Diminished Platelet Adenylate Cyclase Activation by Prostaglandin D₂ in Acute Thrombosis

By Barry Cooper

Prostaglandin D₂ (PGD₂) produced by platelets can inhibit aggregation via activation of platelet adenylate cyclase. PGD₂ activation of adenylate cyclase in platelet membrane fractions was studied in 20 consecutive patients hospitalized with acute deep-vein thrombosis and/or pulmonary embolism. In nine patients, PGD₂-stimulated enzyme activity was decreased at all concentrations of PGD₂ studied. This altered enzyme sensitivity was specific for PGD₂ as basal enzyme activity, and prostaglandin E₁, prostaglandin I₂, and sodium fluoride stimulated adenylate cyclase was normal. The effect of PGD₂ on platelet aggregation and ¹⁴C-serotonin release was also studied in one patient where a four-fold higher concentration of PGD₂ was required to inhibit collagen-induced ¹⁴C-serotonin release. Binding studies using [³H]PGD₂ as a radioligand indicated that this patient's platelets bound 10 fmol PGD₂/10⁶ platelets compared to 30 fmol/10⁶ platelets in a normal control. Five patients had follow-up studies between 2 and 7 mo after their acute thrombotic event, and PGD₂-stimulated adenylate cyclase activity returned towards normal in four. Since PGD₂ is synthesized in platelets at concentrations sufficient to inhibit aggregation and activate adenylate cyclase, diminished platelet sensitivity to this prostaglandin could result in "hyperactivity" and contribute to the thrombosis observed in these patients.

RECENT STUDIES have described qualitative platelet abnormalities that could play a role in the pathogenesis of thrombosis. For example, platelets from diabetic patients with vascular complications have enhanced aggregation with adenosine diphosphate (ADP), epinephrine, and collagen¹² as well as increased synthesis of prostaglandin-E-like material.³ Enhanced sensitivity of platelet aggregation to epinephrine has been reported in patients with type II hyperbetalipoproteinemia,⁴ while increased platelet sensitivity to ADP and collagen has been associated with angina pectoris⁵ and myocardial infarction.⁶ Although interesting, these observations have not conclusively demonstrated a causal relationship between enhanced platelet function measured in vitro and the development of thrombotic complications in these disorders.⁷

Perhaps the most consistent qualitative platelet abnormalities are associated with myeloproliferative disorders, where abnormal aggregation to collagen, ADP, and epinephrine,⁸⁻¹⁰ as well as spontaneous platelet aggregation,¹¹,¹² and circulating platelet aggregates have been described.¹³ Identification of platelet abnormalities are especially important in these disorders, as over 30% of these patients die from an acute thrombotic event.¹⁴⁻¹⁶ We recently reported that the platelets of patients...
with myeloproliferative diseases have a decreased sensitivity to prostaglandin D₂ (PGD₂) as a result of diminished PGD₂-sensitive platelet adenylate cyclase activity.²⁷ PGD₂, along with prostaglandin I₂ (PGI₂) and prostaglandin E₁ (PGE₁), is a potent inhibitor of platelet aggregation via activation of adenylate cyclase.²⁸⁻³³ Since PGD₂ is synthesized by platelets during aggregation at concentrations that can inhibit platelet aggregation,²⁴ we postulated that diminished sensitivity of these platelets to this prostaglandin provides a possible mechanism for unregulated platelet aggregation to occur in vivo with resultant thrombosis. The studies now reported extend these observations to include patients who develop an acute deep-vein thrombosis and/or pulmonary embolism unassociated with a myeloproliferative disease.

MATERIALS AND METHODS

Assay of Adenylate Cyclase

Platelet-rich plasma (PRP) was prepared from 20 ml of venous blood anticoagulated with 13.5% acid citrate dextrose (ACD). The PRP was adjusted to pH 6.5 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, 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 of 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.,²⁶ using ³²P-ATP as substrate and directly measuring the ³²P-cyclic AMP product. Assay mixtures contained 4.0 x 10⁶ dpm α-³²P-ATP (New England Nuclear, 10⁻³⁰ Ci/m mole); 0.10 mM ATP (Sigma Chemical Co., St. Louis, Mo.); 25 mM Tris-HCl (pH 7.4); 5 mM MgCl₂; 2 mM cyclic AMP (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 of 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 pmole 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.²⁷ Prostaglandins were a gift from Dr. John Pike (Upjohn Co., Kalamazoo, Mich.).

Platelet Serotonin Release

Venous blood anticoagulated with 13 mM sodium citrate was mixed with 3.4 µCi/m mole, New England Nuclear, Boston, Mass.) and centrifuged at 160 g for 10 min to prepare PRP. Serotonin release was measured in the PRP by a modification of the method of Jerushalmy and Zucker.²⁸ Forty micrograms of collagen suspension (Worthington) was added to 0.4 ml of stirred PRP in a Payton Dual Channel Aggregometer (Payton Associated, Buffalo, N.Y.). Various concentrations of prostaglandins were added 30 sec prior to the addition of collagen and the reaction stopped after 4 min with 0.1 ml of 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. Aliquots of supernatant (0.10 ml) were added to glass scintillation vials, followed by the addition of 10 ml of Beckman Ready-Solve liquid scintillation cocktail. Samples were counted using a Packard Tricarb liquid scintillation spectrometer.

PGD₂ Binding Assay

PRP was obtained from 200 ml of venous blood anticoagulated with 13.5% ACD, and the washed platelet pellet prepared as described for the adenylate cyclase assay. The platelet pellet was then resuspended in 3.5 ml of assay buffer (138 mM NaCl, 5 mM MgCl₂, 1 mM EDTA, 25 mM Tris-HCl, pH 7.5) to make a platelet concentration of 1⁻¹·5 x 10⁸ platelets/0.10 ml. The binding assay was
performed using \[^{3}H\]PGD\(_{2}\) (70 Ci/m mole) as the radioligand under previously described conditions.\(^{29}\) Incubations were carried out at 20\(^\circ\)C for 20 min in a total volume of 200 \(\mu\)l containing 100 \(\mu\)l of platelet suspension. All binding is expressed as specific binding and represents the difference between radioactivity bound in the presence and absence of 10\(^{-5}\) \(M\) PGD\(_{2}\). \[^{3}H\]PGD\(_{2}\) was kindly provided by Dr. David Ahern (New England Nuclear).

Patients

Twenty consecutive patients at the Peter Bent Brigham Hospital or the West Roxbury VA Medical Center between February 1 and November 1, 1978, with a diagnosis of acute deep venous thrombosis and/or pulmonary embolism were studied. Consenting patients were evaluated if they had a platelet count in the normal range, hemoglobin >9 g/100 ml, and were not taking aspirin, phenylbutazone, sulfinpyrazone, indomethacin, or other drugs known to interfere with platelet function. The experimental protocol was reviewed and approved by the committee for the protection of human subjects at both hospitals.

Laboratory confirmation of an acute thrombosis was obtained for all patients, and equivocal cases were excluded from the study. Ages of the patients ranged from 18 yr to 80 yr, with a mean age of 55 yr. Eleven of the patients had a pulmonary embolism, documented by pulmonary angiogram in 6 cases and an unequivocal lung scan in 5 cases. Twelve patients had acute deep-vein thrombosis of one or both lower extremities documented by venogram in 8 cases and a positive impedance plethysmograph in 4 cases. All patients were studied within 72 hr after the diagnosis of acute thrombosis and after completion of diagnostic tests. Fifteen patients were on heparin at the time of study. Platelets from healthy volunteers, aged 18–60 yr, were used as normal controls.

RESULTS

Adenylate Cyclase Activity

Figure 1 compares the stimulation of adenylate cyclase by PGD\(_{2}\) in normal platelet particulate fractions to the enzyme activation obtained in the 20 patients with acute thrombosis. The dose–response curves demonstrate decreased stimula-

![Fig. 1. Effect of PGD\(_{2}\) on platelet adenylate cyclase activity in normals (○—○) and in patients with acute thrombosis (●—●). Enzyme activity is expressed as the ratio of stimulated to basal activity. Assays were done as described in the Materials and Methods. Results are mean ± SEM for \(n = 15\) (normals) and \(n = 20\) (patients).]
Table 1. Effect of Fluoride, PGE₁, and PGI₂ on Platelet Adenylate Cyclase Activity in Acute Thrombosis

<table>
<thead>
<tr>
<th>Addition</th>
<th>Adenylate Cyclase Activity (Activity Relative to Basal)</th>
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<tr>
<td></td>
<td>Controls</td>
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<tr>
<td>Sodium fluoride (10⁻² M)</td>
<td>7.9 ± 0.4*</td>
</tr>
<tr>
<td>Prostaglandin E₁ (10⁻⁵ M)</td>
<td>27.6 ± 0.9</td>
</tr>
<tr>
<td>Prostaglandin I₂ (10⁻⁴ M)</td>
<td>26.4 ± 0.9</td>
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*Mean ± SEM; n = 15.
†Mean ± SEM; n = 20.

The patients' adenylate cyclase at all concentrations of PGD₂. In the presence of 10⁻⁷ M PGD₂, there was 4.4-fold enzyme activation relative to basal levels in the normal platelets compared to 3.0-fold enhancement in the patients' platelets (p < 0.01). At the highest concentration of PGD₂ tested (10⁻⁵ M), PGD₂ activated adenylate cyclase 14.5-fold, compared to only 11.2-fold in the patients (p < 0.05). In contrast to the decreased stimulation observed with PGD₂, Table 1 indicates that maximal enzyme stimulation by PGE₁ (28.8-fold) and PGI₂ (28.3-fold) in the patients were comparable to control values (27.6-fold by PGE₁ and 26.4-fold by PGI₂). In addition, sodium fluoride stimulated platelet adenylate cyclase 7.9-fold relative to basal in both patients and normal controls. There was also no significant difference in basal enzyme activity in platelets from patients (70 ± 8 pmole cAMP/mg protein/10 min) and normal subjects (78 ± 7 pmole cAMP/mg protein/10 min).

When the patterns of PGD₂-stimulated adenylate cyclase activity were evaluated individually (Fig. 2), 9 of the 20 patients had enzyme activity below the normal range (<2 SD). Two patients had PGD₂-stimulated enzyme activity that appeared qualitatively less than the concurrent control enzyme activity for that day's experiment, although within 2 SD of the normal range. However, platelets from nine patients appeared to have normal adenylate cyclase sensitivity to PGD₂ at all concentrations of the prostaglandin evaluated. Consequently, the abnormal values for the group shown in Fig. 1 may be the result of a subpopulation of patients with acute thrombosis who have diminished sensitivity to PGD₂. However, there were no clinical features of these nine patients that were different from the other patients studied. Only one patient had a history of idiopathic and recurrent acute venous thrombosis. Three patients had their thrombotic event following a traumatic myelopathy; one patient had multiple sclerosis; and four patients had underlying cardiopulmonary disease.

Five of the nine patients with diminished adenylate cyclase sensitivity to PGD₂ were restudied 2–7 mo following their acute thrombotic event. As shown in Fig. 3, enzyme activity in platelets from three of these five patients gradually returned to the normal range. However, adenylate cyclase activity in the platelets from the two patients with the lowest enzyme activity did not return to the normal range. One of these patients had an increase in PGD₂-sensitive enzyme activity from 40% to 80% of normal, while the other patient had been restudied 4 times over a 9-mo interval without improvement in his PGD₂-sensitive enzyme activity. This latter patient is a 42-yr-old man with a prior episode of deep-vein thrombosis 1 yr before his current admission.
Since 15 of the 20 patients were on heparin at the time of study, the effect of heparin on platelet adenylate cyclase was measured. Heparin at final concentrations of 0, 5, 10, 20, and 100 μg/ml was added to the platelet adenylate cyclase assay. Inhibition of adenylate cyclase activity was noted only at 100 μg/ml heparin, with a 40% decrease in both PGE1- and PGD2-stimulated enzyme. The amount of heparin added to plasma in vitro resulting in a 2½-fold prolongation of the activated

![Fig. 2. Platelet adenylate cyclase activation by PGD2 in patients with acute thrombosis. Platelet membranes were incubated in the presence of 10^-6 M PGD2 and compared to basal activity as described in Materials and Methods. Hatched areas represent 95% confidence limits (±2 SD) for 15 normal controls.](image)

![Fig. 3. Serial studies of PGD2-sensitive platelet adenylate cyclase activity in five patients with acute thrombosis. Enzyme activity is expressed as the percent of control activity at 10^-6 M PGD2.](image)
partial thromboplastin time was only 5 µg/ml. It is unlikely that heparin was present in the assay medium, since platelets were washed free of plasma and suspended in buffer prior to measuring enzyme activity. To demonstrate this fact, heparin was added directly to PRP at 5 µg/ml or 100 µg/ml and incubated either for 30 min or for 24 hr at room temperature prior to washing and preparation of the platelet pellet. No inhibitory effect on prostaglandin-sensitive adenylate cyclase was observed under any of these conditions. In addition, 3 of the 9 patients with diminished PGD₂-sensitive adenylate cyclase activity were not on heparin at the time of study, while 9 of the 11 patients with normal PGD₂-activated enzyme were taking heparin. Two patients taking heparin with abnormal PGD₂-sensitive enzyme activity were restudied 10 days and 2 mo after discontinuation of heparin with no increase in the adenylate cyclase activation by PGD₂.

**¹⁴C-Serotonin Release**

In view of the diminished response of platelet adenylate cyclase to PGD₂, the effect of this prostaglandin on intact platelets was studied. The platelets evaluated were obtained from a patient with deep-vein thrombosis whose PGD₂-sensitive platelet adenylate cyclase was only 40% of normal at all concentrations of PGD₂ tested. PRP from the patient and normal controls were incubated with PGD₂, challenged with 40 µg of collagen, and the release of ¹⁴C-serotonin measured. In the absence of prostaglandins, ¹⁴C-serotonin release was normal (Fig. 4). However, higher than normal concentrations of PGD₂ were needed to block the release of
$^{14}$C-serotonin in the patient's platelets when compared to control platelets. Serotonin decreased from 65% to 24% when $2.5 \times 10^{-7}M$ PGD$_2$ was added to normal PRP. Comparable inhibition required the addition of $10^{-5}M$ PGD$_2$ to the PRP from the patient. At $5 \times 10^{-7}M$ PGD$_2$, serotonin release was almost completely inhibited in normal PRP, while 65% was still released from the patient's PRP. Comparable concentrations of PGE$_1$ ($5 \times 10^{-7}M$) decreased serotonin release from 65% to 5% in the patient and normal controls, indicating that the platelet resistance was specific for PGD$_2$ and not a generalized loss of response to prostaglandins.


Platelets from the same patient used to measure $^{14}$C-serotonin release were also used to measure PGD$_2$ binding. As shown in Fig. 5, binding was saturable in normal platelets at 30 fmole PGD$_2$/10$^8$ platelets. In contrast, this patient's platelets bound only one-third the normal amount at PGD$_2$ at saturation (10 fmole ligand/10$^8$ platelets). This binding analysis demonstrates that decreased PGD$_2$ receptor sites on this patient's platelets probably accounted both for the diminished PGD$_2$-sensitive adenylate cyclase activity and for the resistance of his platelets to the inhibitory effect of PGD$_2$ on aggregation.

Fig. 5. Binding of $[^3]H$PGD$_2$ to intact platelets in normal control (— □) and in a patient with acute thrombosis (— ○). Each point represents the mean of triplicate determinations. Platelets were incubated with 4 nM $[^3]H$PGD$_2$ plus 0–10$^{-5}$M unlabeled PGD$_2$ for 20 min at 20°C. Total binding was determined for each point by dividing cpm by the calculated specific activity obtained by diluting 4 nM $[^3]H$PGD$_2$ with a known concentration of unlabeled PGD$_2$. 
These studies provide evidence that there is a platelet defect in some patients with acute thrombosis that is characterized by a diminished response of adenylate cyclase to PGD₂. Additional studies in one patient demonstrated that this biochemical defect was accompanied by resistance of intact platelets to PGD₂, as a fourfold higher concentration of this prostaglandin was required to prevent collagen-induced release of 

\[ ^{14}C \text{-serotonin} \]. Platelets from this same patient were also shown to bind less \[^{3}H\]PGD₂, suggesting that a decrease in PGD₂ receptors was probably responsible for the diminished enzyme sensitivity to this prostaglandin. In addition, enzyme activation by PGD₂ may improve or return to the normal range several weeks following the acute thrombotic event.

Although these findings are similar in many respects to the platelet abnormalities reported by our laboratory in patients with myeloproliferative disorders,¹⁷⁻²⁹ some differences are also apparent. Over 90% of patients with myeloproliferative disorders had this platelet abnormality, but only 50% of patients with acute thrombosis had diminished PGD₂-sensitive enzyme activity. The 60% decrease in adenylate cyclase activity was greater in patients with myeloproliferative disorders than the 25% loss in patients with acute thrombosis. In addition, no patients with myeloproliferative disorders on subsequent study were found to have a return of PGD₂-sensitive enzyme activity to the normal range. In contrast, four of the five patients followed after an acute thrombosis had a gradual return of PGD₂-sensitive enzyme approaching the normal range.

All of the patients thus far studied who have an altered platelet adenylate cyclase activity show diminished cyclase response only to PGD₂. These platelets have a normal quantity of enzyme as indicated by normal basal levels and fluoride-stimulated enzyme activity, as well as normal responses to PGE₁ and PGI₂. These findings suggest that platelets produced by some patients with acute thrombosis, and almost all patients with myeloproliferative disorders have lost specific “binding sites” for PGD₂. This has been directly shown in platelets from patients with myeloproliferative disorders²⁹ and also demonstrated in one patient in the current report. These findings are also consistent with recent data from our laboratory,³⁰ and others,³¹⁻³³ which indicate a common receptor in platelets from PGI₂ and PGE₁ that is separate from PGD₂.

The mechanism for this altered platelet enzyme sensitivity to PGD₂ is not currently known. One possibility is that these platelets have become desensitized or tolerant to the effects of PGD₂. Desensitization is defined as a loss of physiologic responsiveness of a tissue to a hormone and usually follows prolonged exposure of cells to drugs or hormones. This phenomenon has been well described for catecholamines, prostaglandins, and polypeptide hormones in a variety of tissues and is usually associated with a change in the state of tissue receptors.³⁴ Our laboratory recently reported that platelets can become refractory to the effects of epinephrine following exposure to catecholamines for 4 hr, and this desensitization is associated with a 40% decrease of \( \alpha \)-adrenergic receptors.³⁵ Similarly, we recently reported that platelet adenylate cyclase can become desensitized to the effects of prostaglandins by incubation with PGD₂ for 2 hr.³⁶ Under these incubation conditions there is a 40% decrease of the PGD₂-sensitive enzyme with normal fluoride activation and
catecholamine inhibition. If platelets of the patients now reported were exposed to increased concentrations of PGD$_2$ in vivo at sites of thrombus formation, then desensitization of circulating platelets is theoretically possible.

It is unlikely that circulating heparin accounted for the observed effects on the platelets of the patients now reported, even though Amsterdam et al. recently found that heparin inhibited PGE$_1$-stimulated adenylate cyclase in human platelets. Only high concentrations of heparin inhibited prostaglandin-sensitive enzyme activity in vitro, an effect that was noted for PGE$_1$ as well as PGD$_2$. Also, enzyme studies were performed on washed platelets, and platelets separated from plasma containing heparin had normal adenylate cyclase stimulation by prostaglandins. Finally, three of the nine patients with abnormal PGD$_2$-sensitive enzyme were not taking heparin, and enzyme studies were still abnormal in two patients restudied after heparin was discontinued.

The role of altered PGD$_2$-sensitive enzyme activity in the pathogenesis of thrombosis remains to be proven. PGD$_2$ is a potent inhibitor of platelet aggregation, and sufficient PGD$_2$ (17 mM) is synthesized by platelets during aggregation to potentially inhibit aggregation and release. In fact, several authors have suggested that PGD$_2$ might function in this way as a feedback regulator to limit platelet aggregation. A diminished responsiveness to PGD$_2$ might permit unregulated platelet aggregation to occur in certain patients leading to thrombosis. We initially considered this mechanism in our studies of patients with myeloproliferative disorders who have a high incidence of thrombotic complications. We now find that certain patients without myeloproliferative disorders have a similar platelet abnormality coincident with an acute thrombosis. Further prospective clinical and biochemical studies will be needed to determine whether this platelet abnormality initiates thrombosis, is secondary to thrombosis, or is unrelated to thrombotic events.

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