Platelet Resistance to Prostaglandin D₂ in Patients With Myeloproliferative Disorders

By Barry Cooper, Andrew I. Schafer, David Puchalsky, and Robert I. Handin

We investigated the effect of prostaglandin D₂ (PGD₂) on platelet adenylate cyclase activity in 23 patients with myeloproliferative disorders, including eight patients with polycythemia vera, seven with myeloid metaplasia, four with chronic myelogenous leukemia, and four with essential thrombocytopenia. In 20 of these patients there was less activation of platelet adenylate cyclase at all concentrations of PGD₂ studied when compared to normal controls. In 5 of these patients we also studied the effect of PGD₂ on platelet aggregation and the release of ³¹C-serotonin. Each of these patients required ten fold higher than normal concentrations of PGD₂ to inhibit collagen-induced ³¹C-serotonin release. This diminished response was specific for PGD₂; the stimulation of adenylate cyclase by PGE₁ and PGI₂ was normal in these patients’ platelets, as was the inhibition of ³¹C-serotonin release by PGE₁. Aspirin therapy in 5 patients and anticoagulation with sodium warfarin in 1 patient did not correct this platelet abnormality. Five subjects with reactive thrombocytosis who were studied had a normal platelet adenylate cyclase response to PGD₂. Since sufficient PGD₂ is synthesized by platelets to inhibit aggregation, the resistance of these platelets to physiologic concentrations of PGD₂ could contribute to the high incidence of thrombosis in patients with myeloproliferative disorders.

While studying platelet function in several patients with myeloproliferative disorders and recurrent thrombosis, we examined the role of certain prostaglandins (PG) that inhibit platelet aggregation and the release reaction. The relationships between various PG, platelet aggregation, and adenylate cyclase are shown schematically in Fig. 1. Platelet PG endoperoxide intermediates like PGG₂, PGH₂, and the non-PG product thromboxane A₂ modulate aggregation and release and inhibit platelet adenylate cyclase. Another platelet product, PGD₂, is produced in sufficient concentrations in the platelet to activate adenylate cyclase and inhibit platelet aggregation.¹-⁴ PGI₂ (prostacyclin), a newly discovered endothelial cell product, also inhibits aggregation and stimulates adenylate cyclase.⁵-⁷ It has been suggested that PGD₂ and PGI₂ may be important feedback regulators to inhibit platelet activation in vivo. However, the precise role of prostaglandins in the modulation of platelet function is currently not known.

We report here our observations that the platelets of patients with myeloproliferative disorders have a diminished response to the biochemical and physiologic...
PLATELET RESISTANCE TO PROSTAGLANDIN D₂

Fig. 1. Schematic diagram illustrating role of PG production and cAMP levels in mediation of platelet aggregation and release. PGD₂ and PGI₃ may be feedback inhibitors of platelet aggregation via activation of adenylate cyclase. Small dotted arrow, pathway inhibiting adenylate cyclase activity. Other compounds (solid arrows) stimulate adenylate cyclase. Large dotted arrow, inhibition of aggregation; large solid arrow, stimulation of aggregation.

effects of PGD₂. This platelet abnormality offers a possible, although unproven, mechanism for the high incidence of thrombosis in these patients. Moreover, further studies of this platelet defect may help clarify the physiologic role of PGD₂ in platelet metabolism.

MATERIALS AND METHODS

Assay of adenylate cyclase. Platelet-rich plasma (PRP) was prepared from 20 ml venous blood anticoagulated with 3.5% acid citrate dextrose (ACD) (NIH Formula A). The PRP was adjusted to pH 7.5 with additional ACD, and a platelet pellet was prepared by centrifugation at 1500 × g for 10 min. The pellet was washed in buffer containing 8 mM Na₂HPO₄, 2 mM NaH₂PO₄, 10 mM EDTA, 5 mM KCl, and 135 mM NaCl, pH 7.2, and recentrifuged at 1500 × g for 10 min. The supernatant was decanted and the platelet pellet was frozen and thawed in a dry ice acetone bath and suspended in 2 ml ice-cold Tris saline (15 mM Tris-HCl, 138 mM NaCl, pH 7.6).

Enzyme activity of the platelet suspension was measured immediately after thawing by a modification of the method of Salomon et al. using ³²P-ATP as substrate and directly measuring the ³²P cyclic AMP product. Assay mixtures contained 1.5 ± 10³ dpm α-³²P-ATP (New England Nuclear, Boston, Mass.; 10 Ci/mmol); 1.0 mM ATP (Sigma Chemical, St. Louis, Mo.); 25 mM Tris-HCl pH 7.4; 5 mM MgCl₂; 2 mM cAMP (Sigma); 0.1% bovine albumin (Pentex Biochemical, Kankakee, Ill.; fraction V); 10 mM theophylline (Sigma); 1 mM EGTA; and an ATP-regenerating solution consisting of 20 mM creatine phosphate and 1 mg/ml creatine kinase (Sigma). Reactions were initiated by the addition of 20 μl platelet suspension and incubated for 10 min at 37°C at a final volume of 50 μl. Enzyme activity was linear for at least 20 min at protein concentrations up to 4 mg/ml. Data were expressed as nmol cAMP/mg protein/10 min or as the ratio of stimulated to basal activity. In each case, platelet particulate fractions from normal subjects were assayed along with particulate fractions from the patients. Determination of protein was by the method of Lowry et al.²³ Prostaglandins were a gift from Dr. John Pike (Upjohn, Kalamazoo, Mich.). Platelet counts were performed with an electronic particulate counter (Coulter Model F, Hialeah, Fla.).

Platelet aggregation. Venous blood anticoagulated with 13 mM sodium citrate was centrifuged at 160 g for 10 min and the PRP aspirated and adjusted to a count of 250,000/mm³ with platelet-poor plasma. Platelet aggregation was assessed using a standard nephelometric technique in which 0.4-mI aliquots of PRP were stirred at 37°C in a Chronolog Aggregometer (Chronolog, Broomall, Pa.). Aggregation was initiated with 6 μM freshly prepared epinephrine, 1 μM ADP, or 40 μg collagen suspension prepared from bovine Achilles tendon as described by Hovig.²⁴

Platelet serotonin release. Venous blood anticoagulated with 13 mM sodium citrate was mixed with 3.4 μM of ³²C-serotonin (2⁻¹⁵C-5-hydroxytryptamine binoxalate, 44 mCi/mmol, New England Nuclear) prior to centrifugation. Serotonin release was measured in the PRP by a modification of the method of Jerusalmi and Zucker.²⁵ Then 40 μg collagen suspension was added to 0.4 ml stirred PRP in the aggregometer as described above. Various concentrations of PG were added 30 sec prior to the addition of collagen, and the reaction was stopped after 4 min with 0.1 ml saline containing a final concentration of 17 mM EDTA in the PRP. The samples were then centrifuged at 750 g for 20 min at 4°C. Aliquot of supernatant (0.10 ml) were added to glass scintillation vials followed by 1.0 ml Protosol (New England Nuclear). After digestion overnight, 10 ml scintillation fluid made up of 4 g/liter PPO (polyphenylene oxide) and 50 mg/liter POPOP [1.4-bis-(5-phenyloxazoly) benzene] in toluene (Liquifluor, New England Nuclear) was added and vials counted using a liquid scintillation counter (Isocap 300; Searle Analytic, Des Plaines, Ill.).
Patients. Twenty-three patients with myeloproliferative diseases were studied, including eight patients with polycythemia vera, four with Ph+ chronic myelogenous leukemia, four with essential thrombocythemia, and seven with myeloid metaplasia. All consenting patients with these disorders seen at the Hematology Clinic of the Peter Bent Brigham Hospital between March 1 and September 1, 1977, were studied if they had a platelet count > 100,000/mm³ and Hb > 9 g/dl and were not taking any drugs known to interfere with platelet function. The experimental protocol was reviewed and approved by the hospital committee for the protection of human subjects.

Fourteen normal volunteers were also studied, and a normal control was included along with the patient samples. Five hospitalized patients with reactive thrombocytosis selected at random were also studied. This group included a patient with diabetic ketoacidosis, gastrointestinal hemorrhage, and pneumonia; a patient with a fever of unknown origin; a splenectomized patient with immune thrombocytopenia; a patient with diabetes mellitus and pyelonephritis; and a splenectomized patient with thalassemia intermedia.

The clinical and laboratory data on the 23 patients included in this study are summarized in Table 1. The patients were 26–84 yr old, and ten of them were receiving chemotherapy. Of the 23 patients, 8 (34%) had thrombotic episodes, 4 of these patients had unusual events including the Budd-Chiari syndrome, ulnar artery thrombosis, portal vein thrombosis, and mesenteric vein thrombosis. The other 4 had peripheral vascular or cerebrovascular occlusions. At the time of diagnosis, 15 of the 21 patients (71%) had an elevated platelet count. This incidence was similar in the patients with thrombosis, where 7 of 10 events (70%) were associated with an elevated platelet count. At the time of our platelet studies 50% of the patients still had thrombocytosis.

RESULTS

Adenylate cyclase activity. Figure 2 compares the stimulation of adenylate cyclase by PGD₂, PGE₁, and PGI₂ in normal platelet particulate fractions to the

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stimulation obtained with the 23 patients with myeloproliferative disorders. Although PGE₂ is not synthesized in platelets and probably plays no physiologic role in platelet metabolism, this PG was evaluated because of its known ability to stimulate platelet adenylate cyclase. The dose-response curves show decreased stimulation of the patients' adenylate cyclase at all concentrations of PGD₂. In the presence of $10^{-7} \text{M}$ PGD₂ there was a 6.6-fold enzyme activation in normal platelets compared to 2.7-fold enhancement in those of patients ($p < 0.001$). At the highest concentration of PGD₂ tested ($10^{-5} \text{M}$) the PG activated adenylate cyclase 18-fold in controls compared to only 9.3-fold in the patients ($p < 0.001$).

In contrast to the decreased stimulation observed with PGD₂, enzyme stimulation by PGE₁ was normal in platelets from the 23 patients at all doses of the prostaglandin studied (Fig. 2B).

We also evaluated the effect of PGI₂ on adenylate cyclase activity in the last five patients studied. As shown in Fig. 2C, adenylate cyclase stimulation by PGI₂ was normal. In addition, NaF stimulated mean adenylate cyclase equally in the patients [$8.4 \pm 0.6$ (SEM) times baseline levels, $n = 23$] and controls [$7.0 \pm 0.6$ times, $n = 14$]. There was also no significant difference in basal enzyme activity in platelets from patients and normal subjects ($0.1 \text{nmol cAMP/mg protein/10 min}$).

The results in the 23 patients studied were analyzed according to the clinical type of myeloproliferative disease (Fig. 3). Platelets from all but four patients had subnormal adenylate cyclase activation with PGD₂. Eight patients with polycythemia vera were studied, including two whose disease had evolved into myeloid metaplasia; seven were clearly abnormal. All four patients studied with chronic myelogenous leukemia had this enzyme resistance, as did all patients with essential thrombocythemia. These studies were repeated 3-6 mo later in six patients; the diminished response to PGD₂ persisted.
Four of seven patients with myeloid metaplasia also had the defect described, while three of the patients were identical to controls at all doses of PGD₂ studied. These three patients (nos. 17, 21, 22) had platelet counts of 215,000–915,000/mm³ and had had their disease 1–13 yr. It is of interest that none of these three patients had a prior history of thrombosis, while two of the other four patients who had the defect had recurrent thrombotic complications (nos. 18 and 19).

In order to determine whether this platelet abnormality was due simply to an elevated platelet count or to chronic illness, the platelets of 5 hospitalized patients with reactive thrombocytosis were studied. As shown in Fig. 3, platelets from each of these patients had a normal response to PGD₂. In addition, 8 of the 20 patients studied with this diminished response had normal platelet counts, and 1 patient who had a normal adenylate cyclase response (no. 17) had a platelet count of 920,000/mm³. This platelet enzyme defect therefore seems not to be related solely to quantitative platelet abnormalities.

**Platelet aggregation and ¹⁴C-serotonin release.** In view of the diminished responses of platelet adenylate cyclase to PGD₂, we also examined the effect of prostaglandins on intact platelets on the first five patients studied. Each of these patients had normal platelet aggregation patterns in response to 6 μM epinephrine, 40 μg collagen, and 1 μM ADP. When platelets from these five
patients were incubated with PGD$_2$ and then challenged with 40 $\mu$g collagen, higher than normal concentrations were needed to block release of $^{14}$C-serotonin, (Fig. 4A). Serotonin release decreased from 50% to 13% when $5 \times 10^{-6}$ M PGD$_2$ was added to normal PRP. Comparable inhibition required the addition of $5 \times 10^{-7}$ M PGD$_2$ to PRP from our patients. At $10^{-7}$ M PGD$_2$, serotonin release was only 8% in normal PRP, while 42% was released from the patients’ PRP ($p < 0.01$). As shown in Fig. 4B, higher doses of PGE$_1$ were needed to prevent collagen-induced release of serotonin in normal PRP. However, the doses of PGE$_1$ required for inhibition were identical for our patients, indicating that the resistance is specific for PGD$_2$ and not a generalized loss of response to prostaglandins.

Effect of antithrombotic therapy. Five patients were given aspirin (600 mg daily) for 3-4 wk and one patient was subsequently administered sodium warfarin by his physician (A.I.S.) as therapy for peripheral vascular disease with gangrenous toes. In each case, the diminished enzyme sensitivity to PGD$_2$ persisted. As shown in Fig. 5, the patient with polycythemia vera (no. 8) was restudied after 1 and 2 wk of aspirin therapy and again after 3 wk of sodium warfarin, when his prothrombin time was in the therapeutic range. In the presence of $10^{-6}$ M PGD$_2$, platelet adenylate cyclase activity remained 60% of control enzyme activity in spite of the administration of either drug.

DISCUSSION

These studies provide evidence that there is a defect in platelets of patients with myeloproliferative disorders characterized by a diminished response of platelet adenylate cyclase to PGD$_2$. This biochemical defect was accompanied by resistance of intact platelets to PGD$_2$; a tenfold higher concentration of this prostaglandin was required to prevent collagen-induced release of $^{14}$C-serotonin. Since these platelets contain a normal quantity of adenylate cyclase, as indicated by normal basal and fluoride-stimulated enzyme activity, and responded normally to PGE$_1$ and PGI$_2$, our data suggest that the platelets produced by patients with myeloproliferative disorders may have lost specific “binding sites” for PGD$_2$. Recent data have indicated separate receptors on the platelet for PGI$_1$ and PGD$_2$, and using radioligand binding techniques we recently showed that PGE$_1$ and PGI$_2$ bind to a common platelet receptor distinct from PGD$_2$. Our
data on the patients with myeloproliferative disorders also provide indirect evidence supporting the presence of separate receptors for PGD₂ and the other prostaglandins studied (PGE₁, PGI₂).

The platelet abnormality we observed appears to represent an intrinsic membrane abnormality characteristic of all of the myeloproliferative disorders. Changes in platelet membrane glycoproteins have also been recently reported in these patients. All 16 of the patients we studied who had polycythemia vera, chronic myelogenous leukemia, or essential thrombocythemia had the enzyme defect. In addition, 4 of 7 individuals with myeloid metaplasia (a more heterogeneous group of patients) also had the defect. The finding that a diminished platelet response to PGD₂ could occur in several types of myeloproliferative syndromes fits with observations that there may be a common origin for these closely related hematologic disorders involving the stem cell for platelet, erythroid, and myeloid elements.

There have been numerous studies of platelet function in patients with myeloproliferative disorders, since up to 30% of these patients will have a major thromboembolic event. Reported platelet abnormalities include changes in platelet number, presence of circulating platelet aggregates, and abnormalities in platelet morphology and granule contents as well as defective aggregation, release, and lipid peroxidation. Decreased platelet aggregation in response to thrombin, ADP, epinephrine, and collagen has been commonly reported. Berger et al. found some abnormality in aggregation in 81% of 47 cases of polycythemia vera. Impaired aggregation in response to either thrombin, ADP, epinephrine, or collagen was the most common defect. Zucker and Mielke were able to define two groups of patients with myeloproliferative diseases; those patients with recurrent thrombosis or bleeding all had abnormal aggregation, while only one of the patients with no history of thrombosis or bleeding had a platelet abnormality. While the defects in platelet function described to date could explain the bleeding tendency in some patients with myeloproliferative disease, they do not explain the high incidence of thrombosis that should be associated with “hyperfunctioning” platelets. We studied platelet aggregation in five of our patients with significant thrombotic complications and found normal aggregation responses to collagen, epinephrine, and ADP in each case, again suggesting these kinds of abnormalities may not always accompany thrombosis.

If platelet abnormalities play a role in the pathogenesis of thrombosis in these patients, the most likely mechanism is one that increases platelet reactivity. As an example, increased platelet reactivity has been recently described in patients with type II hyperlipoproteinemia and in some patients with diabetes mellitus, two diseases with an increased incidence of thrombosis. Halushka et al. recently reported increased synthesis of PGE-like material by platelets of diabetic patients and suggested that increased oxidation of arachidonic acid may account for the lower threshold of these platelets to aggregating agents. We would like to suggest that the diminished sensitivity of platelet adenylate cyclase to PGD₂ provides a possible, but as yet unproven, mechanism for the high incidence of thrombosis noted in the patients we studied. Sufficient PGD₂ is synthesized by platelets during aggregation to potentially inhibit platelet aggregation and release. In fact, several authors have suggested that PGD₂ might function in this way as a feedback regulator to limit platelet aggregation. The importance of physiologic inhibitors of
the coagulation system has been emphasized by the association of thrombosis with a decrease in plasma antithrombin concentration. A decrease in this soluble inhibitor of several activated coagulation proteins permits excessive activation of coagulation proteins to occur. In a similar fashion, a diminished response to PGD2 might permit unregulated platelet aggregation to occur in patients with myeloproliferative disorders.

Evaluation of our patient population did not allow us to show a statistical correlation between thrombosis and a diminished enzyme response to PGD2. Eight of our patients did have recurrent episodes of thrombosis, including cerebrovascular accidents, the Budd-Chiari syndrome, ulnar artery thrombosis, and mesenteric vein thrombosis, and each of these patients had this enzymatic defect. However, 11 of 15 patients without a prior history of thrombosis also had this abnormality. Nevertheless, this group of patients had an incidence of thromboembolism high enough to warrant careful followup to clarify the association between this platelet abnormality and thrombosis.

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REFERENCES

18. Bolin RB, Okumara T, Jamieson GA: Changes in distribution of platelet membrane


Platelet resistance to prostaglandin D2 in patients with myeloproliferative disorders

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