Platelets Deficient in Glycoprotein IIIb Aggregate Normally to Collagens Type I and III But Not to Collagen Type V

By Beate Kehrel, Antje Kronenberg, Jürgen Rauterberg, Doris Niesing-Bresch, Ulrike Niehues, Joachim Kardoeus, Barbara Schwippert, Diethelm Tschöpe, Jürgen van de Loo, and Kenneth J. Clemetson

The aggregation of platelets induced by collagens is considered an important step in primary hemostasis. Glycoprotein (GP) IIb (GPIIIb, GP IV, CD36) has been proposed as a blood platelet receptor for collagen. Platelets from three healthy blood donors were shown to be clearly deficient in GPIIIb. These platelets aggregated normally in response to type I and III collagens. In addition, platelet factor 4, β-thromboglobulin, and adenosine triphosphate (ATP) secretion in response to type I and III collagens was normal. The findings indicate that GPIIIb is not the major, essential collagen receptor for type I and III collagens. This would explain why all individuals with GPIIIb-deficient platelets examined so far are healthy and, in particular, show no apparent evidence of hemostatic problems. However, in contrast to control platelets, no aggregation and impaired platelet factor 4, β-thromboglobulin, and ATP secretion was observed in response to type V collagen. Therefore, it is postulated that for type V collagen-induced aggregation both GPla/IIa and GPIIIb are essential.

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Platelet interaction with collagen is a crucial event in hemostasis and in the pathogenesis of thrombosis and arteriosclerosis. When the endothelial lining of blood vessels is breached, the membranes of blood platelets interact with the collagen of the connective tissue initiating primary hemostasis. Several structurally distinct types of collagen are well documented. Walls of large arteries contain the two interstitial collagens type I and III in relative abundance.1 In addition, other collagens are present in the vessel wall. Because type V collagen, a secretion product of endothelial cells and smooth muscle cells, is increased in arteriosclerotic plaques,2 type V collagen-induced platelet aggregation may be relevant to development of thrombotic events.

For many years much effort has been devoted to the identification of the platelet collagen receptor. Several platelet proteins, such as fibronectin,3 von Willebrand factor,4 thrombospondin,5 and the zymogen form of platelet collagen factor XIII,6 have been proposed as the collagen receptor. Moreover, a number of platelet membrane proteins have been shown to bind directly to collagen. Chiang and Kang7 described a 65-kD membrane protein from Triton extracts of surface-labeled platelets that bound to insoluble human collagen. Lahav8 isolated a 80-kD surface protein from rabbit platelets with a high affinity for fibrillar bovine type I collagen.

Molecular level studies on platelets with defective collagen-induced aggregation provided evidence for further possible collagen receptors. Moroi et al.10 described a patient with platelets deficient in glycoprotein VI (GPVI) that lack both collagen-induced aggregation and adhesion to type I and III collagen fibrils. Nieuwenhuis et al.11 and Kehrel et al.12 reported unrelated patients with platelets deficient in GPla showing bleeding disorders attributable to a defect in platelet collagen interaction; in addition, the platelets of our patient lacked intact thrombospondin.12 Other independent lines of evidence provide support for GPla playing a key role in platelet collagen interaction.13-15

GPIIIb, also known as GP IV or CD36, has been reported by Tandon et al.16,17 to be a collagen receptor. Purified GP IIIb binds to collagen type I fibrils and Fab fragments of polyclonal antibodies raised against purified GP IIIb inhibit collagen-induced platelet aggregation. Furthermore, soluble GP IIIb competes with membrane-bound GP IIIb and inhibits platelet activation by type I collagen. McGregor et al.18 described the inhibition of platelet aggregation by anti-GP IIIb F(ab)2 in response to low doses of collagen.

GP IIIb was recently reported to be absent from platelets from some healthy Japanese blood donors by Yamamoto et al.19 We now report studies on the aggregation and secretion of GP IIIb-deficient platelets from healthy Japanese donors in response to human and bovine type I, III, and V collagens and equine “Horm” collagen.

Materials and Methods

Blood donors. J.K. (35 years old), T.Y. (38 years old), and Y.A. (33 years old) were three healthy Japanese males with no apparent evidence of hemostatic problems and a normal bleeding time (Simple II: less than 5 minutes). Platelets for control purposes were obtained from healthy female and male adult Japanese and European volunteers. All blood donors denied having taken any medication for at least 14 days before sampling.

Preparation of platelets. Blood was taken by venipuncture of the antecubital vein after informed consent was obtained. Nine parts of blood were anticoagulated with one part of trisodium citrate (0.108 mol/L). Platelet-rich plasma (PRP) was prepared by differential centrifugation at 250g for 10 minutes at room temperature.
**Surface labeling.** Washed platelets were surface-labeled by the periodate/NaBH₄ procedure.

**Dot-blot assay and flow cytometry.** Platelets were screened for GPIIIb deficiency by a dot-blot assay using monoclonal and polyclonal antibodies, and the deficiency was confirmed by flow cytometry as described previously.

**Crossed immunoelectrophoresis.** Absence of GPIIIb in these platelets was confirmed by crossed immunoelectrophoresis combined with immunoblotting of coprecipitated mouse anti-GPIIIb monoclonal antibodies (MoAbs) OKM5 (Ortho Diagnostic Systems, Neckargemünd, Germany) and IOP36 (Immunochem, Mar- seille, France) applying the method of Solum et al with slight modifications. Second-dimensional electrophoresis was performed at a field strength of 1 V/cm for about 18 hours. The coprecipitated MoAbs were passively transferred onto nitrocellulose and stained by alkaline phosphatase-conjugated goat F(ab)₂ antimouse IgG, human and rabbit serum proteins adsorbed (Sigma, Munich, Germany), with p-nitroblue tetrazolium chloride/5-bromo 4-chloro 3-indolyl phosphate p-toluidine (NBT/BCIP; Bio-Rad, Munich, Germany) as a substrate.

**Two-dimensional isoelectric focusing (IEF)/sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).** Two-dimensional IEF/SDS-PAGE of platelet proteins was also used to examine GP deficiencies. Gels were stained with silver. The first-dimension IEF was performed by the horizontal technique using an immobilized pH gradient in the first dimension according to Görg et al adapted to platelet protein analysis as follows. For the electrophoresing, 5-mm-wide strips were cut from a dried 0.5-mm-thick gel with an immobilized linear pH gradient (pH 4 to 9) and rehydrated in a solution containing urea (8 mol/L), Triton X-100 (2% vol/vol), n-octyl β-D-glucopyranoside (2% vol/vol; Pharmacia, Freiburg, Germany), dithioerythritol (DTE; 10 mmol/L), phenylmethylsulfonyl fluoride (8 mmol/L; Boehringer-Mannheim, Mannheim, Germany), and 2-D Pharmalyte pH 3 to 10 (0.1% vol/vol; Pharmacia, Freiburg, Germany). Platelet proteins were solubilized in a lysis solution containing urea (9 mol/L), Triton X-100 (2% vol/vol), DTE (10 mmol/L), and 2-D Pharmalyte pH 3 to 10 (0.1% vol/vol; Pharmacia, Freiburg, Germany). Platelet proteins were solubilized in a lysis solution containing urea (9 mol/L), Triton X-100 (2% vol/vol), n-octyl β-D-glucopyranoside (100 mmol/L; Calbiochem, Frankfurt, Germany), DTE (10 mmol/L), 2-D Pharmalyte pH 3 to 10 (0.1% vol/vol; Pharmacia, Freiburg, Germany), and phenylmethylsulfonyl fluoride (8 mmol/L; Boehringer-Mannheim). Ten microliters of platelet lysate containing 100 μg platelet protein was mixed with 10 μL of Ultro- dix suspension (40 μg Ultrodes [Pharmacia] in 1 mL solubilization solution swollen overnight at 4°C) and applied to the gel strip. After a prerun at 150 V for 1 hour and at 300 V for 1 hour, first-di- mensional electrophoresing was performed at 900 V for 18 hours. Supplementary electrophoresing was performed at 3,000 V for 3 hours using filter paper soaked in distilled water to make contact with the electrodes. For the second dimension, the first-dimen- sional gels were equilibrated in a buffer containing Tris/HCl, pH 6.8 (50 mmol/L), urea (6 mol/L), glycerol (30%), SDS (2%), and DTE (65 mmol/L), and treated afterwards with iodoacetamide (260 mmol/L) in equilibration buffer. They were placed on top of a hori- zontal 7.5% acrylamide SDS separation gel (with a 4.5% stacking gel) and electrophoresis was performed in Tris/glycine/SDS buffer (Tris at 25 mmol/L, glycine at 192 mmol/L, and SDS at 1%) starting with 20 mA for 60 minutes and then with 30 mA for about 3 hours.

**Collagen purification.** Human and bovine type I and III colla- gens were purified from fetal skin and type V collagen from placenta after limited pepsin digestion.

**RESULTS**

Platelets from J.K., T.Y., and Y.A. were found to be deficient in GPIIIb by screening using a dot-blot assay.

**Flow cytometry.** Absence of GPIIIb was confirmed by flow cytometry as described previously for T.Y. and J.K. and as shown for Y.A. in Fig 1.

**Crossed immunoelectrophoresis.** Crossed immunoelec- trophoresis applied to proteins of control platelets showed an arc with low anodal mobility that was identified as...
GPIIb by immunoblotting (Fig 2A). No GPIIb-related arc was seen in the analysis of platelets from J.K., Y.A. (Fig 2B), or T.Y.

Two-dimensional IEF-SDS-PAGE/surface-labeling. Fluorograms of two-dimensional IEF-SDS-PAGE separations of whole platelets surface-labeled by the periodate NaB₃H₄ method detect GPIIb in control platelets (Fig 3A) most readily, but no spot at the position of GPIIb is observed in Japanese Nak⁺⁻ donor J.K. (Fig 3B). There is an additional small spot slightly above the position of GPIIb in this donor’s platelet proteins.

IEF-SDS-PAGE/silver staining. Two-dimensional IEF-SDS-PAGE of proteins from control platelets followed by silver staining showed prominent spots (Fig 3C) corre-
sponding to GPIIIb as identified by immunoblotting. No GPIIIb-related spots were found in the gel of platelets from J.K., Y.A. (Fig 3D), and T.Y.

Western blots. To further confirm the apparent absence of GPIIIb, protein blots were performed using polyclonal anti-GPIIIb antibody. A strong positive reaction corresponding to an 88-kD band was seen with control platelets, but this band was not observed with Japanese Nak’s donor Y.A. (data not shown).

Platelet aggregation. All aggregation experiments were performed at least twice and with all agonists dose-response relationships were studied. Aggregation of the GPIIIb-deficient platelets from all three donors was normal in response to ADP (2 μmol/L), calcium ionophore A23187 (1.5 μmol/L), epinephrine (4 μmol/L), thrombin (0.15 U/mL), arachidonic acid (1 mmol/L), ristocetin (1 mg/mL), and wheat germ agglutinin (60 μg/mL).

The response to bovine methylated type I and type III collagens was normal as shown by the aggregation patterns of representative GPIIIb-deficient platelets from donor Y.A. (Fig 4B and D) as well as the response to equine “Horm” collagen (1 μg/mL), human type I collagen (30 μg/mL), and human type III collagen (20 μg/mL). In contrast to normal platelets, aggregation of GPIIIb-deficient platelets from all three donors could not be induced by human or bovine type V collagen at concentrations up to 200 μg/mL. (Fig 5B). With this type of collagen, the threshold concentration inducing full aggregation was 60 μg/mL (mean) as estimated with 12 controls (range, 20 to 80 μg/mL).

Platelet secretion. GPIIIb-deficient platelets responded with normal ATP release (Fig 4B and D) to bovine methylated collagens type I and type III. Secretion of α-granule from GPIIIb-deficient platelets induced by bovine methylated type I and type III collagens studied by measuring the release of βTG (Fig 6A and B) and PF4 into the plasma did not differ from normal platelets.

In accordance with the aggregation results, both ATP and α-granule secretion in response to bovine type V collagen of GPIIIb-deficient platelets was significantly impaired (Figs 5B and 6C).

DISCUSSION

Using several different methods, we showed that platelets from three healthy Japanese blood donors were deficient in

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**Fig 4.** Aggregation and ATP release in response to bovine methylated type I and type III collagens of control (A and C) and GPIIIb-deficient platelets from representative donor Y.A. (B and D) monitored on a lumi-aggregometer.
GPlllb-deficient platelets from control (A) and GPlllb-deficient platelets from representative donor Y.A. (B) monitored on a lumi-aggregometer.

Our findings that GPIIlb-deficient platelets aggregate normally with type I and III collagens indicate that this GP does not have an essential role as aggregation receptor for these collagens. Thus, the interpretation by Tandon and Jamieson17 and McGregor et al18 of their observation that Fab fragments of a polyclonal antibody against GPIIlb inhibited collagen-induced aggregation and that GPIIlb in solution competed with membrane GPIIlb for collagen decreasing platelet aggregation should be reconsidered.

Tandon and Jamieson17 reported that Fab fragments of anti-GPIIlb antibodies inhibited aggregation induced by ADP and epinephrine and McGregor et al18 also found inhibition of aggregation induced by low doses of thrombin. This indicates that the inhibitory effect is rather general and not confined to collagen, which favors the idea that GPIIlb is involved in signal transduction as proposed by Schüpp et al.23 This is supported by the observation by Aiken et al34 that the MoAb OKM5 induces platelet aggregation and secretion but also inhibits binding of fibrinogen, fibronectin, and von Willebrand factor to thrombin-activated or phorbol mystrate acetate-activated platelets. The activation effect requires intact IgG, whereas, for the inhibition effects, F(ab)_2 fragments suffice. This implies that it is necessary to bring Fc receptors into proximity to GPIIlb by cross-linking with intact antibody.24 A role of GPIIlb in transducing some collagen-induced signals could therefore possibly explain the apparent contradictions in the interpretation of the results obtained with anti-GPIIlb antibodies.

Although GPIIlb-deficient platelets aggregated and secreted normally in response to type I and III collagens, they did not respond to type V collagen. GPIIlb may therefore be critically involved with this collagen either as a direct receptor or in transmitting a signal arising from type V collagen.

Type V collagen differs from type I and III in many respects. It occurs in the extracellular matrix of nearly all connective tissues at concentrations far below those of the interstitial collagens type I and III. However, several investigators have shown abnormal high levels of type V collagen, especially in inflamed or fibrotic tissues.35 Because it is a normal constituent of the subendothelial matrix of blood vessels and was found in enhanced density on the luminal surface of rat aortas, mainly along the cell walls, after experimentally increased blood pressure,36 it could be relevant to pathophysio-logic blood cell/vessel wall interactions.

Recently, it has been found that the GPIa-deficient platelets from the patient published by Nieuwenhuis et al11 showed no response to type V collagen (Kehrel and Nieuwenhuis, unpublished data), similar to GPIIlb-deficient platelets. Flow cytometric analysis with the antibody Gi9 against GPIa showed normal amounts of GPIa in the GPIIlb-deficient platelets (data not shown). Therefore, the presence of both GPIa/IIa and GPIIlb are essential for type V collagen-induced platelet activation, which could possibly involve the bridging of these receptors by this type of collagen.

Our results indicate that the properties of GPIIIb are not
completely consistent with those described by Santoro as typical for a collagen receptor. GPIIIb-deficient individuals have no hemostatic disorders and GPIIIb-deficient platelets are only defective in collagen type V-induced aggregation and secretion.

In conjunction with our previous findings that GPIIIb is probably not the major, essential receptor for thrombospondin, the normal collagen-induced aggregation and secretion may explain why all individuals with GPIIIb-deficient platelets examined so far including this report are healthy and in particular show no apparent evidence of hemostatic disfunctions. Yamamoto et al reported a high rate (between 3% and 11%) of Japanese blood donors with platelets lacking GPIIIb (Nak*− platelets). It is hard to imagine that such a large part of the Japanese population could lack the essential platelet receptor for both thrombospondin and collagen without serious hemostatic consequences.

The observation of Tandon et al that platelets lacking GPIIIb show reduced adhesion to collagen could be explained by the hypothesis of Morton et al that platelet adhesion to collagen may involve sites in the collagen molecule distinct from those more directly associated with aggregation and may only have marginal effects on physiologic processes.

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