Interaction of Platelets and Fibrin with Injured Rabbit Aortic Neointima

Effect of Prostaglandin I\textsubscript{2} and Heparin

Burt Adelman, Michael B. Stemerman, and Robert I. Handin

Following aortic balloon deendothelialization, rabbits develop a neointima composed of smooth muscle cells and extracellular connective tissue. Injury of this neointima with a balloon catheter results in the accumulation of platelet aggregates and fibrin on the vessel surface. We studied platelet attachment and secretion following injury of the neointima and also the effect of prostaglandin I\textsubscript{2} (PGI\textsubscript{2}) and heparin on these events. Platelet factor 4 was detected within the neointima by indirect immunofluorescence 30 minutes after neointimal injury. By using \textsuperscript{51}Cr-labeled platelets, it was possible to quantify total platelet attachment following neointimal injury. When animals were sacrificed 30 minutes after reinjury, there were 4.46 \times 10^6 platelets/cm\textsuperscript{2} of aortic surface in animals injured 10 days after initial balloon deendothelialization, and 3.75 \times 10^6 platelets/cm\textsuperscript{2} of aortic surface in animals injured 29 days after initial injury. In these two groups, infusion of 850 ng/kg/min PGI\textsubscript{2}, along with a single infusion of 2500 units of heparin, inhibited fibrin deposition and reduced platelet attachment by 71% and 76%, respectively. Although infusion of heparin alone prevented fibrin deposition, neither heparin nor PGI\textsubscript{2} individually reduced platelet attachment as profoundly as did their combined use.


Platelets are important both in the pathogenesis of vascular disease and in subsequent ischemic complications.\textsuperscript{1-3} On the basis of these observations, clinical trials of anticoagulant and antiplatelet drugs have been undertaken to prevent thrombus formation and to retard the development of atheroma. Unfortunately, the results of these clinical trials have been disappointing with few successful outcomes.\textsuperscript{4-6} This lack of success may be due to the inability of existing drugs to sufficiently suppress platelet reactivity or to the advanced nature of the vascular lesions encountered in symptomatic patients. In addition, because human studies are focused on clinical events rather than on actual changes occurring on the surface of atherosclerotic blood vessels, these trials do not contribute to a better understanding of the pathobiology of atherosclerosis. In contrast, experimental animal models permit careful study of the pathogenesis of atherosclerosis and facilitate the study of specific therapeutic interventions. Most experimental techniques use physical or biochemical means to induce vascular injury.\textsuperscript{7} The interaction of blood components with the subendothelium then initiates smooth muscle cell proliferation and intimal thickening. In most models, this initial injury is exacerbated by repeated injury or concurrent dietary modification.\textsuperscript{8}

In previous studies, we have used a rabbit model of vascular disease in which the aortic endothelium is removed with a balloon catheter because the sequence of platelet and vascular responses that follow injury have been particularly well described.\textsuperscript{9} By 30 minutes after deendothelialization, the aorta was covered by a layer of adherent platelets\textsuperscript{10} that presumably released the platelet-derived growth factor (PDGF). This released protein may initiate the proliferation of smooth muscle cells that produce a multilayered myointima composed of smooth muscle cells and extracellular matrix.\textsuperscript{5,8,11,12} The entry of platelet proteins into the vessel wall has been confirmed directly by studies showing the movement of platelet factor 4 (PF-4), another \(\alpha\)-granule protein,
into the vessel wall following endothelial removal. In addition, we have previously shown that infusion of 850 ng/kg/min of prostaglandin I₂ (PGI₂), which completely inhibits platelet aggregation ex vivo, prevents platelet adhesion to exposed subendothelium and secretion of PF-4 into the underlying vessel wall. In contrast, aspirin and other nonsteroidal antiplatelet agents, which only block secondary platelet aggregation and release, do not inhibit adhesion.

A second balloon catheter injury, which damages the neointima, results in a more complicated hemostatic reaction characterized by nonhomogeneous platelet attachment and fibrin deposition, with large platelet aggregates suspended in a fibrin net. In electron micrographs of the reinjured neointima, Stemerman demonstrated that those platelets closest to the vessel conformed to the highly irregular exposed neointimal connective tissue. Fibrin strands were intermixed with the platelet thrombi and were often seen to be interposed between the exposed neointimal connective tissue and adhering platelets. In addition, fibrin strands were seen beneath the exposed neointimal surface, penetrating as much as 1 μ into the connective tissue. These findings are never seen in vessels of animals injured only once.

In the present study, we have investigated the effect of PGI₂ and heparin on platelet adhesion and fibrin formation following injury of rabbit aortic neointima. We report that use of either agent alone has little effect but that combined use of these drugs almost completely suppresses platelet and fibrin deposition. This complex response to injury closely mimics events that occur on the surface of atherosclerotic vessels in human disease. Our data suggest that interruption of both platelet activity and fibrin formation may be necessary to prevent further proliferation of smooth muscle cells and the development of thrombotic complications.

Methods

Materials

Male New Zealand white rabbits weighing 2.8 to 3.2 kg were obtained from ARI Breeding Laboratories, Avon, Massachusetts, and allowed free access to rabbit chow and water. PGI₂ was kindly supplied by John Pike of the Upjohn Company, Kalamazoo, Michigan. Unfractionated heparin was obtained from Abbott Laboratories, North Chicago, Illinois. Adenosine diphosphate (ADP) was obtained from Sigma Chemicals, St. Louis, Missouri and acid-soluble collagen from Hormon-Chemie, Munich, Germany. Fluorescein-conjugated rabbit antisheep IgG was obtained from Cappel Laboratories, Cochranville, Pennsylvania. The 51Cr used for labeling platelets was obtained from New England Nuclear, Boston, Massachusetts. All other chemicals used were reagent grade obtained from standard distributors.

Platelet Studies

Platelet counts on platelet-rich plasma (PRP) were performed on a Coulter Counter Model F (Coulter, Hialeah, Florida). When platelet counts were performed on whole blood, the technique outlined by Brecher and Cronkite was followed. Platelet aggregation studies were performed on PRP obtained from whole blood drawn into 1/10 volume 3.8% sodium citrate and centrifuged at 300 x g at room temperature for 10 minutes, using a standard nephelometric technique. Aliquots of PRP were warmed to 37°C and stirred at 800 rpm in a Chronolog dual-channel aggregometer (Chronolog Corporation, Broomall, Pennsylvania). The final concentration of ADP was 2.9 μM and of collagen 12.5 μg/ml.

Balloon Deendothelialization

Rabbits were anesthetized with 6 mg/kg intravenous sodium pentobarbital and 5 mg intravenous diazepam supplemented with ether. The entire aorta of each animal was denuded of endothelium according to a modification of the Baumgartner balloon deendothelialization method. In rabbits to be reballoononed at a later date, the femoral arteriotomy site was closed and the animals returned to their cages. Balloon injury was repeated 10 days, 29 days, and 50 days after initial deendothelialization. The second ballooning was performed in the same manner as the first except that the opposite femoral artery was exposed.

Morphologic Techniques

Immunofluorescent staining for the detection of PF-4 antigen was performed on aortic sections obtained 30 minutes after neointimal injury in animals subjected to balloon deendothelialization 10, 29, and 50 days previously. The immunofluorescent technique used for the detection of PF-4 antigen in aortic sections has been described in detail elsewhere. Briefly, 4-μ sections of frozen, nonfixed rabbit aorta were mounted on glass slides and fixed in cold acetone. Sections were incubated with sheep anti-rabbit PF-4 IgG for 30 minutes and then washed in phosphate-buffered saline. A second incubation was then performed using fluorescein-labeled rabbit anti-sheep IgG for 30 minutes. The sections were again washed and then coverslipped for viewing with a Zeiss II epifluorescent microscope.

Animals from which specimens were to be examined by transmission electron microscopy and light microscopy were perfused at 100 mm Hg with 0.1 M cacodylate-buffered 2.5% glutaraldehyde, pH 7.4, through a 16-gauge catheter placed in the carotid artery. The further processing of the tissue and preparation of specimens for light and electron microscopy have been previously described. For examination, standard sections were taken from each aorta at the level of the right renal artery, the celiac artery, the eighth intercostal artery, and the fourth intercostal artery.
Adhesion of 51Cr-labeled Platelets

Quantitation of platelet attachment to the vessel surface was studied in animals undergoing neointimal injury 10 and 29 days after initial balloon deendothelialization. The technique for quantitating platelet attachment to the vessel surface has been previously described. Briefly, 60 minutes before neointimal injury, 8 x 10^5 51Cr-labeled platelets were infused intravenously into each animal. Thirty minutes after injury, each animal was sacrificed by pentobarbital overdose and perfused-fixed as described above. The fixed aorta was cut into 1-cm rings of known area and the associated radioactivity for each ring determined in a Beckman Gamma 8000 gamma counter (Beckman Instruments, Menlo Park, California). Total platelet attachment per cm^2 of aortic surface was then calculated by dividing the cpm/cm^2 of the total aorta by the specific activity of the circulating platelets at the time of sacrifice (cpm/platelet).

Infusion of PGI

The individual and combined effects of PGI and heparin on platelet and fibrin attachment after neointimal injury were studied in animals injured 10 and 29 days after initial balloon deendothelialization. So that this data could be expressed in a quantitative fashion, each animal was infused with 51Cr-labeled platelets as described above.

Animals Receiving Only PGI

PGI in 0.1 M Tris buffer, pH 9.0, was infused into the jugular vein via a 16-gauge plastic catheter connected to a constant-infusion pump (Sage pump model 341, Orion Research Incorporated, Cambridge, Massachusetts). Before initiation of the PGI infusion, each animal was given an intravenous infusion of 3 mg/kg aminophylline. Five minutes after the aminophylline infusion, blood for aggregation studies was drawn from the femoral arteriotomy site and the PGI infusion begun. Each animal received 850 ng/kg/min PGI. After 30 minutes, a second arterial blood specimen was obtained for repeat aggregation studies to document the PGI effect. This dose of PGI produced 100% inhibition of platelet aggregation in response to ADP and 85% inhibition in response to collagen. Animals were balloon-injured within 15 minutes of the second aggregation study, and the PGI infusion was continued until the animals were sacrificed 30 minutes after injury.

Animals Receiving Only Heparin

In these animals, a single intravenous injection of 2500 units of heparin was given 30 minutes before balloon injury. One half-hour after neointimal injury, these animals were sacrificed. The whole blood-clotting time checked before sacrifice was always greater than 1 hour.

Animals Receiving Both PGI and Heparin

These animals were given both PGI and heparin in the manner described above. Platelet aggregation studies and the whole blood-clotting time were checked as above, and the animals were sacrificed 30 minutes after neointimal injury. Heparin infusion did not alter platelet counts. The average platelet count before heparin administration was 428,000/µl and approximately 30 minutes later was 481,000/µl. This small rise in platelet count reflects the administration of the labeled platelets.

Statistical Analysis

All data were expressed as a mean and analyzed using the Newman-Keuls test.

Results

Quantitative Analysis of Platelet Attachment Following Neointimal Injury

Quantitative platelet attachment to the injured neointimal surface was studied in rabbits balloon-deendothelialized 10 and 29 days before neointimal injury and sacrificed 30 minutes after injury. Multiple 1-µm sections from the thoracic and abdominal aortic segments of each animal confirmed the presence of a thickened neointima. As shown in Table 1, there was an average of 4.46 x 10^6 platelets/cm^2 of aortic surface in animals reinjured at 10 days and 3.75 x 10^6 platelets/cm^2 in animals reinjured after 29 days.

Table 1. Effect of Heparin and Prostaglandin I on Platelet Adhesion Following Neointimal Injury

<table>
<thead>
<tr>
<th>Drug treatment</th>
<th>Attached Platelets/cm² x 10⁶</th>
</tr>
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<tbody>
<tr>
<td>A. 10-day</td>
<td>4 No drug 4.46 ± 0.95</td>
</tr>
<tr>
<td>B. 10-day</td>
<td>5 Heparin 2.70 ± 0.88</td>
</tr>
<tr>
<td>C. 10-day</td>
<td>6 PGI 2 2.50 ± 0.62</td>
</tr>
<tr>
<td>D. 10-day</td>
<td>5 Heparin + PGI 2 1.42 ± 0.28</td>
</tr>
<tr>
<td>E. 29-day</td>
<td>7 No drug 3.75 ± 1.36</td>
</tr>
<tr>
<td>F. 29-day</td>
<td>5 Heparin 2.65 ± 0.85</td>
</tr>
<tr>
<td>G. 29-day</td>
<td>6 PGI 2 4.57 ± 1.35</td>
</tr>
<tr>
<td>H. 29-day</td>
<td>5 Heparin + PGI 2 0.89 ± 0.42</td>
</tr>
<tr>
<td>29-day non-reinjured</td>
<td>5 No drug 0.29 ± 0.16</td>
</tr>
</tbody>
</table>

B vs A p < 0.005.  
C vs A p < 0.005.  
D vs A p < 0.001.  
D vs C p < 0.025.  
D vs B p < 0.05.  
F vs E not significant.  
G vs E not significant.  
H vs E p < 0.001.  
H vs G p < 0.001.  
H vs F p < 0.025.  
Values are expressed as means ± sd.
Figure 1. Platelet and fibrin adhesion following neointimal injury. A. Section taken from the aorta of an animal sacrificed 30 minutes after injury in which extensive platelet and fibrin masses are seen overlying the neointima (bar = 10 μ). B. Electron micrograph of a platelet-fibrin mass in which platelets (arrow) can be seen in close association with fibrin (arrowhead) (bar = 1 μ).
Platelet attachment was also studied in a group of animals that were sacrificed without reinjury 29 days after initial balloon deendothelialization. In these animals, platelet attachment was low, averaging $0.29 \times 10^8$ platelets/cm$^2$ of aortic surface. This level of attachment is not different from levels we have previously reported in animals with intact endothelial cells.$^{13}$

Sections from the aorta of animals reinjured 10 and 29 days after balloon deendothelialization were examined by light and transmission electron microscopy. In all animals examined, the extent of neointimal thickening correlated with the time after initial injury. After the second injury, platelet attachment along the aortic surface was heterogeneous (Figure 1).

PF-4 antigen was detected by indirect immunofluorescent staining within the vessel wall of animals from each group sacrificed 30 minutes after reinjury. Figure 2 shows a representative section from one such animal. The most intense fluorescence was noted within the neointimal portion of the vessel with little material appearing beyond the internal elastic lamina.

Effect of Heparin and PGI$_2$ on Platelet Attachment Following Neointimal Injury in the Rabbit

The effects of PGI$_2$ and heparin on platelet attachment 30 minutes following balloon injury of the rabbit aortic neointima are summarized in table 1. In the animals reinjured 10 days after the initial balloon deendothelialization, infusion of 850 ng/kg/min PGI$_2$ resulted in a 44% reduction of platelet attachment when compared to identical animals reinjured but not given PGI$_2$ ($p < 0.005$). In all animals, this dose of PGI$_2$ resulted in near total inhibition of ex vivo platelet aggregation. Administration of a single dose of 2500 units of heparin 30 minutes before balloon reinjury resulted in a 38% reduction of platelet attachment compared to the nontreated, reinjured animals ($p < 0.005$). This dose of heparin prolonged the whole blood-clotting time to over 1 hour in all animals. The combined use of these same doses of PGI$_2$ and heparin resulted in a 71% reduction of platelet attachment ($p < 0.001$) when compared to nontreated-reballooned animals.

Identical experiments were performed in animals reinjured 29 days after initial deendothelialization. In

Figure 2. Indirect immunofluorescent staining for PF-4 antigen following neointimal injury. Bright fluorescent staining is seen in the neointimal portion of this section taken from the aorta of an animal sacrificed 30 minutes after injury (bar = 100 $\mu$m).
these animals, neither heparin nor PGI$_2$ alone significantly reduced platelet attachment 30 minutes after injury. However, the combined use of both agents reduced platelet attachment by 76% ($p < 0.001$).

Microscopic examination of specimens from each of these treatment groups confirmed the attachment of platelets to the vessel wall in animals that had received either heparin or PGI$_2$ alone. Animals receiving PGI$_2$ displayed areas of fibrin formation that contained some platelet aggregates. However, animals treated with heparin had no evidence of fibrin deposition. Only the animals treated with the combination of agents had uniform reduction of attached platelets and absence of fibrin along the reinjured surface. Representative micrographs are shown in Figure 3.

Discussion

Our studies demonstrate that: 1) platelet attachment following reinjury of previously deendothelialized rabbit aortas is a heterogeneous process characterized by platelet aggregate formation and fibrin deposition over areas of neointimal fibromuscular thickening; 2) a secreted platelet α-granule protein (PF-4) enters the thickened vessel wall as it does after primary injury; and 3) combined use of PGI$_2$ and heparin is most effective in inhibiting platelet attachment to the injured neointima.

The ability of the damaged neointimal surface to initiate both platelet adhesion and fibrin formation suggests that the neointima is more thrombogenic than normal subendothelium, which only attracts a

Figure 3. Effect of PGI$_2$ and heparin on platelet and fibrin attachment to the injured neointima. A. Platelet-fibrin masses on the neointimal surface 30 minutes after injury in an animal given no drug treatment. B. Section from an animal given 850 ng/kg/min PGI$_2$; platelets and fibrin are still present. C. Section from an animal given 2500 units of heparin; here platelets are present in a monolayer along the surface, but fibrin is absent. D. Section from an animal given both PGI$_2$ and heparin; here only a rare platelet is present along the surface (bar = 10 μ).
monolayer of platelets after deendothelialization. In previous studies, we have demonstrated that infusion of 650 to 850 ng/kg/min PGI₂ to balloon-deendothelialized rabbits completely inhibited ex vivo platelet aggregation and reduced platelet attachment to the aortic subendothelial surface by 63%. In the present study, the inhibitory effect of PGI₂ and heparin on platelet attachment to the injured neointima was found to correspond to the timing of the second injury. The individual use of these agents was effective in animals reinjured 10 days after initial balloon deendothelialization. However, combined use of PGI₂ and heparin dramatically reduced platelet attachment in both groups of animals.

In all these studies, aminophylline, a phosphodiesterase inhibitor, was used to augment the effect of PGI₂. Other studies have demonstrated an enhancing effect of aminophylline on in vivo PGI₂-induced platelet inhibition when the dose of PGI₂ was below 50 ng/kg/min. The effect of phosphodiesterase inhibitors given alone on platelet attachment to the reinjured neointima is not known. However, Cazenave et al. have shown that, in vitro, the phosphodiesterase inhibitor dipyridamole can partially inhibit platelet attachment to collagen-coated surfaces.

The increased resistance of the 29-day neointima to antithrombotic agents may result from a time-dependent increase in neointimal content of the thrombogenic material or the presence of more potent thrombogenic substances. Some data already exist to support the hypothesis that the increased thrombogenicity of the neointima is related to its composition. The smooth muscle cells that make up the neointima produce extensive amounts of connective tissue. The collagen species produced by smooth muscle cells are different from those that endothelial cells produce and secrete into the native subendothelium. Subendothelial collagen is primarily Type IV and V (AB₂), whereas smooth muscle cells produce Types I and III, in addition to IV and V. Collagen Types I and III are effective platelet-aggregating agents in vitro; however, basement membrane collagen Types IV and V (AB₂) do not promote platelet aggregation as readily. Thus, the altered proteoglycan nature of the neointimal surface may result from its smooth muscle cell constituents, producing collagen types that are highly thrombogenic. Smooth muscle cells of the neointima also produce various proteoglycans that may affect platelet and fibrin activation. In addition, altered blood flow over the injured neointima may enhance the deposition of platelets and fibrin. In particular, microscopic static regions may exist along the neointimal surface in which hemostatic materials may be sequestered from the usual high flow of the aorta.

Although animals in the 29-day reinjury groups demonstrated increased resistance to the effects of antithrombotic agents, the actual number of platelets that attached to the vessel surface was not greater than that observed in the 10-day reinjury group. Factors such as blood flow and vessel wall-initiated fibrinolysis may limit the absolute size of the thrombus that forms following neointimal injury. In addition, experimental design may also account for the observed differential effects of PGI₂ and heparin in the 10- and 29-day groups. In these studies, each group of animals was handled sequentially; therefore, subtle environmental factors may have been responsible for the observed differences in platelet responsiveness to the administered drugs.

Following neointimal injury, local release of PDGF may further activate smooth muscle cell proliferation and cause further intimal thickening. At present, it is not possible to directly follow the fate of the PDGF in vivo. However, in previous studies, we have demonstrated that PF-4, a related α-granule protein, enters the vessel wall following balloon deendothelialization of the rabbit aorta. In this study, we further demonstrate that PF-4 can be detected within the neointima of reinjured animals. This observation again suggests that secreted platelet proteins, including PDGF, are available to vascular smooth muscle cells following injury.

This report provides new insights into the thrombogenic nature of the fibromuscular neointima. We have documented that this surface is highly thrombogenic when reinjured and that platelet aggregation on it results in the release of a platelet α-granule protein that penetrates the vessel wall. In addition, we have shown that only by the combined use of PGI₂ and heparin in large doses can we markedly alter the thrombogenic nature of the reinjured neointima and maximally reduce platelet deposition and fibrin formation. Because our model mimics events that might occur on human atherosclerotic plaques, our observations may explain the varied effects of antiplatelet therapy in advanced vascular disease.

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