Platelet-Associated Factor XIII as a Marker of Platelet Activation in Patients With Peripheral Vascular Disease

Dana V. Devine, Gail Andestad, Diane Nugent, and Cedric J. Carter

In the past several years, monoclonal antibodies have been developed that distinguish between resting and activated platelets in vitro. These antibodies recognize epitopes expressed on membrane proteins or soluble proteins, such as factor XIIIa and thrombospondin, that bind only to activated platelets. We have used fluorescence flow cytometry to determine whether three such antibodies can detect platelet activation in patients with severe peripheral vascular disease (PVD). Using two activation-specific monoclonal antibodies and a polyclonal antiserum to factor XIII α-chain, we have examined the platelets from PVD patients, age-matched control subjects who were free of detectable PVD, and unmatched control subjects. Cells analyzed as platelets were identified by their light-scatter profile and their reactivity with monoclonal anti-glycoprotein Ib. The platelets of patients with PVD showed no increase in binding of activation-dependent 1B3 (directed against a 180-kD membrane protein) compared with age-matched control subjects (p=0.780). Similarly, there was no difference between PVD patients and control subjects in activation-dependent CD63 expression. Conversely, the binding of anti-factor XIII α-chain was significantly higher than in the control groups (p<0.001). These data suggest that the detection of soluble factors that bind to activated but not resting platelets may be of use in the detection of pathological in vivo platelet activation.

KEY WORDS • platelet activation • factor XIII • CD63 • peripheral vascular disease

On exposure to a physiological agonist such as thrombin or adenosine diphosphate (ADP), the platelet undergoes a series of biochemical changes that cause it to become "activated," acquiring those changes necessary for hemostasis. These processes include the reorganization of membrane proteins to induce the expression of receptors on glycoprotein (GP) IIb/IIIa for adhesive proteins such as fibrinogen. When platelets are fully stimulated, their granules fuse with the canalicul system of the platelet membrane, facilitating the release of their contents into the surrounding plasma.

Over the past several years, a number of antibodies have been described that react with activated but not resting platelets. Generally, these antibodies recognize either a conformational change in a protein that is normally found on the platelet surface or a novel protein that is not found on the surface of a resting platelet. This latter group includes antibodies to granule membrane constituents such as GMP-140 and CD63 (p53) as well as antibodies to soluble proteins that adhere to activated platelets such as factor XIIIa, thrombospondin, and the soluble form of CD63 (p53). Although the application of activation-dependent antibodies to in vitro studies of platelet activation is a powerful technique, its clinical utility is less certain.

Activation-dependent monoclonal antibodies have been used successfully to show platelet activation during adult respiratory distress syndrome (ARDS) and cardiac surgery; however, these are acute conditions presenting a strong stimulus to platelet activation. We wished to determine whether activation antigens on platelets could be detected by flow cytometry in a chronic condition, such as peripheral vascular disease (PVD), in which progressive activation of platelets has been implicated in the pathogenesis of the disease. To this aim, we have studied a cohort of patients with PVD, age-matched control subjects, and unmatched normal donors. Platelets were reacted with a panel of monoclonal and polyclonal antibodies that detect the activation-dependent expression of a 180-kD platelet membrane protein, CD63, and platelet-associated factor XIIIa. Whereas the activation-dependent monoclonal antibodies against CD63 and p180 failed to distinguish between diseased and nondiseased groups, platelet-associated factor XIII levels were significantly elevated in patients with PVD.

Methods

Patients

This study was approved by the Ethics Review Committee of the University of British Columbia and the...
University Hospital. Informed consent was obtained from all study participants before blood collection. The first study group consisted of three types of individuals: 1) Twenty-nine patients with severe PVD were studied (19 men and 10 women; age range, 43–80 years; mean age, 64.5 years). These were hospital inpatients, of whom 17 had a history of femoropopliteal bypass surgery, and 12 were candidates for the procedure; none had evidence of sepsis. All patients had severe PVD, defined as ankle–brachial pressure ratios <0.5, 13–15 Twenty-six were smokers, and all had evidence of other vascular disease. Five had type II diabetes mellitus. 2) An age-matched control group consisted of 10 patients (three men and seven women; age range, 63–84 years; mean age, 72.6 years). These were outpatients who were classified as free of PVD as determined by strain-gauge plethysmography and arterial Doppler studies. None of these control subjects had diabetes mellitus. In keeping with the age group, among the control subjects were two individuals with hypertension, one with congestive heart failure, and one with a remote stroke. 3) Blood from normal donors was tested in parallel with the two previous groups. Nineteen healthy volunteers were selected from hospital staff (five men and 14 women; age range, 22–46 years; mean age, 33.2 years). A second cohort of 26 patients was studied that contained 10 women and 16 men (age range, 34–82 years; mean age, 67.1 years). Sixteen of these patients had a history of femoropopliteal bypass surgery, and all were smokers. All had evidence of other vascular disease; seven had diabetes mellitus. PVD severity was stratified on the basis of ankle–brachial pressure ratios into severe (ratio, <0.5) or moderate (ratio, 0.5–0.7), 13–15 and patients were categorized accordingly before the platelet studies. Physicians performing clinical evaluation remained unaware of the results of the platelet studies.

**Verification of Specificity of Polyclonal Antiserum**

Preliminary studies demonstrated that Western blot alone was insufficiently sensitive to identify anti–factor XIII antibody binding to whole-plasma proteins immobilized after sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE); no bands were seen. Therefore, specificity of the antiserum to factor XIII was studied by a combination of immunoprecipitation and Western blot analysis. Platelet lysate prepared as described 18 or normal plasma was reacted with protein G–Sepharose (Pharmacia) to remove Ig's. After removal of the protein G–Sepharose, factor XIII was immunoprecipitated from the samples by use of polyclonal anti–factor XIII a-chain or anti–factor XIII b-chain antiserum (Calbiochem) and more protein G–Sepharose. Anti–factor XIII a-chain should immunoprecipitate its antigen from both platelets and plasma, whereas anti–factor XIII b-chain should precipitate factor XIII only from plasma. After electrophoresis by SDS-PAGE under nonreducing conditions, samples were transferred to nitrocellulose paper and probed with either anti–factor XIII a-chain or b-chain antiserum followed by peroxidase-conjugated F(ab')2 goat anti-rabbit IgG (Jackson Immunoresearch). Thus, each anti–factor XIII immunoprecipitate was transferred to nitrocellulose, and the blots were probed with both anti–factor XIII a-chain and anti–factor XIII b-chain antibody. With this approach, the identification of the antigen in the immunoblot by the same antibody used for the immunoprecipitation verified that the material was present in amounts sufficient to be detected by this method. The pattern of bands identified or bands missing when a blot was probed with the antiserum directed against the other chain of factor XIII then defined the specificity of the precipitating antibody.

**Platelet Preparation**

In studies of platelet activation, care must be taken to ensure that any activation detected in the assay reflects the in vivo situation and is not a venipuncture or handling artifact. Assay validation for the detection of platelet-associated factor XIII included an analysis of sampling parameters, including collection medium, collection vessel, and time to processing. Other investigators have used cocktails of platelet inhibitors or collection directly into fixative to inhibit platelet activation during processing. In the assessment of platelet-associated factor XIII, thrombin generation must be blocked, because factor XIII is activated by thrombin in the presence of calcium. Therefore, our collection procedure focused on the chelation of calcium and the inhibition of thrombin. Blood was collected in several different ways: 1) by syringe directly into 1% paraformaldehyde after discarding the first 3 mL of blood in the line; 2) by syringe directly into an anticoagulant cocktail of 5 μmol/L prostaglandin E1, 10 mmol/L EDTA, and sufficient d-phenylalanyl-l-prolyl-l-arginine chloromethyl ketone (PPACK) to inhibit 10 IU/mL thrombin; 3) by syringe directly into EDTA; and 4) into EDTA Vacutainer tubes. Platelet-rich plasma (PRP) was isolated by centrifugation of the whole blood at 80g for 15 minutes. A platelet pellet was obtained by centrifuging the PRP for 3 minutes at 1,000g.
After the method analysis described above was performed, the following protocol was used for the patient studies. Blood was drawn into 10% disodium EDTA (pH 7.4) to a final concentration of 5.4 mmol/L with a 21-gauge butterfly needle and plastic syringe. To minimize variation in collection and handling, all specimens were drawn by the same phlebotomist; samples were not used if the collection procedure was difficult. All samples were processed within 30 minutes of collection. Platelet pellets were isolated as described above. The pellet was washed once in phosphate-buffered saline containing 0.015 mol/L EDTA and 2% bovine serum albumin (PBS-EDTA-BSA) at pH 7.4 and then fixed in PBS containing 1% paraformaldehyde. Platelets were resuspended in PBS-EDTA-BSA to a concentration of approximately 5 x 10^8/mL.

Detection of Activation Antigens by Flow Cytometry

Washed, fixed platelets were incubated 1:1 for 45 minutes at room temperature with antibodies diluted in PBS-EDTA-BSA to the previously determined optimum binding concentration. Murine monoclonal antibodies to GP Ib and 31A were used as positive and negative controls, respectively. Murine monoclonal antibody 1B3 was tested against normal donors, age-matched control subjects, and patients with PVD; anti-CD63 was tested on platelets from patients with PVD and normal control subjects. Polyclonal rabbit anti-factor XIII a-chain antiserum was tested against all groups in parallel with nonimmune rabbit serum. The latter was used as a control for nonspecific staining in the assessment of anti–factor XIII binding. To determine the maximum possible 1B3 binding, an aliquot of each subject's platelets was stimulated to express the activation-dependent epitope on the 180-kD protein by incubation for 30 minutes with 5 IU/mL α-thrombin. Aliquots of untreated platelets were also tested for 1B3 binding to identify any in vivo expression of this marker. After washing three times in PBS-EDTA-BSA, these aliquots were run in parallel with untreated platelets and served as a positive control for the demonstration of 1B3 binding by the platelets of each individual studied.

After the primary antibody incubation, samples were washed three times in PBS-EDTA-BSA and reacted 1:1 with fluorescein-conjugated F(ab')2 anti-IgG of appropriate species specificity at saturating concentration for 45 minutes at room temperature. Specimens were then washed two times in PBS-EDTA-BSA. Single-color fluorescence and laser light scatter were measured on a Coulter EPICS Profile flow cytometer equipped with an argon laser delivering 25 mW at 488 nm. Fluorescence emission was detected with a 575-nm band-pass filter, and all readings were taken at a constant voltage of 1,300 V. The instrument was controlled daily by use of Immuno-check fluorospheres (Coulter).

Fluorescence histograms were analyzed by setting a gate at the right shoulder of the negative antibody control and determining both a percentage of the total cells and a weighted fluorescence value (WFV) for those cells found in channels to the right of the gate. The WFV was calculated by multiplying the mean channel fluorescence by the percentage of total cells counted falling within the region. In this way, the positive events were weighted with respect to their degree of fluorescence within the region defined as positive (after Reference 19). Net fluorescence for binding of anti-factor XIII a-chain was determined by subtracting the nonimmune rabbit WFV from the serum factor XIII WFV. Antibody binding data for the three groups were analyzed by analysis of variance and multiple-comparison testing.

Results

Assay Validation

The specificity of the antiserum to factor XIII a-chain was established by demonstrating that the antibody precipitated a protein corresponding to the molecular weight of factor XIII a-chain from platelet lysates and normal plasma. Antiserum to factor XIII b-chain precipitated no protein from platelet lysate; however, the precipitate from plasma showed single-band reactivity in immunoblot at 74 kD with anti–factor XIII a-chain and 80 kD with anti–factor XIII b-chain antisera. Since the antiserum to factor XIII a-chain immunoprecipitated and immunoblotted only a single band of 74 kD from whole platelet lysate, we believe that it is monospecific for platelet factor XIII a-chain in its platelet reactivity.

Before analysis of patient samples, the blood collection procedure was validated against other methods of collection and anticoagulation. Collection of blood into the various permutations of EDTA-based anticoagulant cocktails gave similar results. There was no statistically significant difference in the amount of platelet-associated factor XIII or CD63 measured in 10 experiments. The collection of blood directly into fixative caused an increase in platelet-associated factor XIII. This was most likely a result of the fixation of plasma factor XIII to the platelet surface, because >70% of platelets in four experiments were also positive for factor XIII b-chain compared with <2% of unfixed platelets (data not shown). The immediate fixation of whole blood for these studies was ruled out. Because we were concerned that the continued manipulation of the platelets during the processing could cause activation, platelets were fixed in 1% paraformaldehyde after the first wash. A time-course study indicated that platelets must be processed from the whole blood within 3 hours of collection or there is a decrease in the percentage of factor XIII-positive platelets compared with platelets processed immediately after collection (data not shown).

PVD Studies

Representative histograms of the binding of anti-factor XIII a-chain antiserum to the platelets of a patient with severe PVD and a healthy normal donor are shown in Figure 1. Monoclonal antibody to GP Ib was used to identify the population of signals as platelets. The interaction of platelets with irrelevant negative-control monoclonal antibodies failed to give detectable fluorescence signals. The interaction of any donor platelets with normal rabbit serum gave a weak fluorescence signal that was subtracted from the anti–factor XIII fluorescence signal. In the first experimental group, a monoclonal antibody specific for activated platelets was tested along with a polyclonal antiserum to factor XIII a-chain. This monoclonal antibody, 1B3, recognizes an activation-dependent epitope on a
180-kD platelet protein. The comparison among monoclonal antibody binding for the three groups studied demonstrated no statistically significant differences (Table 1). Antibody 1B3 failed to distinguish between platelets from diseased and nondiseased patients \((p=0.780)\). That this technique could detect the binding of 1B3 to activated platelets was verified by the observation that weighted fluorescence values were approximately twofold higher after thrombin stimulation. In addition, the expression of 1B3 binding sites on platelets after thrombin stimulation could be readily seen in the fluorescence histograms (Figure 2).

A second set of patients with PVD was studied by use of anti-factor XIII a-chain antibody to another activation-dependent antigen, CD63. For analysis, the patients were stratified by disease severity based on ankle–brachial pressure ratios and clinical course into moderate (0.5–0.7) or severe (<0.5) PVD. No increase in the expression of CD63 was seen in these patients; however, the platelet-associated factor XIII was significantly increased in both the moderate and severe PVD groups compared with the disease-free daily controls (Table 2). Although the average percentage of platelets expressing factor XIII was higher in the severe PVD group than in the moderate PVD group, the difference was not statistically significant.

Between the first and second studies, the flow cytometer used in these investigations underwent an instrument upgrade. This resulted in an increased sensitivity and concomitant increase in the mean fluorescence values for positive cells. Therefore, the absolute values for WFVs cannot be compared between the PVD patient groups. Valid comparisons may be made only among patients and control subjects within study 1 or study 2. Statistically significant in-

### Table 1. Binding of Monoclonal Antibody 1B3 and Polyclonal Antibody to Factor XIII a-chain to Platelets of Patients With Peripheral Vascular Disease, Age-Matched Control Subjects, and Daily Control Subjects

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Study group</th>
<th>Weighted fluorescence value ± SD</th>
<th>Range</th>
<th>(n)</th>
<th>ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Factor XIII</td>
<td>PVD</td>
<td>3.9±6.0</td>
<td>0.04–29.1</td>
<td>29</td>
<td>(p=0.001^*)</td>
</tr>
<tr>
<td></td>
<td>AMC</td>
<td>0.6±0.9</td>
<td>0.0–2.4</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DC</td>
<td>0.8±2.3</td>
<td>0–12.19</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>1B3</td>
<td>PVD</td>
<td>4.2±2.1</td>
<td>1.3–9.1</td>
<td>19</td>
<td>(p=0.780)</td>
</tr>
<tr>
<td></td>
<td>AMC</td>
<td>3.5±1.1</td>
<td>2.3–5.9</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DC</td>
<td>4.4±2.3</td>
<td>1.6–11.4</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>1B3 after thrombin</td>
<td>PVD</td>
<td>15.0±8.5</td>
<td>6.1–35.7</td>
<td>19</td>
<td>(p=0.070)</td>
</tr>
<tr>
<td></td>
<td>AMC</td>
<td>9.4±2.8</td>
<td>5.3–13.9</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DC</td>
<td>12.0±4.6</td>
<td>4.5–22.4</td>
<td>21</td>
<td></td>
</tr>
</tbody>
</table>

ANOVA, analysis of variance; PVD, peripheral vascular disease; AMC, age-matched control subjects; DC, daily control subjects.

\(^*\)PVD results were significantly different from AMC and DC by multiple comparisons test with the Bonferroni correction.
report data on patients with PVD that indicate that in this group of patients, a subpopulation of platelets expresses surface-bound factor XIII, whereas platelets from age-matched control subjects or other normal donors do not. The platelets from this same group of patients failed to show any significant binding in comparison with controls of monoclonal antibodies specific for activation-dependent expression of CD63 or the protein p180.

When platelets are activated by thrombin, factor XIIIa but not factor XIII can bind to the platelet surface.\(^5\) The capacity of platelets to bind factor XIIIa in vitro also can be induced by collagen or epinephrine but not by ADP (A.D. Cox and D.V. Devine, unpublished data). Factor XIIIa does not bind to resting platelets. We have previously used flow cytometry and anti-factor XIII a-chain antibody to study the in vitro binding of factor XIII to platelets.\(^20\) Since factor XIII must be cleaved to factor XIIIa to bind to the surface of an activated platelet, platelet-associated factor XIIIa is a marker of both thrombin generation and platelet activation.

The cause for the discrepancy in the expression of the activation antigens studied is unknown. The number of activation antigen sites is similar among the markers studied; there are \(10^8\) to \(10^9\) factor XIIIa binding sites per platelet,\(^4\) and the p180 epitope is expressed at an equivalent order of magnitude.\(^16\) Although thrombin probably mediates the activation of factor XIII, the agonists mediating platelet activation in PVD remain to be identified. Since there is no known inhibitor of factor XIIIa, the binding of this protein to nonadherent platelets in flowing blood need not occur in the immediate vicinity of the site of platelet activation. It is possible that the association of the platelet with factor XIIIa occurs in the very early stages of platelet activation when the platelet has undergone shape change but not release. Such platelets do not necessarily continue to full release. Thus, the expression of platelet-associated factor XIII may represent an earlier stage of platelet activation than that measured by antibodies such as anti-CD62 (GMP-140) or anti-CD63, which require granule release. If this is so, the measurement of platelet-associated factor XIII may provide a different and perhaps more sensitive assay for the detection of in vivo platelet activation.

The origin and molecular form of the platelet-associated factor XIII detected in these studies have not been determined. Factor XIII is found in plasma and in platelets; however, the subunit structure differs with the

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### Table 2. Binding of Monoclonal Antibody Against CD63 and Polyclonal Antibody to Factor XIII \(\alpha\)-chain to Platelets of Patients With Severe or Moderate Peripheral Vascular Disease or Disease-Free Normal Control Subjects

<table>
<thead>
<tr>
<th>Group</th>
<th>Factor XIII (% positive)</th>
<th>Factor XIII WFV</th>
<th>CD63 WFV</th>
<th>ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>Daily control subjects</td>
<td>3.2</td>
<td>5.1</td>
<td>27.0</td>
<td>28.3</td>
</tr>
<tr>
<td>Moderate PVD (n=9)</td>
<td>11.9</td>
<td>8.6*</td>
<td>54.1</td>
<td>38.0*</td>
</tr>
<tr>
<td>Severe PVD (n=17)</td>
<td>17.1</td>
<td>14.4*</td>
<td>82.5</td>
<td>80.1*</td>
</tr>
</tbody>
</table>

WFV, weighted fluorescence value; ANOVA, analysis of variance; PVD, peripheral vascular disease. Data are expressed as percent platelets in positive channels or as a WFV.

*PVD results were significantly different from daily control subjects by multiple comparisons test with the Bonferroni correction.
source. The plasma form of factor XIII is a heterotetramer composed of two catalytic a-chains and two b-chains, whereas platelet factor XIII contains only two a-chains. Platelet-associated factor XIII may be either plasma factor XIIIa generated by thrombin cleavage or platelet factor XIIIa bound back to the surface of activated platelets. We have previously demonstrated that washed platelets weakly express factor XIIIa after stimulation with thrombin without the addition of exogenous factor XIIIa.20 Although this factor XIIIa is presumably of platelet origin, it is unlikely that the platelet has a mechanism to move this protein through the plasma membrane from its cytoplasmic location.21 It is therefore probable that platelet-associated factor XIII either is of plasma origin or is derived from lysed platelets. The mechanism of the association between platelets and factor XIIIa is unexplored. There may be a specific receptor for factor XIIIa on the platelet surface. It is also possible that factor XIIIa is bound to a known factor XIIIa substrate such as fibrinogen, fibronectin, or factor V, which are themselves platelet-associated.20,22,23

It is clear that monoclonal antibodies to activation-dependent proteins or epitopes will detect major, acute platelet activation in ARDS and cardiopulmonary bypass.9,10 In our study, two activation-dependent monoclonal antibodies did not detect platelet activation in the subtle chronic condition of PVD. This is consistent with the observations of Galt et al,24 who failed to find increased CD62 expression on the platelets of patients with peripheral arterial occlusive disease. The ability to detect activated circulating platelets is of interest for several reasons. From the scientific perspective, it may provide evidence as to the ongoing relative importance of platelets in thrombosis and atherosclerosis. From the clinical point of view, it could prove useful both in defining prognosis and in the selection of therapy.

Acknowledgments
We wish to thank Drs. D. Lynn Doyle, Peter Fry, and York Hsiang, Department of Vascular Surgery, University of British Columbia, for facilitating our access to patients for this study. We also thank Katherine Serrano for technical assistance and Dr. Charles S. Greenberg, Duke University, for helpful discussion.

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Platelet-associated factor XIII as a marker of platelet activation in patients with peripheral vascular disease.
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doi: 10.1161/01.ATV.13.6.857
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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