The interaction of human thrombospondin (TSP) with GPIa-IIa and GPIIb-IIIa was studied. The binding for both proteins became time-independent after 60 min. A 7-fold excess concentration of unlabelled GPIa-IIa added either initially, or after time-dependent binding, resulted in a 50% inhibition of GPIa-IIa bound to TSP. GPIa-IIa and GPIIb-IIIa specifically bound TSP since: (a) the binding of GPIIb-IIIa to TSP was dependent on the presence of 1 mM MgCl₂ and 1 mM CaCl₂, whereas binding of GPIa-IIa was ion-independent. (b) The binding was saturable, with dissociation constants of 0.69 ± 0.17 µM and 3.77 ± 1.02 µM for GPIa-IIa and GPIIb-IIIa respectively. (c) GPIIb-IIIa and GPIa-IIa did not significantly bind to BSA. (d) GPIIb-IIIa bound fibrinogen ion-specifically, whereas little or no binding of GPIa-IIa was detectable. (e) Both GPIIb-IIIa and GPIa-IIa bound collagen in an ion-independent manner. (f) GPIIb-IIIa did not compete with GPIa-IIa for binding to TSP. (g) Binding of GPIa-IIa to TSP was inhibited with anti-(GPIa-IIa) (6F1), whereas mouse IgG and anti-(GPIIb-IIIa) (AP-2) had no effect. (h) The interaction of GPIIb-IIIa with TSP is 5.5-fold more favourable than that of GPIa-IIa, suggesting that GPIa-IIa may be a preferred binding protein for TSP-mediated platelet adhesion.

INTRODUCTION

Thrombospondin (TSP) is a major platelet-secreted protein, that was first purified in its non-denatured state by Lawler and co-workers in 1978 [1]. Since then a great number of studies have been published describing its structure, biological function and tissue distribution. For example, TSP has been found to be a multi-domain protein much like fibronectin with binding sites for heparin [2], collagen [3], and fibrinogen [4]. A number of studies have demonstrated that TSP promotes cell-substratum adhesion of a variety of cells including fibroblasts [5], endothelial cells [5,6], epithelial cells [5] and various tumour cell lines such as melanoma cells [5-8], small-cell carcinoma cells [8], and osteosarcoma cells [9]. Recent studies have also postulated a role for TSP in cell proliferation [10] and angiogenesis [11].

A major interest of ours has been to define a role for TSP in haemostasis. Toward this end, we have observed that TSP promotes platelet aggregation [12] and platelet adhesion [13]. A possible mechanism which might account for our previous observations would require secreted TSP to bind to platelet receptors and cross-link platelets to themselves and to exposed collagen fibrils in the damaged vessel. A similar mechanism, which proposes that TSP stabilizes platelet aggregates by cross-linking platelet-bound fibrinogen, has been proposed previously to explain the role of TSP in platelet aggregation [14].

Our previous studies have identified two potential platelet TSP-biding proteins which could function to bind secreted TSP. The first is GPIIb-IIIa, the platelet fibrinogen receptor, which reversibly binds TSP in vitro [15] and antibodies against which inhibit TSP-promoted platelet aggregation and adhesion [13,15]. The second is GPIa-IIa, a platelet collagen receptor [16]. It was shown that human platelets from a patient with a bleeding disorder, showing a deficiency of glycoprotein Ia, failed to respond to collagen [17] and displayed a decreased platelet adhesion to subendothelium [18]. We have shown that anti-(GPIa-IIa) monoclonal antibodies (mAbs) completely inhibited the TSP-dependent adhesion of normal and thrombasthenic platelets [13]. This inhibition was dose-dependent and complete inhibition could be achieved with as little as 2 µg/ml of antibody, while no more than 50% inhibition of adhesion could be achieved with anti-(GPIa-IIa) mAb at concentrations in excess of 50 µg/ml. These results suggested that the interaction of GPIa-IIa with TSP must be more favourable than the interaction of GPIIb-IIIa with TSP, despite the fact that platelets contain approx. 50000 GPIIb-IIIa receptors as measured by anti-(GPIIb-IIIa) mAb binding [19], and only 800 GPIa-IIa receptors as measured by anti-(GPIa-IIa) mAb binding [20].

The aim of the present study was to demonstrate that TSP could interact with GPIa-IIa in a specific and favourable manner using an in vitro binding assay. Our results show that TSP can specifically interact with GPIa-IIa and that this interaction is more favourable than the interaction of TSP with GPIIb-IIIa. These results suggest that GPIa-IIa could function as the physiologically preferred platelet TSP-binding protein.

EXPERIMENTAL

Materials

All reagents, unless specified otherwise, were purchased from Sigma. Reagents for SDS/PAGE were obtained from Bio-Rad. Iodobeads were purchased from Pierce. Na¹²⁵I was purchased from Amersham. mAb Gi9, specific against human platelet GPIa-IIa, and mAb CD41a, specific against platelet GPIIb-IIIa, were purchased from Amac. mAb AP-2 was kindly provided by Dr. Thomas J. Kunicki, The Blood Center of Southeastern Wisconsin, Milwaukee, WI, U.S.A. AP-2 is directed against epitopes on both GPIIb and GPIIIa [21]. mAb 6F1, specific for human platelet GPIIb-IIa [20], was kindly provided by Dr. Barry S. Collier (Department of Medicine, State University of New York, Stony Brook, NY, U.S.A.). Microtitre plates, Removawell

Abbreviations used: TSP, thrombospondin; PGE₁, prostaglandin E₁; BCA, bicinchoninic acid; mAb, monoclonal antibody.

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Immulon 4, were purchased from Dynatech. CNBr-activated Sepharose was purchased from Pharmacia.

**TSP purification**

TSP was purified from Ca²⁺ ionophore (A23187)-activated human platelets as described previously [4].

**Protein assays**

Protein concentrations were determined by the bicinchoninic acid (BCA) protein assay, adapted for microtitre plates, as described by Pierce. BSA was used as the standard.

**PAGE**

SDS/PAGE was performed using the Pharmacia Phast Gel system. Gels were stained, dried on to paper, and autoradiograms were prepared from the dried gels using intensifying screens (DuPont Cronex Lightning Plus screens mounted in Spectroline Cassettes, Reliance X-Ray, Oreland, PA, U.S.A.). Kodak X-Omat-AR film was used and developed according to the instructions provided with the film. Films were exposed overnight at −70 °C.

**Protein labelling**

Purified GPIIb-IIIa, GPIIb-IIa, and TSP were labelled with ¹²⁵I using Iodobeads as described previously [15]. Briefly, 50 µg of purified protein dissolved in octylglucoside buffer (see method for purification of GPIIb-IIa and GPIIb-IIIa) was incubated with one Iodobead for 5 min. Unreacted iodide was removed on a small column of Sephadex G-25 equilibrated in octylglucoside buffer as previously described [22]. The specific radioactivities of protein obtained from typical labelling experiments ranged between 5 × 10⁶ and 1 × 10⁷ c.p.m./µg. When comparing the binding of GPIIb-IIIa with GPIIb-IIa, specific radio-activities were adjusted to the same value (1 × 10⁶ c.p.m./µg).

**Purification of GPIIb-IIa and GPIIb-IIIa**

GPIIb-IIa and GPIIb-IIIa were purified by immunoaffinity chromatography from platelet detergent extracts. Briefly, 5 units of washed platelet pellets, obtained after the centrifugation step following ionophore stimulation to obtain TSP [4], were dissolved in 10 ml of octylglucoside buffer (50 mM Tris/HCl, pH 7.35, containing 150 mM NaCl, 100 mM octylglucoside, 100 µM leupeptin and 1 mM phenylmethanesulphonyl fluoride). Undissolved material was removed by centrifugation of the detergent extract at 4 °C for 30 min at 10 000 g. Platelet detergent extract (10 ml) was first passed through an anti-(GPIIb-IIIa) mAb-Sepharose column (prepared by coupling 2 mg of CD41a to 1 ml of CNBr-activated Sepharose according to the instructions provided by Pharmacia) and then over an anti-(GPIIb-IIa) mAb-Sepharose column (prepared by coupling 2 mg of G9 to 1 ml of CNBr-activated Sepharose). Antibody-treated columns were washed with 10 ml of binding buffer, containing 0.5 M NaCl, and eluted with 100 mM glycine, pH 2.7, containing 50 mM octylglucoside. Fractions (0.5 ml) were collected into tubes containing 10 µl of 1 M Tris/HCl added to the collection tubes to neutralize the solution. Peak protein fractions were pooled and concentrated.

Approx. 2 mg of GPIIb-IIIa and 100 µg of GPIIb-IIa were isolated from 5 units of washed platelets.

**Binding assays**

Samples (50 µl) of radiolabelled GPIIb-IIIa or GPIIb-IIa, containing approx. 100 000 c.p.m. and dissolved in binding buffer (50 mM Tris/HCl, pH 7.35, containing 150 mM NaCl, 50 mM octylglucoside, 100 µM leupeptin and 1 mM phenylmethanesulphonyl fluoride) were incubated for 1 h in detachable microtitre wells (Immulon 4 Removawell) which had been previously coated overnight at 4 °C with 50 µl of a 40 µg/ml TSP solution in 20 mM Bis-Tris-propane buffer, pH 6.5, and blocked for 1 h with 200 µl of 1% (w/v) BSA. Wells were then washed three times by aspiration with 200 µl of binding buffer and bound radioactivity levels determined by counting each well in a γ-counter.

Wells were saturated with 1 µg of adsorbed protein under the conditions of our protein-coating procedure, as determined from radioactive protein studies and the BCA protein assay (results not shown). The amount of radioactive protein bound ranged from 5 to 10% of input radioactivity. Radioactive protein bound to control BSA-coated wells was, in all experiments, less than 5% of that bound to TSP or glycoproteins, and was subtracted from experimental values given for test proteins unless otherwise stated. For SDS/polyacrylamide gel analysis, bound GPIIb-IIa was eluted from immobilized TSP by incubation of washed wells with 50 µl of SDS/PAGE sample buffer.

**RESULTS**

**Analysis of purified GPIIb-IIa and GPIIb-IIIa**

The platelet integrins, GPIIb-IIa, and GPIIb-IIIa, were purified from washed human platelets by antibody-affinity chromatography. The purified integrins were radiolabelled with [¹²⁵I]iodine and typically specific radioactivities of 10⁶ c.p.m./µg were obtained. The silver-stained as well as the radiolabelled proteins, were characterized on SDS/polyacrylamide gels (Figure 1). The low-molecular-mass impurities in GPIIb-IIa are probably proteolytic breakdown products, because they immunoprecipitate with anti-(GPIIb-IIa) antibody (results not shown). However, they do not interfere with the binding experiments as GPIIb-IIa, which eluted from the binding plates, contained very little of the lower-molecular-mass material (compare lanes 2 and 3 in Figure 1). The major polypeptides bound are the unlagged α and β chains of GPIIb-IIa. The estimated apparent molecular masses obtained for the purified proteins on our gel system (130 and 97 kDa for GPIIb-IIIa and 150 and 120 kDa for GPIIb-IIa) were consistent with published values [19,20]. In addition, SDS/polyacrylamide gel analysis of the 6F1 and AP-2 immunoprecipitates of labelled GPIIb-IIa and GPIIb-IIIa revealed the same bands as shown in Figure 1 (results not shown).

**The requirements for binding of GPIIb-IIa and GPIIb-IIIa to immobilized TSP, collagen and fibrinogen**

The binding of GPIIb-IIa to TSP became time-independent after 60 min and could be partially reversed with the addition of a 7-fold excess of unlabelled GPIIb-IIa at time zero or after binding had proceeded for 45 min (Figure 2). GPIIb-IIIa could not prevent the binding of GPIIb-IIa to TSP or displace bound GPIIb-IIa after binding had proceeded for 45 min, indicating that GPIIb-IIa and GPIIb-IIIa do not share the same binding domains on TSP (Figure 3). The bivalent ion requirements for binding of GPIIb-IIa and GPIIb-IIIa to TSP differ significantly (Table 1). For
example, maximal binding of GPIIb-IIIa to TSP requires 1 mM CaCl₂ and 1 mM MgCl₂, whereas the binding of GPI-Il is ion-independent. The ion dependence for the interaction of GPIIb-IIIa and GPIIb-IIIa with TSP is the same whether TSP is immobilized and fluid-phase glycoprotein is bound, or whether glycoprotein is immobilized and fluid-phase TSP is bound (results not shown); this suggests that the binding of TSP to glycoprotein is not dependent on conformational changes in protein caused by adsorption to microtitre wells. Both GPIIb-IIIa and GPIIa-IIa were found to bind collagen in an ion-independent manner (Table 2). In contrast, GPIIb-IIIa bound fibrinogen in an ion-dependent manner.

The effect of anti-(GPIb-IIa) antibody on the binding of GPIIb-IIa to TSP
To establish further the specificity of the interaction of GPIIb-IIa with TSP, we assessed the capacity of anti-(GPIIb-IIa) (6F1) to block binding. mAb 6F1 has previously been shown to block TSP-dependent platelet adhesion [13]. We found that 6F1 blocked the binding of GPIIb-IIa to TSP. For example in the presence of 10 μg/ml of AP-2, an mAb raised against GPIIb-IIIa, 1430±200 c.p.m./well was bound while 1600±300 c.p.m./well was bound for the buffer control. Elution of 125I-GPIIb-IIa bound to TSP revealed that the majority of the bound counts represented GPIIb-IIa and not small amounts of contaminating proteins present in the starting material (compare lanes 2 and 3 in Figure 1). These results not only confirm that GPIIb-IIa binds TSP specifically, but...
Table 2 Comparison of the effect of bivalent cations and EDTA on the binding of 125I-labelled GPIa-IIa and GPIb-IIIa to TSP, collagen, fibrinogen and BSA

The binding of GPIa-IIa and GPIb-IIIa to immobilized TSP, rat tail type-I collagen (coll), human fibrinogen (FG) and BSA was compared. Binding buffer contained either 2 mM EDTA or 1 mM CaCl\(_2\) and 1 mM MgCl\(_2\). The concentration of radiolabelled integrins was 30 \(\mu\)g/ml. Values given are the means of two duplicate determinations and are representative of a typical experiment.

<table>
<thead>
<tr>
<th>Fluid-phase 125I-protein bound (c.p.m.)</th>
<th>Solid phase</th>
<th>EDTA</th>
<th>Mg(^{2+})/Ca(^{2+})</th>
<th>EDTA</th>
<th>Mg(^{2+})/Ca(^{2+})</th>
</tr>
</thead>
<tbody>
<tr>
<td>GPla-IIa</td>
<td>TSP</td>
<td>1310 ± 60</td>
<td>1130 ± 81</td>
<td>200 ± 12</td>
<td>870 ± 32</td>
</tr>
<tr>
<td></td>
<td>Coll</td>
<td>1400 ± 82</td>
<td>660 ± 66</td>
<td>740 ± 72</td>
<td>980 ± 42</td>
</tr>
<tr>
<td></td>
<td>FG</td>
<td>150 ± 42</td>
<td>280 ± 28</td>
<td>10 ± 6</td>
<td>1000 ± 21</td>
</tr>
<tr>
<td></td>
<td>BSA</td>
<td>10 ± 5</td>
<td>10 ± 5</td>
<td>10 ± 5</td>
<td>10 ± 5</td>
</tr>
</tbody>
</table>

Table 3 The effect of fluid-phase TSP on the binding of GPIa-IIa to solid-phase TSP

TSP-coated microtitre wells were incubated for 1 h at room temperature with 50 \(\mu\)l of a 1.5 \(\mu\)g/ml solution of 125I-labelled GPla-IIa in binding buffer containing various concentrations of TSP, mouse IgG and BSA. The protein-GPla-IIa solutions were preincubated for 30 min before addition to TSP-coated wells. Wells were washed and the bound radioactivity counted. Values are the means of three determinations ± S.E.M. Percentage inhibition was calculated as [c.p.m. (\(\mu\)g/ml TSP)] - c.p.m. (X \(\mu\)g/ml TSP)]/c.p.m. (0 \(\mu\)g/ml TSP) X 100, where X is 5, 10, 20, or 40.

<table>
<thead>
<tr>
<th>Protein ((\mu)g/ml)</th>
<th>TSP</th>
<th>IgG</th>
<th>BSA</th>
<th>Percentage inhibition by TSP (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2230 ± 335</td>
<td>2480 ± 463</td>
<td>2180 ± 286</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>1160 ± 40</td>
<td>2430 ± 305</td>
<td>2005 ± 329</td>
<td>48</td>
</tr>
<tr>
<td>10</td>
<td>1092 ± 87</td>
<td>2307 ± 152</td>
<td>1920 ± 192</td>
<td>51</td>
</tr>
<tr>
<td>20</td>
<td>969 ± 48</td>
<td>1988 ± 286</td>
<td>1965 ± 126</td>
<td>70</td>
</tr>
<tr>
<td>40</td>
<td>332 ± 44</td>
<td>2040 ± 180</td>
<td>1918 ± 207</td>
<td>85</td>
</tr>
</tbody>
</table>

Figure 4 Concentration dependence of the binding of 125I-labelled GPIa-IIa and GPIb-IIIa to TSP

Binding was performed to immobilized TSP in binding buffer containing 1 mM CaCl\(_2\) and 1 mM MgCl\(_2\). The closed and open circles depict the binding obtained with GPla-IIa and GPIb-IIIa respectively. Specific radioactivities for both proteins were adjusted to the same value (10\(^5\) c.p.m./\(\mu\)g). Values given are the means of two duplicate determinations and are representative of a typical experiment. The line drawn was computer generated by a least-squares-fit to a rectangular hyperbola with calculated dissociation constants of 0.69 ± 0.17 \(\mu\)M (± S.E.M.) for GPla-IIa and 3.77 ± 1.02 \(\mu\)M (± S.E.M.) for GPIb-IIIa with correlation coefficients of 0.933 and 0.938 respectively (GraphPad Inplot, Version 3.1, San Diego, CA, U.S.A.).

**DISCUSSION**

The identity of the cell receptor mediating TSP-dependent cell-adhesive interactions has been of great interest to us because of our previous work showing that antibodies raised against the platelet fibrinogen receptor, GPIIb-IIIa, inhibited TSP-dependent platelet aggregation [12] and TSP-dependent melanoma cell adhesion [23], and that GPIIb-IIIa could bind TSP in vitro [15]. Similar observations were made by Lawler and co-workers who showed that GPIIb-IIIa-like integrin receptors could be isolated by TSP-affinity chromatography from endothelial and smooth muscle cells [24] and from platelets [25]. However, the specific functional consequence of these binding interactions remained unclear since GPIIb-IIIa and (GPIIb-IIIa)-like integrin receptors display a broad reactivity with a number of adhesive proteins, notably fibrinogen [26]. In addition, several studies have failed to show an interaction of GPIIb-IIIa with TSP in vitro. For example, Leung and Nachman [27], using an enzyme-linked immunosassay, could demonstrate an interaction between GPIIb-IIIa and fibrinogen but not between GPIIb-IIIa and TSP. Similarly, Pytel et al. [28], using GPIIb-IIIa incorporated into liposomes, could not demonstrate an interaction with TSP.

Non-integrin TSP-cell binding proteins have also been described. Human lung small-cell carcinoma has been shown to bind TSP through a two-chain receptor having molecular masses of 80000 and 105000 [29]. Human lung carcinoma cells contain a 50000 molecular-mass CSVTCG-specific TSP receptor [30]. Platelet GPIV or CD36 has been referred to as the platelet TSP receptor because platelet GPIV was found to bind TSP in vitro [31] and GPIV was found to mediate platelet-monocyte interactions in a TSP-dependent manner [32]. However, the presence

also lend support to our previous studies showing that GPIIa-IIa may function as a platelet TSP-binding protein [13].

To establish that binding of GPIIa-IIa to solid-phase TSP is the same as binding to fluid-phase TSP, fluid-phase TSP was evaluated for its capacity to inhibit binding of GPIIa-IIa to solid-phase TSP. We found that increasing concentrations of fluid-phase TSP competed for the binding of solid-phase TSP, whereas IgG and BSA had no effect (Table 3). Therefore, fluid-phase and solid-phase TSP bind GPIIa-IIa.

**Comparison of the binding constants for the interaction of GPIIa-IIa and GPIIb-IIIa with TSP**

We previously observed that TSP-mediated platelet adhesion could be completely inhibited with anti-(GPIIa-IIa) mAbs but only partially inhibited with anti-(GPIIb-IIIa) mAbs [13]. These results suggested that the interaction of GPIIa-IIa with TSP was more favourable than the interaction of GPIIb-IIIa with TSP. To test this hypothesis, the apparent dissociation constant \(K_d\) for the interaction of TSP with GPIIb-IIIa was measured and compared with that obtained for GPIIa-IIa (Figure 4). We found that the \(K_d\) value for the interaction of GPIIa-IIa with TSP was 0.69 \(\mu\)M and that for the interaction of GPIIb-IIIa with TSP was 3.77 \(\mu\)M. These results indicate that GPIIa-IIa binds TSP 5.5-fold more favourably than GPIIb-IIIa.
or absence of CD36 in malaria-parasitized erythrocytes had no effect on TSP binding, suggesting that CD36 is not a TSP receptor in this system [33]. Platelets deficient in GPIb bound TSP to the same extent as normal platelets containing GPIb, suggesting that GPIb is not a TSP receptor in this system as well [34].

In an effect to identify and characterize platelet TSP receptors further, we recently developed a rapid and sensitive cell-adhesion assay [35]. We have used this assay to identify possible platelet and cell-adhesion receptors. In a recent study aimed at identifying potential platelet TSP receptors, we discovered that antibodies against the platelet fibrinogen receptor, GPIIb-IIIa, and GPIIb-IIa, the collagen receptor, inhibited TSP-mediated platelet adhesion [13].

To establish further that GPIIb-IIIa and GPIIIa-IIia could function as platelet-adhesion receptors, as suggested from our earlier antibody studies, we undertook the present study comparing the binding interactions of GPIIb-IIia and GPIIIa-IIia with TSP using an in vitro binding assay. Our results indicate that both GPIIb-IIia and GPIIIa-IIia interact with TSP in a reversible and saturable manner. The binding interaction between TSP and GPIIb-IIia differs significantly from that of GPIIIa-IIia. GPIIb-IIia interacts with TSP in an ion-independent manner, whereas GPIIIa-IIia requires Ca\(^{2+}\) and Mg\(^{2+}\). Although Mn\(^{2+}\) has been shown to promote fibronectin-receptor-mediated adhesion and binding [36], in this system Mn\(^{2+}\) did not potentiate the binding of either purified GPIIb-IIia or GPIIIa-IIia to TSP. This result is consistent with the observation that Mn\(^{2+}\) does not promote collagen- and fibrinogen-dependent platelet aggregation (G. P. Tuszyński and M. A. Kowalska, unpublished work). Although Mg\(^{2+}\) has been shown to promote and Ca\(^{2+}\) to inhibit binding of GPIIb-IIia to collagen [37], we observed no bivalent-cation-dependent binding of GPIIb-IIia in our studies in vitro. A possible explanation for this difference is that we studied binding of GPIIb-IIia in detergent solution, whereas previous studies measured binding of GPIIb-IIia incorporated into lipid vesicles. These lipid vesicles could alter bivalent-cation requirement for binding. However, under physiological conditions bivalent-cation binding of GPIIb-IIia may not occur as plasma has high levels of Ca\(^{2+}\). Therefore the non-cation-dependent interaction between GPIIb-IIia and its ligands would be favoured.

GPIIb-IIia binds to different domains on TSP as GPIIIa-IIia neither competes with the binding of GPIIb-IIia to TSP nor displaces bound TSP. These latter observations suggest that TSP can bind to both GPIIb-IIia and GPIIIa-IIia on the platelet surface. Finally, the binding interaction between GPIIb-IIia and TSP is 5.5-fold more favourable than the binding interaction between GPIIb-IIia and TSP, suggesting that GPIIb-IIia may function as a high-affinity platelet TSP-binding protein.

The number of GPIIb-IIia sites on platelets has been reported to be 800 per platelet [20]. This number is consistent with the number of TSP molecules binding to non-stimulated platelets which was reported by Wolff et al. [38] to be 760 per platelet in the absence of added bivalent cations. In addition, consistent with the bivalent-cation-independent binding observed in our studies in vitro, these authors observed little or no cation dependence for binding of TSP to unstimulated platelets, finding that in the presence of EDTA, prostaglandin E\(_2\) (PGE\(_2\)) and theophylline, 760 molecules per platelet were bound. Our previous studies showing inhibition of TSP-dependent platelet adhesion by anti-GPIIb-IIia were done using PGE\(_2\)-treated platelets [13].

Our results may help to explain the haemorrhagic diathesis of a patient who suffered a bleeding tendency, characterized by frequent petechial bleeding, post-operative hemorrhages and severe menstrual bleeding episodes, necessitating blood trans-

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