Increased Plasma Concentrations of Platelet Factor 4 in Coronary Artery Disease

A Measure of In Vivo Platelet Activation and Secretion

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SUMMARY Previous studies have shown that there is both a significant shortening in platelet survival and a measured hyperactivity to platelet-aggregating agents in patients with documented coronary artery disease compared with control groups. We used a recently described radioimmunoassay for the platelet-secreted protein platelet factor 4 (PF4) to study 162 patients with documented coronary artery disease.

There was a significant increase in plasma PF4 concentrations in patients with documented coronary artery disease compared with angiographically normal patients (8.7 vs 16 ng/ml, respectively, n = 121), but as in previous studies of platelet survival, we could not correlate elevated plasma PF4 concentration and the severity or site of the coronary artery disease. In addition, there was no correlation with left ventricular function, serum cholesterol or the type of angina. Patients with confirmed acute myocardial infarction had no significant difference in mean plasma PF4 concentrations compared with similar groups of coronary disease patients who had prolonged chest pain or chronic stable angina. Coronary artery bypass grafting in a subgroup of patients did not affect the mean plasma PF4 concentration during 1 year of follow-up after bypass surgery, but medical therapy for angina with increasing doses of propranolol and nitrates significantly reduced PF4 concentration in another subgroup of patients who were not considered to be candidates for surgical therapy.

INCREASING EVIDENCE implicates platelets in the development and progression of atherosclerosis. Hyperplasia of smooth muscle, the earliest pathologic change in atherosclerotic lesions, occurs in tissue cultures in response to platelet-derived growth factor, and atherosclerosis can be prevented in some animal models by inducing thrombocytopenia. In patients with ischemic heart disease, in vivo platelet activation has been inferred from increases in platelet turnover as measured with chromium-51 platelet-survival studies and by hyperaggregability in response to known aggregating agents. Platelet survival studies require platelet manipulation to attach the radioactive label and 7–8 days to perform, while platelet aggregation studies look only at platelet behavior in vitro. These shortcomings make it difficult to evaluate large groups of patients undergoing several interventions and introduce the artifacts of platelet handling in vitro.

Several sensitive radioimmunoassays have been developed for specific platelet proteins that are contained in the platelet α granules and secreted during the platelet release reaction. Measurements of plasma concentrations of these proteins in carefully prepared cell-free plasma can be used as a marker for in vivo platelet secretion serially in large numbers of patients. There have been preliminary reports of elevated concentrations of several of these proteins in patients with coronary artery disease, but a systematic study to determine the sensitivity and specificity of this test in ischemic heart disease has not been done. We used a radioimmunoassay for one of these proteins, platelet factor 4 (PF4), to demonstrate that (1) patients with documented coronary artery disease have increased plasma concentrations of PF4 compared with patients with normal coronary angiograms; (2) there is no difference in the mean plasma concentrations of PF4 when comparing patients with chronic stable angina, unstable angina, and acute myocardial infarction; (3) there is no difference in PF4 concentrations in patients before and after coronary artery bypass grafting (CABG), whereas PF4 decreases to normal in symptomatic patients undergoing medical management for their angina symptoms.

Methods

Blood Collection

Venous blood samples for PF4 determinations were drawn by our previously described technique. Briefly, a 21-gauge scalp vein needle was inserted into a tourniquet in place, the first 10 drops of blood were discarded and a venous blood sample was drawn into a plastic syringe. The blood was then immediately transferred to an iced polypropylene tube containing EDTA (final concentration [f.c.] 5 mM), N602 dibutyryl adenosine 3'5' cyclic monophosphoric acid.
(f.c. 1 mM) and prostaglandin E\textsubscript{2} (f.c. 1 μg/ml). Samples were transported to the laboratory on ice and centrifuged at 4°C at 12,000 g for 10 minutes within 15–30 minutes from the time of collection. Platelet-poor plasma was then removed and stored frozen until assayed for PF4. We have carefully standardized this collection technique to minimize in vitro artifactual release.\textsuperscript{11} We recently compared our additives and processing techniques with other published methods being used\textsuperscript{10,12-14} and found no significant differences between methods when analyzed by our radioimmunoassay.

**Patient Population**

In the first part of the study, blood samples were obtained from serial patients scheduled for diagnostic cardiac catheterization. All samples were drawn the day before the catheterization after written and verbal consent had been obtained. Patients were questioned about their medications during the previous 2 weeks and all inpatient medications were recorded. The patient’s history, physical examination, and catheterization reports were reviewed by one of the authors. Significant narrowing of a coronary artery was defined as at least a 50% decrease in luminal diameter. Normal angiograms were defined as those in which there was no evidence of atherosclerosis. Subcritical disease was defined as narrowing of a coronary artery of less than 50% of the luminal diameter. All patients with valvular disease that was not due to ischemic coronary artery disease were excluded from the study because valvular disease alone has been reported to shorten platelet survival and elevate plasma concentration of PF4.\textsuperscript{10,12} To determine the effects of therapy on the degree of platelet activation, serial blood samples were obtained during follow-up clinic visits on subgroups of the catheterization patients who had either undergone CABG or were followed in the Audie Murphy VA Hospital Cardiology Clinic. Referred patients were not available for follow-up studies.

In the second part of the study, plasma PF4 samples were obtained from a separate group of patients with documented coronary artery disease to determine whether acute myocardial ischemia or infarction influenced the degree of platelet activation and PF4 release. Patients admitted to the coronary care unit for the evaluation of chest pain were included in the study if they had a positive coronary angiogram, a past hospitalization for an acute myocardial infarction at the VA Medical Center, or previous coronary artery bypass grafting. Blood samples were drawn within 24 hours of admission to the coronary care unit and then serially during hospitalization. All patients were evaluated with serial cardiac enzymes including CPK and LDH isoenzymes, SGOT, and serial ECGs. The diagnosis of acute myocardial infarction was made if the patient had two of the following criteria: typical ischemic chest pain, typical enzyme changes or typical persistent electrocardiographic changes. The plasma PF4 concentrations in these two groups of patients with unstable angina or acute myocardial infarction were then compared with PF4 concentrations in a group of chronic angina patients who were attending a routine cardiology outpatient clinic.

**Assay Methods**

Plasma and serum PF4 concentrations were measured using our previously reported radioimmunoassay method.\textsuperscript{11} This assay detects as little as 165 pg in a 250-μl plasma sample, for a lower limit of detection of 660 pg/ml. Platelet counts were performed in EDTA-anticoagulated, platelet-rich plasma using a Coulter model C Thrombocounter. Serum cholesterol was measured by a conventional enzymatic method as part of the admitting chemistry profile.

**Statistical Analysis**

All PF4 concentrations were converted to natural logarithms before statistical analysis to obtain normally distributed values. Groups were compared by means of one-way analysis of variance and Dunnett test. The correlation between plasma PF4 and the number of abnormal coronary vessels, left ventricular ejection fraction, serum cholesterol, type of angina, and specific vessel involved were determined by a one-way analysis of variance or a first-order linear regression analysis. The effect of coronary artery bypass grafting or medical therapy for angina was evaluated in subgroups of patients by a paired t test. All values are given as the geometric mean and 95% confidence interval (CI\textsubscript{95}).

**Results**

**Part One**

To determine whether significant platelet activation and PF4 release occur in coronary artery disease, plasma concentrations of PF4 were compared in patients with normal coronary angiograms and in patients with one-, two-, and three-vessel coronary artery disease (fig. 1). Patients with significant coronary artery disease had a significantly higher plasma PF4 (16 ng/ml; CI\textsubscript{95} 13–19 ng/ml) than patients with normal coronary angiograms (8.7 ng/ml; CI\textsubscript{95} 5.9–13 ng/ml) (p < 0.02). Seven patients who had subcritical narrowing were analyzed separately and found to have a plasma PF4 concentration that was intermediate between the other two groups (12.3 ng/ml; CI\textsubscript{95} 6.1–25 ng/ml). The results were further analyzed by determining the correlation between plasma PF4 concentrations and the following characteristics: number of obstructed vessels, left ventricular function as measured by the ejection fraction determined at left ventricular angiography, serum cholesterol concentrations, type of angina, and site of specific vessel involvement.

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\*Kindly provided by Dr. John Pike (Upjohn Co., Kalamazoo, Michigan).
Number of Obstructed Vessels

There was no significant correlation between mean PF4 plasma concentrations and the number of vessels involved: one-vessel disease, 12 ng/ml (CI = 9–17 ng/ml); two-vessel disease, 23 ng/ml (CI = 17–32 ng/ml); and three-vessel disease 15 ng/ml (CI = 11–20 ng/ml).

Ejection Fraction

The patients who had documented coronary artery disease were divided into three groups by ejection fraction (<35%, 35–60%, >60%). There was no statistical correlation between the severity of the left ventricular dysfunction and the degree of platelet activation and PF4 secretion (table 1). The group with an ejection fraction less than 35% is very small, possibly accounting for the lack of effect seen.

Serum Cholesterol

A linear regression analysis was performed between plasma PF4 values and serum cholesterol in all patients with documented coronary artery disease (n = 96) and a subgroup of these patients (n = 37) who were younger than 55 years of age. Correlation coefficients were 0.049 and 0.123, respectively.

Table 1. The Relationship Between Plasma Concentrations of Platelet Factor 4 and Subgroups of Patients with Coronary Artery Disease

<table>
<thead>
<tr>
<th>Ejection fraction (%)</th>
<th>PF4 (geometric mean; ng/ml)</th>
<th>95% confidence limits</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 35</td>
<td>9</td>
<td>25</td>
</tr>
<tr>
<td>35-60</td>
<td>48</td>
<td>15</td>
</tr>
<tr>
<td>&gt; 60</td>
<td>48</td>
<td>15</td>
</tr>
<tr>
<td>Serum cholesterol (mg %)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 203</td>
<td>23</td>
<td>18</td>
</tr>
<tr>
<td>203–231</td>
<td>19</td>
<td>11</td>
</tr>
<tr>
<td>232–263</td>
<td>27</td>
<td>18</td>
</tr>
<tr>
<td>&gt; 263</td>
<td>37</td>
<td>16</td>
</tr>
<tr>
<td>Type of angina</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>11</td>
<td>24</td>
</tr>
<tr>
<td>Atypical</td>
<td>13</td>
<td>14</td>
</tr>
<tr>
<td>Chronic</td>
<td>58</td>
<td>14</td>
</tr>
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<td>Unstable</td>
<td>17</td>
<td>17</td>
</tr>
<tr>
<td>New</td>
<td>7</td>
<td>21</td>
</tr>
<tr>
<td>Months after CABG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before</td>
<td>39</td>
<td>16</td>
</tr>
<tr>
<td>1 month</td>
<td>30</td>
<td>17</td>
</tr>
<tr>
<td>2 months</td>
<td>21</td>
<td>11</td>
</tr>
<tr>
<td>3–6 months</td>
<td>15</td>
<td>32</td>
</tr>
<tr>
<td>6–12 months</td>
<td>18</td>
<td>12</td>
</tr>
</tbody>
</table>

Abbreviations: PF4 = platelet factor 4; CABG = coronary artery bypass graft.

(p > 0.3). Geometric means for all patients divided into four groups of serum cholesterol concentrations are shown in table 1. Comparison of these groups by a one-way analysis of variance also failed to show a positive correlation between plasma concentrations of PF4 and serum cholesterol values. This quartile distribution was chosen because Cohn et al. found a significant correlation between serum cholesterol and severity of coronary artery disease in these groups.

Angina Pattern

To determine whether the type of angina correlated with the degree of platelet activation and secretion, patients were classified into one of five groups: no angina, new angina of less than 2 months' duration, atypical angina, unstable angina (angina at rest or an increased frequency or severity of chronic stable angina), and chronic stable angina. Mean plasma PF4 concentrations were again not significantly different between these groups (table 1).

Vessel Involvement

To determine whether the specific vessels involved correlated with the degree of platelet activation, all patients were analyzed by the site of their lesion (fig. 2). Some groups were small, but mean PF4 concentrations were not significantly different between groups.
patients undergoing anatomic or medical therapy when compared by one-way analysis of variance. Thus, platelet activation was not influenced by the anatomic site of the lesion.

**Medical and Surgical Therapy**

Serial samples were analyzed in catheterization patients undergoing coronary artery bypass grafting or intensive medical therapy to determine the effects of either surgical or medical therapy on platelet activation and secretion. There was no significant difference in the mean plasma PF4 concentrations before and 4–5 weeks after coronary artery bypass grafting (mean 4.4 weeks). When additional samples were obtained and analyzed over the next year, no significant trend was apparent (table 1). These patients were not restudied to determine graft patency during the follow-up period. Another subgroup of patients, however, who were only treated medically, had a significant decrease in mean plasma PF4 values as antianginal therapy was optimized \( (p < 0.05) \). Nine patients had marked decreases to normal, four remained normal, and two had modest increases in PF4 values (fig. 3). In these patients, the mean increase in propranolol was 68 mg every 6 hours and in isosorbide dinitrate, 19 mg every 6 hours. Only a small group of patients had no change in their therapy, but there was a slight decrease of plasma PF4 concentrations in two, a marked increase in two, and one remained normal. Geometric mean plasma concentrations of PF4 for these five patients increased from 20 ng/ml \( (CI_{95} 12–35 \text{ ng/ml}) \) to 22 ng/ml \( (CI_{95} 8–60 \text{ ng/ml}) \).

When we analyzed the entire population of catheterized patients for the effect of antiplatelet drugs on precatheterization PF4 values, one-way analysis of variance showed no differences. All patients were assigned to one of the following groups: no antiplatelet drugs, aspirin, propranolol, aspirin and propranolol, or other antiplatelet drugs. We did not further subdivide groups for nitrates, because nitrate use was common in all groups. More than 60% of patients in the propranolol groups were also receiving isosorbide dinitrate.

**Associated Illnesses**

All subgroups described above were comparable for age, incidence of hypertension, peripheral vascular disease, and diabetes.

**Part Two**

In the second part of the study, we attempted to correlate clinical presentation with PF4 values in patients with known coronary artery disease. These were patients with acute myocardial infarction, patients with chest pain requiring hospitalization to rule out myocardial infarction and ambulatory patients with chronic stable angina. Mean plasma PF4 concentrations were significantly increased in all three groups of patients with coronary artery disease compared with normal subjects, but there was no difference between groups by one-way analysis of variance (fig. 4). All three groups were comparable for age, sex, incidence of previous myocardial infarction, previous coronary artery bypass graft surgery, diabetes mellitus, hyper-
PF4 (ng/ml)

+AMI  -AMI  OPD

180  -  160  -  140  -
120  -  100  -  80  -
60  -  40  -  20  -
40  -  20  -
0

Figure 4. Plasma concentrations of platelet factor 4 (PF4) were measured in three groups of patients in an attempt to correlate platelet activation and clinical presentation. Group (+AMI) consisted of patients admitted to the coronary care unit who fulfilled the criteria for an acute myocardial infarction (see Methods). Group (-AMI) consisted of patients admitted to the coronary care unit with chest pain who did not fulfill the criteria for an MI. Group (OPD) consisted of patients with documented coronary artery disease and stable angina who were followed in the outpatient department. Groups were compared using a one-way analysis of variance.

tension, peripheral vascular disease, cerebrovascular accidents, propranolol therapy and aspirin ingestion.

Group 1

Group 1 included 14 patients admitted to the coronary care unit after a prolonged episode of chest pain and found to have an acute myocardial infarction by the previously described criteria. Eight of the patients had transmural infarctions and six had subendocardial infarctions. When these patients were followed serially during hospitalization, no consistent changes were noted. Eight of the 14 patients had initial values that were greater than 2 standard deviations above the mean PF4 concentration of normal volunteers; in two of these patients, PF4 concentration gradually decreased toward normal, and in the other six patients, PF4 concentrations remained elevated for up to 6–8 days of observation. Six of the 14 infarct patients had normal PF4 concentrations in their initial samples; in two of these patients PF4 values increased further during follow-up and in the other four PF4 values remained normal.

Group 2

Group 2 included 14 patients with known coronary artery disease who were admitted to the coronary care unit for evaluation of prolonged chest pain but failed to meet the criteria for an acute myocardial infarction. Only some of these patients had serial PF4 determinations. The remainder either underwent further invasive evaluation or were discharged for further outpatient follow-up. Serial PF4 concentrations showed the same variability in this group as in the acute infarction group.

Group 3

Group 3 was composed of 13 patients with documented coronary artery disease and stable angina pectoris who were being followed in the outpatient cardiology clinic and who were being seen at a routine appointment on the day that the sample was obtained. Serial PF4 measurements were not obtained from these patients.

Discussion

Platelet activation in coronary artery disease may have several mechanisms. These include direct platelet activation due to atherosclerotic lesions in the coronary arteries, which might produce defects in endothelial prostacyclin production; abnormalities of local blood flow, producing platelet injury related to shear stress; and activation of coagulation with thrombin production and thrombus formation. Alternatively, platelet activation may be a remote effect of myocardial ischemia, caused by alterations of pH, lactate production, plasma catecholamine concentrations or local release of ADP. Studies to investigate these hypotheses require a test that can be done repeatedly and in large groups of patients. The measurement of plasma concentration of PF4 is such a test.

In this study we have shown that measuring plasma concentrations of PF4 is a valuable approach to the study of platelet activation in patients with coronary artery disease. Previous studies in which platelet activation was demonstrated in coronary artery disease used either chromium-51 platelet survival or comparisons of threshold concentrations of standard aggregating agents. Survival studies require that platelets be isolated and radioactively tagged before they are reinjected and measurements are made. Platelets are easily activated during in vitro handling, introducing another variable that may affect survival measurements. In addition, these studies require 7–8 days of serial sampling and are best suited to long-range studies on small groups of patients. Aggregation studies also have limitations that make them less useful than the PF4 radioimmunoassay used in this study. Previous studies have inferred platelet hyperactivity in coronary artery disease from demonstration of reduced thresholds for aggregation with standard aggregating agents. An aggregation study from a single patient can require 3–4 hours of technician time to establish threshold concentrations for multiple aggregating agents. Using the PF4 radioimmunoassay we studied more than 100 patients undergoing cardiac catheterization and approximately 50 patients with angina or myocardial infarction in a relatively
short time. These assays are rapid and sensitive and have as their major advantage the fact that in vivo platelet hyperactivity is being evaluated rather than indirectly inferred.

These tests are exquisitely sensitive to artifactual elevations of PF4 caused by errors in blood collection and processing. We have carefully standardized our collection and processing technique, but we must still question whether elevated PF4 values in individual patients are artifactual. We have confirmed that our collection technique is reproducible enough that there is no significant difference in PF4 values in two samples drawn on separate days from normal subjects. In addition, there are no residual platelets in the platelet-poor plasma that are measurable by Coulter counter, no change in the plasma PF4 after freezing and thawing of the sample, and no correlation between the plasma PF4 and platelet count in normal subjects. Although there is a variation in PF4 concentrations in normal subjects and patients followed serially, review of our data shows that normal subjects only occasionally have an elevated plasma PF4 value and patients with elevated plasma PF4 only occasionally have a normal PF4 value. One patient with valvular disease who was not included in this analysis had PF4 values of 234, 44, 39 and 46 ng/ml when followed serially, which clearly suggests consistent degrees of platelet activation and secretion.

The results of this study may be compared with previously reported studies in which platelet survival or aggregation were studied. Elevated PF4 and shortened platelet survival have been found in 50–68% of patients with documented coronary artery disease. Neither measure of platelet hyperactivity can be correlated with the extent or site of coronary vessel involvement. Both the current study and an early study by Steele et al. demonstrated no correlation between abnormal platelet behavior and the patient’s lipid status. This in contrast to an expanded study by Steele and Rainwater in which the incidences of shortened platelet survival in patients with hyperbetalipoproteinemia were 79% and 53%, respectively. In addition, they showed that platelet survival improved significantly in 15 patients when there was correction of hypertriglyceridemia. Further studies must be completed before definite conclusions can be reached.

We could not show a difference in mean plasma PF4 concentrations between patients with documented acute myocardial infarctions, unstable angina and chronic angina. Handin et al., using serial plasma concentrations of PF4 in patients admitted to a coronary care unit with chest pain, found that patients with documented infarctions had acute elevations in PF4 concentrations when compared with the control group. The control group, however, consisted of all patients admitted with chest pain who did not have an acute myocardial infarction and the group was not further subdivided for the presence or absence of documented coronary artery disease. The statistically significant difference in PF4 values could therefore be explained by the presence or absence of coronary artery disease. The authors serially sampled nine of their patients with myocardial infarctions and found that after 1 week in the hospital, the PF4 level remained elevated in the majority of patients. Analysis of their serial data reveals the same heterogeneity of response that we reported in groups 1 and 2. This difference in study design probably explains the differences in the results between the two studies.

Coronary artery bypass grafting had no effect on measured PF4 concentrations in 39 patients in this study. The same result was reported by Steele and coworkers, who measured platelet survival in 16 patients. In contrast, Harker et al. reported a significant improvement in platelet survival in nine patients who underwent bypass grafting. We found a significant reduction in plasma PF4 concentrations in a small subgroup of patients who had their anginal symptoms treated by increasing doses of propranolol and nitrates. The present study was not a prospective trial to evaluate the effect of β blocker or nitrates in comparison with placebo, but these are exciting preliminary results that must be evaluated further. The propranolol effect is similar to the results reported by Frishman et al., who showed that propranolol therapy (80 mg/day) normalized the aggregation thresholds to ADP in angina patients when compared with placebo. In contrast, Steele et al. found no effect of propranolol (160 mg/day) on platelet survival times in 10 patients studied before and after drug administration. Both propranolol and organic nitrate vasodilators affect in vitro platelet function. A reduction in plasma PF4 could therefore be due either to a direct effect on platelet function or to an improvement in myocardial blood flow or function.

The stimulus for platelet activation in coronary artery disease is unknown and the results from studies in which platelet activity has been measured are preliminary. Studies of the role of the platelet in atherosclerosis are important, and measurement of plasma PF4 is a useful tool for this kind of research. Doyle et al. suggested that there is a significant correlation between PF4 measurements and the more laborious platelet turnover studies in coronary disease patients. We evaluated a number of variables in more than 150 coronary patients to begin to understand associated events. This approach will not establish a cause-and-effect relationship between variables, but will provide a scientific basis for the design of future studies to explore the role of the blood platelet in atherosclerosis and its clinical manifestations.

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