy veins might benefit less from prolonged inhibition of platelet deposition than do the diseased veins and coronary arteries of man; hence, these results may not be directly applicable to aortocoronary bypass grafting in human patients.

In man, an atherogenic diet, lipoprotein profile, rate of reendothelialization, or recurrent endothelial damage may justify use of these drugs over a long period. When the small lumen at the distal anastomoses is considered, the decision not to treat may be the riskier choice. Nevertheless, this study of the course of platelet deposition during the early and late periods after grafting with that in control vein specimens (figures 3 and 4), which could not be done accurately without normalization because of variation from animal to animal with respect to amount of injected radioactivity, blood volume, and platelet count. Since all the data in the control and treated groups were obtained and analyzed in the same way, they must all be affected in the same way, without lessening the validity of comparisons.

Although labeled platelets do not aggregate to the same extent as undisturbed platelets, we believe that the labeling causes only partial degranulation, rather than irreversible damage, and that they recuperate within 24 hr after administration and participate in the thrombotic process. The small number of platelet aggregates that formed were removed by centrifugation before intravenous administration. On biodistribution, performed in most of our experimental animals, less than 1.5% of total In-labeled platelets were found in the lung tissue. Furthermore, we have recently measured platelet adhesion to various synthetic graft materials by techniques using the scanning electron microscope and 111In-labeled platelets.23 The order of platelet adhesion to Dacron and Gore-Tex (Teflon) grafts and other reference materials is similar except that the tracer technique is five to 100 times more sensitive. Since platelet thrombosis is a surface reaction, we believe this interaction could be best represented by the number of adherent platelets per unit surface.
grafting in untreated and treated dogs indicates that significant platelet deposition occurs within a few days after aortocoronary bypass surgery. It is essential that inhibitors of platelet adhesion be present in this critical period. Such treatment may not be necessary for longer than 30 days after aortocoronary bypass grafting, since — at least in dogs — the graft reendothelializes, as demonstrated by our scanning electron microscopic studies. The advantage of the tracer technique is that it estimates total platelet deposition over a wider surface area, whereas the electron microscope reveals only the superficial layer of platelets on a smaller selected area and underestimates the total number of platelets adhering. As the graft reendothelializes, both platelet adhesion and graft permeability to blood-borne molecules decrease and, hence, the likelihood of thrombosis and
atherosclerosis decreases. Consequently, we suggest that inhibition of platelet deposition is indeed beneficial during the intraoperative and the immediate postoperative period. As reendothelialization takes place, the need for antiplatelet therapy wanes. At the time of complete reendothelialization, it should be possible to discontinue administration of antiplatelet drugs without consequent graft occlusion.

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