Measurement of GPV Released by Activated Platelets Using a Sensitive Immunocapture ELISA – Its Use to Follow Platelet Storage in Transfusion

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Summary

Thrombin, the most potent platelet agonist, plays a central role in haemostasis and in the occurrence of thrombotic events. This agonist activates platelets by cleaving the PAR G-protein coupled receptors and by binding to glycoprotein (GP) Ib and also cleaves GPV at the platelet surface to liberate the soluble 69 kDa fragment GPVf1. Monoclonal antibodies (MoAbs) to GPV were developed as tools to study the mechanism of platelet GPV cleavage and measure release of GPV in pathological situations. Specificity of the MoAbs for GPV was confirmed by flow cytometry and immunoprecipitation of proteins from human platelets and Dami megakaryocytic cells. A sensitive immunocapture sandwich ELISA for soluble GPV was developed using two MoAbs recognizing different epitopes of GPV and purified platelet or recombinant GPV as reference protein. This ELISA was employed to determine the mean plasma concentration of GPV in 100 normal individuals (17.3 ng/ml), to demonstrate the dose-dependent release of GPVf1 from washed platelets stimulated with thrombin and to follow the progressive release of GPVf1 during storage of therapeutic platelet concentrates. The present report describes a sensitive GPV ELISA of direct application to survey the processing and storage of platelet concentrates for transfusion and of potential value to monitor platelet activation in thrombotic states.

Introduction

The surface of a damaged, atherosclerotic blood vessel focuses the formation of active α-thrombin from plasma prothrombin and the recruitment and activation of blood platelets (1, 2). Platelets and thrombin play a central role in the genesis of thrombotic events in arteries such as myocardial infarction and stroke. Thrombin, the most potent known platelet agonist, leads to strong platelet secretion and aggregation and to the expression of platelet procoagulant activity.

Thrombin cleaves two main platelet surface proteins: protease activated receptor 1 (PAR-1) and glycoprotein V (GPV). PAR-1 (65 kDa) belongs to the seven transmembrane domain G-protein coupled receptor family and is present at 1,800 copies/platelet (3-5). GPV (82 kDa) is a platelet specific protein present at 12,000 copies/platelet and belongs to the leucine-rich family (6-9). GPV is a type I transmembrane protein which associates non covalently with GPIbα (145 kDa), GPIbβ (26 kDa) and GPIX (20 kDa) to form the platelet GPIb-V-IX complex (7, 10). Whereas PAR-1 is known to be involved in transducing the thrombin signal, gene inactivation studies in mice suggest that other platelet receptors might be implicated and one candidate is GPIbα (10, 11). The function of GPV has not yet been defined, but this protein might also participate in platelet activation through its association with GPIbα (12, 13).

PAR-1 and GPV are cleaved at nM thrombin concentrations, releasing a short soluble NH2-terminal peptide (PAR-1S) and a 69 kDa NH2-terminal fragment (GPVf1), respectively. These peptide fragments reflect the direct action of thrombin on platelets and as such represent attractive markers of thrombotic events of potential clinical value. Glycocalicin (100 kDa), an extracellular portion of GPIbα, is released by proteases such as calpain and leukocyte elastase (14). GPVf1 and glycocalicin both contain leucine-rich repeats of 24 amino acids, fifteen and seven repeats respectively, which are believed to confer a stable structure (15). Plasma glycocalicin levels have been found to be increased in diseases such as cirrhosis and leukemia but not significantly changed in patients with acute myocardial infarction (16).

The aim of the present study was to develop a sensitive test to quantitate circulating GPVf1. Until now, the detection and quantification of GPV has been hampered by a lack of essential reagents such as antibodies and purified reference protein. In this report, we describe the production of monoclonal antibodies against platelet GPV and of recombinant soluble GPV and the development of an immunocapture sandwich ELISA for GPV. This assay was used to determine plasma levels of GPV in normal individuals and to follow the liberation of GPVf1 after stimulation of platelets by thrombin and during storage of therapeutic platelet concentrates.

Materials and Methods

Materials. All products used in cell culture were of tissue culture grade and endotoxin free. Dulbecco's Modified Eagle Medium (DMEM), NCTC-135 medium, fetal bovine serum (FBS, Myoclone Superplus), Hepes, 8-azaguanine, HT supplement, HAT supplement, PEG-4000, DMSO and Glutamax II were purchased from Life Technologies, Cergy Pontoise, France and insulin, pyruvate, oxaloacetic acid, PMSF and PGI_2 from Sigma, St Louis, Mo, USA. Purified human α-thrombin was provided by the Etablissement de Transfusion Sanguine de Strasbourg, France. Recombinant hirudin was a generous gift of Dr A. Pavirani, Transgène SA, Strasbourg, France. Aprotinin, leupeptin and Nutridoma-CS medium were obtained from Boehringer Mannheim, Germany and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse F(ab)'2 antibodies and rabbit anti-mouse IgG from Jackson Immunoresearch, West Grove, Pa, USA.

Animals. Female Balb/c mice, 6-8 weeks old, and New Zealand white rabbits were purchased from the Centre de Production Animale, Olivet, France.

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Cells. The Dami cell line was obtained from the ATCC (Rockville, MD, USA), while the murine myeloma cell line P3×63.Ag8.653 was a generous gift of Dr J. E. K. Hildreth, The Johns Hopkins University School of Medicine, Baltimore, MD, USA. Hybridomas and myeloma cells were cultured in HY medium (DMEM supplemented with 10% FBS, 10% NCTC-135, 10 mM Hepes, 0.2 U/ml insulin, 0.45 mM pyruvate, 1 mM oxaloacetate and 2 mM L-glutamine), at 37° C in a humidified 5.5 % CO_2 atmosphere.

Preparation of washed human platelets. Platelet rich plasma was prepared from acid-citrate-dextrose anticoagulated blood obtained from aspirin free healthy volunteers and platelets were washed by sequential centrifugation as previously described (17). The cells were finally suspended in Tyrode's buffer, pH 7.35, containing 5 mM Hepes, 0.35% human serum albumin and 2 μg/ml apyrase and adjusted to 3.10^5 platelets/μl.

Purification of platelet and recombinant GPV. GPV was purified from platelets according to a published procedure (18). Washed platelets (2×10^{12}) were incubated at 37° C for 16 h in 10 mM Hepes, pH 7.6, containing 300 mM NaCl, 1 mM EDTA and 0.01 mM PMSF. The extract was centrifuged at 11,000 g for 20 min, the supernatant was brought to 40% saturation with ammonium sulfate and centrifuged at 11,000 g for 20 min and this supernatant was then brought to 60% saturation with ammonium sulfate and centrifuged at 11,000 g for 20 min. The resulting pellet was dissolved in 50 mM potassium phosphate, pH 6.8, containing 1 mM EDTA (Buffer A) and frozen at -80° C until further purification. Pooled platelet extracts were dialyzed against Buffer A and concentrated in a 10,000 molecular weight cut-off Amicon concentrator (Millipore, Saint Quentin en Yvelines, France). This sample was applied to a Superose 13 10/30 gel filtration column (Pharmacia, Uppsala, Sweden) and eluted with Buffer A at 0.5 ml/min. Glycoprotein fractions were detected by SDS-PAGE and periodic acid Schiff staining and applied to a Mono Q-HR anion exchange column (Pharmacia) equilibrated with Buffer A and the flow-through fraction was collected and applied to a wheat germ agglutinin-Sepharose affinity column (Pharmacia) in Buffer A. Proteins were eluted with 2.5% (wt/vol) N-acetyl glucosamine in Buffer A, dialyzed against PBS and stored frozen at -80° C. The HPLC system was a Waters 650 apparatus (Millipore), elution was monitored at 280 nm and protein concentrations were determined using the BCA protein kit (Pierce Chemicals, Rockford, IL, USA). Protein purity was controlled on a Phast electrophoresis system (Pharmacia) and the final yield from 2×10^{12} platelets was 200 μg of GPV.

Recombinant GPV was purified from culture supernatants of CHO transfected cells. CHO dihydrofolate (DHFR) negative cells (CHO DUK-, ATCC 9096-CRL) were transfected with full length GPV in the pTG2328 expression vector and a permanent cell line (CHOT5 1B) was obtained by successive amplification with methotrexate. These cells, which had negligible surface expression but secreted a 82 kDa soluble GPV (≈ 0.4 μg/ml), were grown in serum free CHO-S-SFM-II medium (Life Technologies) containing 1.2 μM methotrexate. CHOT5 1B supernatants (4 l) were concentrated 10 times using a Minitan-S ultrafiltration apparatus (Millipore) and then recirculated for 40 h at 0.2 ml/min through a 1 ml HiTrap NHS-activated column (Pharmacia) coupled with 9.5 mg V.1 MoAb. After preelution with 10 mM phosphate buffer, pH 8, GPV was eluted with 0.1 M triethylamine, pH 11.5, rapidly neutralized with 1/20 volumes of 1 M phosphate buffer at pH 7 and dialyzed against PBS. Purity of GPV was estimated to be greater than 95% on SDS-PAGE gels and recovery was approximatively 45%.

Production of polyclonal and monoclonal antibodies. Polyclonal antibodies against GPV were raised in rabbits using the following protocol. The animals were injected subcutaneously with 100 μg purified platelet GPV in complete Freund's adjuvant and then twice with 100 μg GPV in incomplete Freund's adjuvant. After three days, three booster injections of 100 μg GPV were given 3 to 4 days apart in the ear vein. Blood was drawn 4 to 7 days after the last injection and weekly thereafter and immunoglobulins were purified from serum by chromatography on HiTrap Protein G columns (Pharmacia).

Female Balb/c mice, 6-8 weeks old, were injected intraperitoneally with 10^9 washed human platelets in Tyrode's buffer or 50 μg purified platelet GPV in complete Freund's adjuvant. At three week intervals the mice received two additional injections of 10^9 platelets or 50 μg GPV. One week after the third injection, the mice were injected intraperitoneally and intravenously with 10^8 washed human platelets in PBS or 50 μg GPV in a mixture (1 vol/3 vol) of complete and incomplete Freund's adjuvant . Four days later, the spleen of one mouse was removed and fused with the myeloma cell line P3x653.Ag8 in 5% PEG containing 0.5% DMSO as previously described (19, 20). Fused cells were resuspended in HY medium supplemented with 20% FBS, HAT and Nutridoma-CS and seeded in eight flat bottomed 96-well plates (Falcon, Becton Dickinson Co, Lincoln Park, NJ, USA). The fusion was screened by flow cytometry as described below, hybridomas secreting antibodies of interest were subcloned twice by limiting dilution and the final hybridoma clones were isotyped using

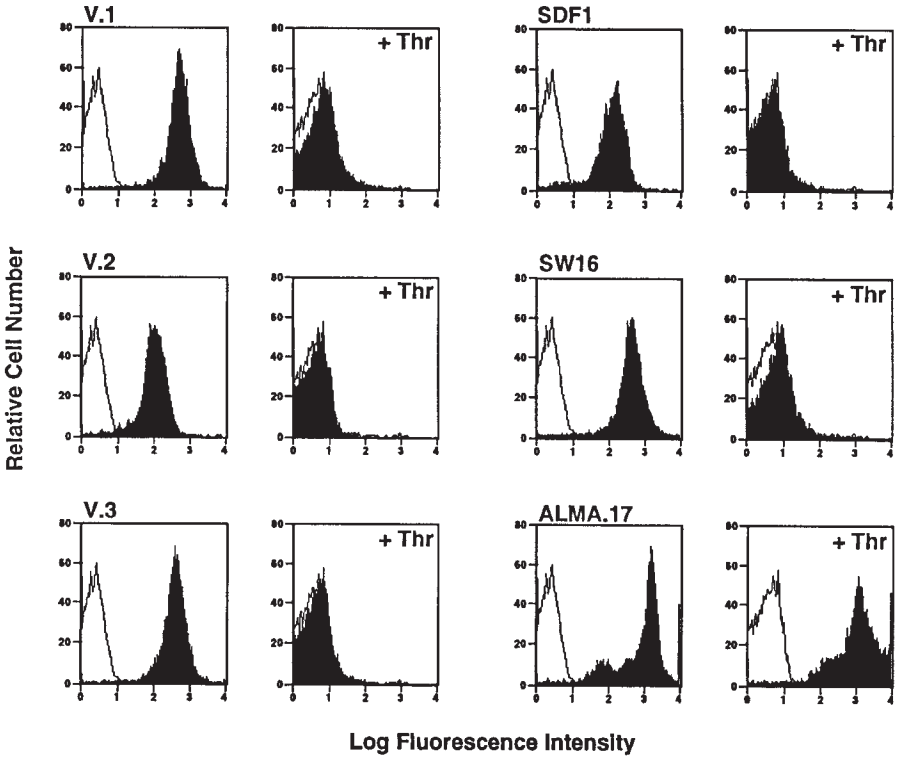

Fig. 1 Flow cytometric analysis of MoAbs binding to resting platelets or platelets treated with thrombin. MoAbs V.1, V.2, V.3 and SDF.1 bound to resting platelets but lost reactivity when platelets were stimulated with 5 U/ml human α-thrombin (+Thr). This behavior was identical to that of SW16, a reference anti-GPV MoAb directed against the GPVf1 fragment released by thrombin cleavage. ALMA.17, an anti-GPIIb-IIIa MoAb, bound equally to resting platelets and platelets treated with thrombin. Reactivity to MOPC21, an IgG1 negative control, is presented in each panel as an open histogram

an isotyping kit (Boehringer Mannheim). Culture supernatants from the final clones were collected, treated with 0.02% sodium azide and stored at 4° C. The hybridomas secreting relevant antibodies were expanded into ascites in Balb/c mice previously primed with 0.5 ml incomplete Freund's adjuvant. IgG MoAbs V.1, V.2, V.3 and V.5 were purified from ascites on HiTrap Protein G columns, while the IgM MoAb SDF.1 was purified by dialysis of the ascites fluid against 20 volumes of distilled water, centrifugation at 5,000 rpm for 20 min, dissolution of the precipitate in 0.5 ml 0.5 M NaCl and addition of 0.5 ml 0.1 M phosphate buffer, pH 7.4.

Flow cytometry. Analyses were carried out on suspensions of washed human platelets (2×10^5 platelets in 100 µl), after stimulation or not with human α-thrombin at 37° C for 5 min followed by neutralization with 40 U/ml hirudin. The suspensions were incubated with hybridoma culture supernatants (100 µl) at 22° C for 30 min and then with 1 µg GAM-FITC at 22° C for 30 min in the dark. After each incubation, the platelets were washed in 10 mM Hepes, pH 7.6, containing 150 mM NaCl and 1 mM EDTA. The cells were finally resuspended in the same buffer and analyzed on a FACSort fluorescence cytometer using Cell Quest software (Becton Dickinson, San Jose, CA, USA). Light scatter and fluorescence intensity were recorded for 10,000 platelets on a logarithmic gain.

Immunoprecipitation. Immunoprecipitations were performed as previously described (21). Washed platelets (50 µl) in Tyrode's buffer were vectorially labeled with ^{125}I and lysed in TEN buffer (50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 150 mM NaCl) containing 1% Triton X-100 and protease inhibitors (0.5 mM PMSF, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 0.5 µg/ml chymostatin, 0.5 µg/ml antipain) for 2 h at 0° C. The labeled lysate was incubated with MoAb for 1 h at 0° C and then with 10 µg rabbit anti-mouse IgG for 1 h at 0° C. After addition of formalin fixed Staphylococcus aureus (Cowen strain I, SaC) to a final concentration of 2%, the mixture was incubated for a further 30 min at 0° C, pelleted and washed in TEN lysis buffer. The pellets were taken up in SDS-PAGE sample buffer and heated to 100° C for 3 min, after which SaC was removed by centrifugation and the eluted proteins were analyzed by SDS-PAGE and autoradiography.

Enzyme immunoassays: selection of MoAbs. F(ab')$_2$ fragments of goat anti-mouse IgG Fc (100 µl of a 5 µg/ml solution in 50 mM carbonate buffer, pH 9.5) were adsorbed onto 96-well micotiter plates (Maxi-Sorb, Nunc) by incubation overnight at 4° C. After one wash with PBS-T (PBS containing 0.05% Tween-20), the plates were blocked with 200 µl of 1 % BSA in PBS and incubated at 37° C for 30 min. All subsequent incubations were carried out for 1 h at room temperature and each was followed by washing three times with PBS-T. The wells were treated first with 100 µl MoAb added as either pure culture supernatant, a 1 : 200 dilution of ascites, or 5 µg/ml purified Ig in PBS-T. This was followed by successive incubations with purified GPV (200 ng/ml in PBS-T), 100 µl rabbit anti-GPV serum (1 : 500) and finally 100 µl peroxidase-conjugated goat anti-rabbit Ig (Bio-Rad). Bound antibody was detected by addition of a chromogenic substrate (OPD, *o*-phenylenediamine dihydrochloride, Pierce) and measurement of the optical densities of the wells at 490 nm in a THERMOmax microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Enzyme immunoassays: quantification of GPV. Purified MoAb V.1 IgG (5 µg/ml in 50 mM carbonate buffer, pH 9.5) was adsorbed onto polyvinyl 96-well plates (Maxi-Sorb, Star-well, Nunc) by incubation overnight at 4° C. The wells were blocked with BSA as described above and washed once with PBS-T. GPV standards were diluted in PBS-T containing 0.1% BSA, 100 µl aliquots were added to triplicate wells and the plates were incubated for 1 h at room temperature. After washing, 100 µl biotinylated MoAb V.3 (0.5 µg/ml in PBS-T containing 0.1% BSA) was added to the wells and incubation was continued for 1 h at room temperature. Bound V.3 was detected by addition of peroxidase-conjugated streptavidin (1 : 2,000 in PBS-T containing 0.1% BSA) and chromogenic revelation with OPD.

Measurement of soluble GPV in plasma, serum and platelet concentrates. Blood (5 ml) was collected into Diatubes (Becton Dickinson) from 100 individuals at the end of a blood donation lasting less than 10 min and the samples were kept on ice. Plasma was obtained within one hour of blood collection by centrifugation at 14,000 g for 15 min in Eppendorf tubes followed by removal of the upper 2/3 of the plasma layer and was stored at -80° C until assay. This procedure was previously found to minimize platelet activation and PF4 secretion. Serum was prepared from blood (80 ml) collected into a glass tube, left at room temperature for 4 h and centrifuged at 1,510 g for 10 min. Plasma samples (100 µl) were assayed in the GPV ELISA at dilutions of 1 : 20 to 1 : 40 and serum samples at dilutions of 1 : 1,000 to 1 : 2,000.

Platelet concentrates (4 to 6×10^{11} platelets) obtained from 6 individuals by apheresis on an MCS+ apparatus (Haemonetics, USA) and deleukocytation with a Haemonetics 994 FP kit were stored at 22° C on a horizontal shaker. Samples (10 ml) were withdrawn aseptically on the day of collection (D_0) and on days 2, 4, 6, 8 and 13. Plasma was isolated by centrifugation of 1 ml aliquots at 14,000 g for 15 min and stored at -80° C. The plasma samples were assayed in the GPV ELISA at dilutions of 1 : 100 to 1 : 500 and their PF4 content was determined using a commercial ELISA (Asserachrom PF4, Stago, France). Surface expression of GPV and P-selectin on platelets was assayed in parallel by flow cytometry. Platelet samples were treated with 1 µM PGI_2 and 8 U/ml hirudin to block any further platelet activation during processing of the samples and then washed once in PBS. Aliquots of washed platelets (2×10^7) were incubated with 2 µg primary antibody (V.1 or CLB-Thromb6, Immunotech) in PBS followed by GAM-FITC and analyzed on a FACSort fluorescence cytometer.

Data analysis. Values for each measured variable were expressed as the mean ± standard deviation, while statistical differences were determined by variance analysis using the Statistic General Manova software.

Results

Development and characterization of anti-GPV MoAbs. Anti-GPV MoAbs were obtained by immunization of Balb/c mice in two ways, by injection of washed human platelets or of GPV purified from human platelets. In both cases, the hybridomas were screened by flow cytome-

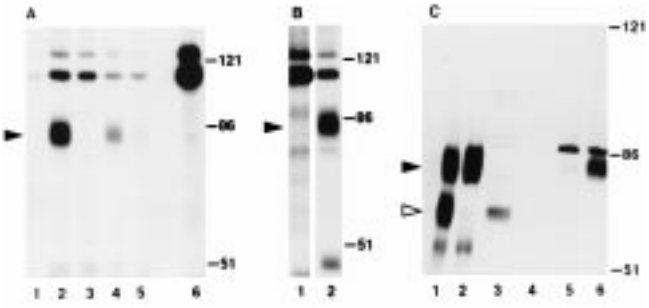

Fig. 2 Characterization of anti-GPV MoAbs by immunoprecipitation and Western blotting. A) Resting platelets were surface labeled with ^{125}I and protein lysates were immunoprecipitated with irrelevant MoAb GM10.1 (lane 1), MoAbs V.1 (lane 2), V.2 (lane 3) and V.3 (lane 4) raised against purified GPV and MoAbs SDF.1 (lane 5) and SDF.2 (lane 6) raised against human platelets. A broad band centered at 82 kDa characteristic of glycosylated GPV (closed arrowhead) was precipitated by V.1, V.3 and SDF.1 (lanes 2, 4, 5). Immunoprecipitation with V.2 was less efficient and didn't show a visible band (lane 3). SDF.2 immunoprecipitated the GPIIb-IIIa complex, which reacts strongly with ^{125}I and is seen as a contaminating doublet in lanes 1-5. B) ^{125}I-labeled platelets were treated with 5 U/ml thrombin (lane 1) or left untreated (lane 2) and lysate proteins were immunoprecipitated with MoAb V.1. The 82 kDa band (closed arrowhead) was lost after thrombin treatment, confirming its identity with GPV and suggesting that V.1 is directed against the NH2-terminal GPVf1 fragment. C) Western blot analyses with MoAb V.5 of platelet lysates (lanes 1-2), Dami cell supernatants (lanes 3-4) and Dami cell lysates (lanes 5-6), treated with 10 U/ml thrombin (lanes 1, 3 and 5) or saline (lanes 2, 4 and 6). V.5 labeled a 82 kDa band (closed arrowhead) in resting platelets and Dami cells, while thrombin treatment generated a 69 kDa GPVf1 fragment (open arrowhead) seen in platelet lysates (lane 1) and Dami cell supernatants (lane 3). All experiments were performed under reducing conditions

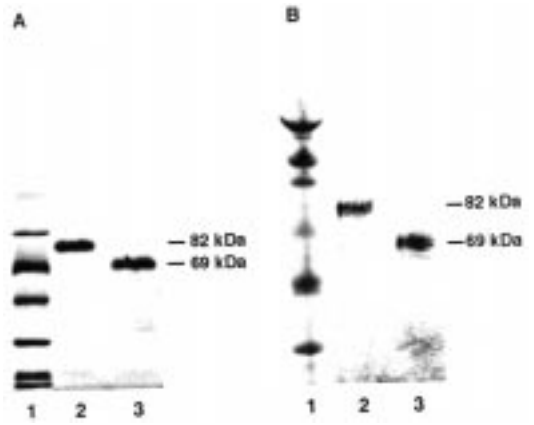

Fig. 3 SDS PAGE of platelet and recombinant purified GPV. GPV was purified by several chromatographic steps from fresh platelet concentrates or by single step affinity chromatography from CHOT51B cell supernatants as described in the Methods Section. Platelet (panel A) and recombinant (panel B) purified GPV were separated on a non reduced 4-15% gel without treatment (lane 2) or after treatment with 10 U/ml thrombin (lane 3). Proteins are stained with Coomassie blue and lane 1 corresponds to molecular weight markers

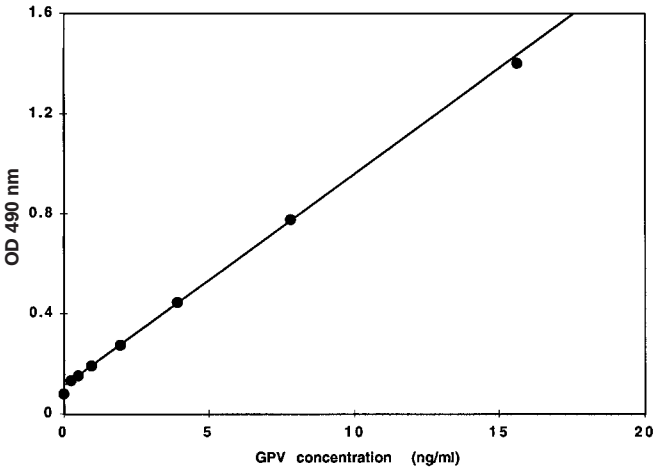

Fig. 5 Standard curve of the GPV ELISA. The GPV ELISA was performed as described in the Methods Section using V.1 as the capturing antibody, serial dilutions of purified recombinant GPV and biotinylated V.3 as the detecting antibody. Concentrations of GPV in unknown samples were calculated from the linear portion of the curve. The results presented here are from one typical calibration experiment carried out in duplicate

try for differential binding to resting platelets and platelets stimulated with thrombin. Four MoAbs, V.1, V.2, V.3 and SDF.1, showed strong binding to resting platelets but lost reactivity after treatment of the platelets with thrombin (Fig. 1), behavior similar to that of the known anti-GPV MoAb SW16 (7). These MoAbs were used to immunoprecipitate GPV from lysates of platelets surface labeled with ^{125}I. Clones V.1, V.3 and SDF.1 precipitated a surface protein of approximatively 82 kDa (Fig. 2A) which was cleaved by thrombin (Fig. 2B), suggesting reactivity against the GPVf1 fragment. Other clones were able to recognize GPV in solid phase binding assays but not by flow cytometry and in particular one such clone V.5 recognized GPV and the GPVf1 fragment on Western blots of platelets and the Dami cell line (Fig. 2C).

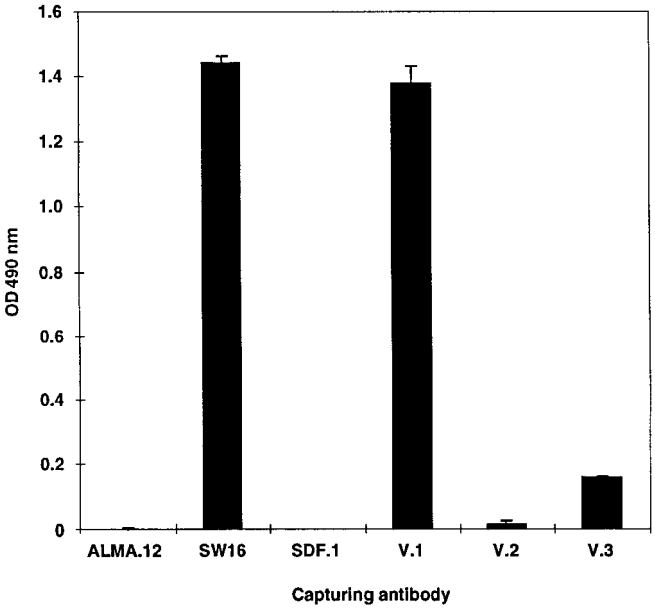

Fig. 4 Capturing efficiency of anti-GPV MoAbs. MoAbs SW16, SDF.1, V.1, V.2 and V.3 against GPV and ALMA.12 against GPIbα were coated on 96 well plates and tested for capture of purified platelet GPV. Bound GPV was detected by addition of a polyclonal anti-GPV rabbit antibody followed by peroxidase-coupled GAR and OPD chromogenic substrate and measurement of optical densities at 490 nm. V.1 and SW16 had the highest capturing efficiency and V.3 intermediate efficiency whereas SDF.1, V.2 and ALMA.12 were ineffective. V.1 was selected for further development of the GPV ELISA. Results are from one experiment representative of three separate experiments performed in triplicate

Purification of platelet and recombinant soluble GPV. Soluble GPV was obtained from fresh platelet concentrates or in a recombinant form after transfection of CHO cells with the GPV cDNA. Despite the presence of the transmembrane segment, recombinant GPV was secreted in the cell supernatant probably after enzymatic cleavage during intracellular processing. Both the native and recombinant proteins were purified to homogeneity and appeared as a single 82 kDa band which decreased to 69 kDa after thrombin treatment (Fig. 3). Platelet and recombinant soluble GPV were subsequently used as reference in the development of a GPV ELISA.

Capturing efficiency of anti-GPV MoAbs. As a first step in the development of a GPV immunocapture ELISA, we tested the capturing ability of anti-GPV MoAbs. GAM-IgG Fc was employed to immobilize the MoAbs and bound purified GPV was detected with an anti-GPV rabbit serum. The results of Fig. 4 indicate that MoAbs V.1 and SW16 had the highest capturing efficiency. Secondly, we tested the ability of biotinylated V.2 and V.3 to detect GPV captured by V.1. Since biotinylated V.3 was able to detect GPV captured by V.1 whereas biotinylated V.2 was not (data not shown), we decided to use V.1 as the capturing MoAb and biotinylated V.3 as the detecting MoAb in our GPV sandwich ELISA.

Development of a GPV sandwich ELISA. In further experiments, we found that use of V.1 plated at 5 μg/ml and biotinylated V.3 added at 0.5 μg/ml provided the best conditions for a good signal to noise ratio (data not shown). Using these MoAb concentrations, we were able to establish a titration curve linear between 0.24 ng/ml and 16 ng/ml GPV (Fig. 5). Titration curves were originally obtained with GPV purified from human platelets and subsequently using purified recombinant GPV. The detection limit of the assay was 0.044 ng/ml. Precision was determined by testing three plasma pools containing 10.5, 18.2 and 32.5 ng/ml GPV seven times on the same day and on five different

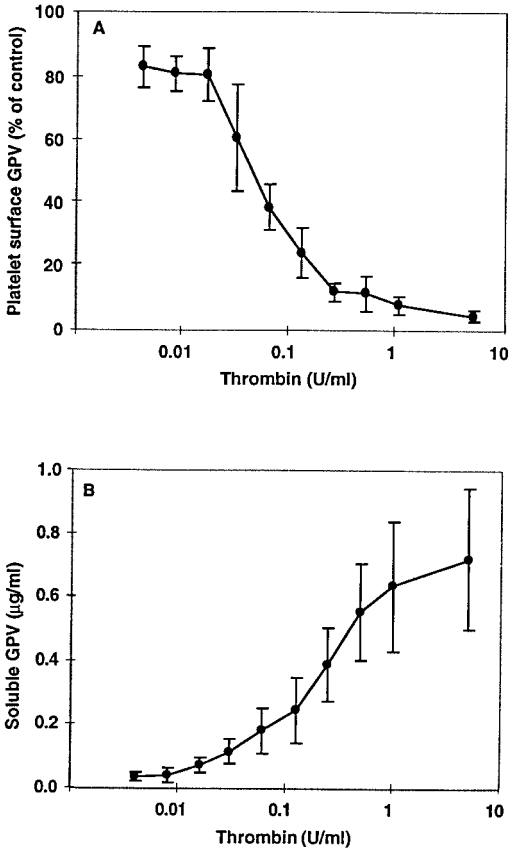

Fig. 6 Cleavage of GPV from the surface of platelets treated with increasing concentrations of thrombin. Washed platelets in Tyrode's albumin buffer were treated with 0.004 to 8 U/ml α-thrombin for 10 min at 20° C, followed by hirudin to block any further enzymatic activity. Platelet surface GPV was analyzed by flow cytometry using MoAb V.1 (A), while soluble GPV secreted into the supernatant was determined by GPV ELISA (B). Thrombin induced a dose-dependent decrease of surface GPV (A) and a concomitant release of soluble GPV into the supernatant (B). Flow cytometric data are expressed as the mean fluorescence intensity (%) with unstimulated platelets taken as 100%. ELISA calibration curves were determined using recombinant GPV as reference protein and concentrations of soluble GPV are given in µg/ml. Values are the mean ± SD from three separate experiments performed in duplicate

days. In these tests, the intra-assay coefficients of variation were 7%, 5% and 12%, respectively, while the inter-assay coefficients of variation were 14%, 13% and 5%, respectively. Assays without V.1 capturing MoAb showed no detectable absorbance due to non specific binding of GPV at solution concentrations of up to 25 ng/ml. The mean recovery was 90% as determined by addition of known concentrations of recombinant GPV. Finally, addition of increasing quantities of plasma samples gave dose-response curves running parallel to the calibration curve, which confirmed the absence of interference from plasma components (data not shown).

Release of GPVf1 from platelets treated with thrombin. The cleavage of platelet GPV by thrombin was followed by measuring the accumulation of soluble GPV in platelet supernatants by GPV ELISA and the parallel disappearance of cell surface GPV by flow cytometry. Platelets treated for 5 min at 22° C with increasing concentrations of purified human α-thrombin progressively lost surface GPVf1 (Fig. 6) with an EC50 of 0.06 U/ml (0.5 nM). This was accompanied by the progressive appearance of GPVf1 in the supernatant, with maximum release at ≈ 10 U/ml and an EC_{50} of 0.2 U/ml (1.8 nM). Since levels of soluble GPV in the supernatant were not modified by centrifugation at 100,000 g, there was no contribution from platelet microvesicles. Western blot analysis of supernatants and the persistence of flow cytometric reactivity to the GPVf2 specific MoAb 1D9 (22) (data not shown) confirmed that cleavage occurred at or near the thrombin cleavage site.

Plasma and serum levels of GPV in normal individuals. Plasma levels of GPV in 100 normal individuals as determined by GPV ELISA (Table 1) displayed a mean value of 17.3 ± 6.3 ng/ml (range 4.3–31.9) with no significant age or sex differences. Serum from 7 different individuals contained 1.2 ± 0.17 µg/ml GPV.

Release of soluble GPV during storage of platelet concentrates. In order to determine whether GPV could be a useful index of platelet activation by thrombin or leukocyte proteases, we followed the cleavage of GPV during storage of therapeutic platelet concentrates. Apheresis concentrates from 6 donors were tested for surface expression of GPV and P-selectin by flow cytometry and for liberation of soluble GPV and PF4 by ELISA. Cleavage of GPV was readily detected after 2 days storage (Fig. 7) and steadily increased up to day 13, when the majority of the protein had been removed from the platelet surface. In contrast, platelet exocytosis as measured by P-selectin exposure or PF4 release was less pronounced and progressed more slowly. The sharp decrease in surface GPV and increase in P-selectin on day 13 was accompanied by a decrease in GPVf2 and GPIbα suggesting calpain activation (data not shown). During the first 8 days of storage, surface GPIIb-IIIa, GPIbα and GPVf2 levels were stable and comparable to those of day 0, while platelet aggregation in response to thrombin and agglutination in response to ristocetin were also preserved until day 8 but significantly decreased by day 13 (data not shown).

Discussion

Studies of the structure and function of platelet GPV have until now been hampered by a lack of specific reagents. Only two monoclonal antibodies against human GPV have been previously described, MoAb SW16 developed by immunization against human platelets which reacts with GPVf1 (7) and MoAb 1D9 obtained by immunization against purified GPV which recognizes GPVf2 (22). In this report, we describe five new MoAbs directed against the GPVf1 fragment. Three (V.1, V.3 and SDF.1) reacted strongly with resting platelets but lost reactivity after their stimulation by thrombin in a manner similar to SW16, one (V.2) displayed weak labeling of GPVf1 lost after fixation and one (V.5) recognized only denatured GPV. None of these antibod-

Table 1 GPV level in plasma from normal human subjects

Age	Men (ng/ml)	Women (ng/ml)	Average (ng/ml)
≤ 30	19.8 ± 7.9 (n = 17)	14.6 ± 4.3 (n = 13)	17.5 ± 7.0 (n = 30)
31-50	16.9 ± 6.5 (n = 37)	16.9 ± 6.4 (n = 16)	16.9 ± 6.4 (n = 53)
>50	18.4 ± 4.9 (n = 10)	17.6 ± 4.9 (n = 7)	18.0 ± 4.8 (n = 17)
Average	17.9 ± 6.7 (n = 64)	16.2 ± 5.4 (n = 36)	17.3 ± 6.3 (n = 100)

Each 5 ml of blood was collected in Diatube and processed within 1 h as described in the Methods section. Plasma values were determined by ELISA by comparing with a standard curve and performed in duplicate for each sample. Results are shown as mean values ± SD

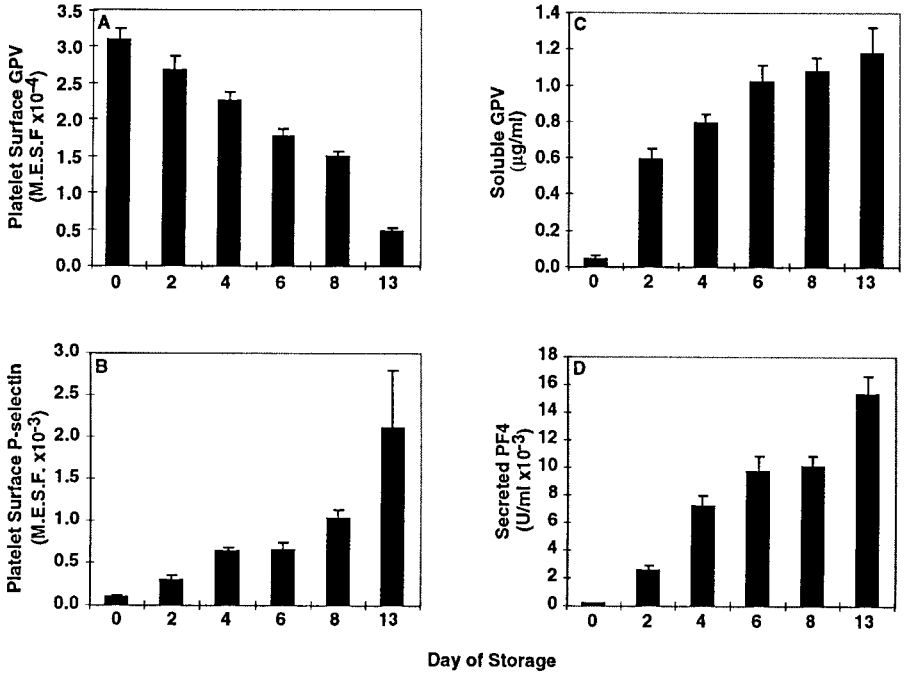

Fig. 7 Soluble GPV is gradually released during storage of therapeutic platelet concentrates. PRP samples were drawn aseptically from six apheresis units over a storage time of 13 days. Platelets were analyzed by flow cytometry for surface GPV using MoAb V.1 (panel A) and for P-selectin exposure using MoAb CLB-Thromb6 (panel B). Plasma supernatants were analyzed by ELISA for soluble GPV (panel C) and for secreted PF4 (panel D). Storage was accompanied by the progressive disappearance of platelet surface GPV and a parallel increase in soluble GPV in plasma, together with platelet degranulation leading to P-selectin exposure and PF4 secretion. Day 0 corresponds to the day of preparation of the apheresis unit and results are expressed as mean values ± SEM

ies competed for binding to GPV, which would suggest the recognition of distinct epitopes. All were without effect on platelet aggregation induced by thrombin, ADP or collagen or on agglutination induced by ristocetin.

Using a combination of the V.1 and V.3 MoAbs, it was possible to develop an immunocapture sandwich ELISA for GPV. The assay was shown to be specific, accurate and sensitive with a detection level of 0.044 ng/ml and a linear response up to 16 ng/ml GPV and was not influenced by the presence of plasma proteins. This method would be suitable for the automatic testing of large series of samples and to our knowledge represents the first quantitative assay to measure release of GPV from platelets.

In the plasma of normal individuals, soluble GPV was present at a mean concentration of 17 ng/ml or 207 pM but was undetectable in undiluted urine. When measuring markers of platelet activation in plasma, care must be taken to avoid artefactual activation during sample processing and to completely eliminate platelets and platelet debris. In our laboratory, we routinely employ a one step centrifugation procedure which ensures minimal cellular activation and avoids platelet contamination by selective removal of the upper plasma layer. Using this procedure, we found no significant differences in plasma levels of GPV in samples collected into 3.8% citrate, 10 mM EDTA, ACD or ACD and hirudin containing anticoagulants, or into Diatubes®. Although plasma levels did not directly correlate with age or sex, there was a tendency to higher values with increasing age when results were normalized for platelet count. We have not determined the *in vivo* half-life of GPV, but this may be estimated (16) to be about 4 days if one assumes 12,000 copies/platelet and 50% loss of GPV during a 10 day platelet life span.

Using washed platelets we showed a dose-dependent release of soluble GPV in the supernatants after thrombin activation. Thrombin cleavage occurs at position 460 and generates a 69 kDa fragment. Additional fragments of GPV can be removed from the platelet surface by enzymatic cleavage. Leukocyte elastase cleaves at un unknown position to release a 73 kDa fragment and calpain cleaves near the transmembrane domain to liberate the 82 kDa GPVs fragment (14). From the literature there is uncertainty as to whether thrombin cleaves exclusively at position 460 or also indirectly by activating endogenous platelet calpain (14, 23). To discriminate between calpain and thrombin cleavage we performed flow cytometric analysis using the MoAb 1D9, which is directed against the GPVf2 portion of GPV left attached to the platelet surface (22) and did Western blotting on the platelet supernatants. Using these assays we showed that treatment with up to 5 U/ml thrombin for 5 min only cleaved at the thrombin site. Disappearance of GPV from the platelet surface is due to proteolysis but has also been shown to redistribute to the surface connected canalicular system (24). As platelets treated with thrombin displayed only minimal decreases in surface GPIbα and GPIX, internalization of the GPIb-V-IX complex would however appear to be minimal in our experiments.

Soluble GPV released by thrombin reached a plateau value of 0.7 µg/ml in platelet suspensions containing 3×10^5 cells/µl. This corresponds to 8.5 pmol GPV produced by 3×10^8 platelets, which is slightly higher than the value of 6 pmol GPV estimated on the basis of 12,000 copies/platelet. The discrepancy may be due to some release of GPV from intracellular stores (25) and/or to possible overestimation of the concentration of purified GPV in the standard solution. Nevertheless, these results indicate that GPV was fully released into the supernatant at sufficiently high thrombin concentrations. Soluble GPV was present in serum at a concentration (1.2 µg/ml or 14.6 nM) compatible with complete cleavage from the platelet surface and Western blots revealed two major bands of 69 and 82 kDa, most likely corresponding to GPVf1 and GPVs, respectively suggesting a combined action of thrombin and calpain during the clotting process.

One application of potential interest is the quantification of soluble GPV in therapeutic platelet concentrates. As platelets undergo progressive lesions leading to loss of function during the preparation and storage of concentrates (26), a suitable test to monitor these changes and help predict the functional status of the cells would be of value in efforts to develop concentrates with longer shelf life. Our ELISA tests revealed progressive accumulation of GPV in the plasma supernatants of platelet concentrates, which was already evident after two days storage. These results confirm and extend those of previous studies where the disappearance of GPV from the platelet surface during storage was

followed by flow cytometry (27). Although GPV could be released into the supernatant not only by thrombin or calpain but also by the proteases of contaminating leukocytes, the stability of GPIb and GPVf2 on the platelet surface over one week of storage strongly favors thrombin cleavage. Thrombin generation during storage of platelet concentrates has in fact been previously documented by determination of fibrinopeptide A levels (28). Soluble GPV could also originate from platelet microvesicles. However, the production of microvesicles was only observed after very long storage times.

In vitro results and preliminary data in an animal model of thrombosis (29) favor the use of plasma GPV as a marker of thrombosis but formal proof depends on future clinical studies. Thrombin concentrations (~ 1 nM) able to induce robust platelet responses *in vitro* only generate partial (25%) and delayed GPV shedding from the platelet surface. Nevertheless, a combination of high local thrombin concentrations and longer exposure times in the vasculature, and a good stability in plasma could contribute to increased GPV levels in pathological settings. Despite the lack of *in vivo* data, GPVf1 may be expected to have a significant plasma half-life in view of its *in vitro* stability in plasma and serum, its large size and its sequence homology with platelet glycocalicin. The present ELISA will be suitable for large scale testing and could offer advantages over existing tests. Flow cytometric detection of platelet activation markers such as P-selectin, CD63 or activated GPIIb-IIIa requires rapid processing of samples by experienced operators and careful standardization to allow detection and quantification of a small number of activated platelets. Furthermore, a potential disadvantage of monitoring activated cells is their possible disappearance from the circulation following activation. The detection of platelet activation in plasma has traditionally relied on the determination of PF4 and β-thromboglobulin levels (30). These assays are very sensitive but must be performed carefully to avoid artefactually high levels due to inadequate sampling and centrifugation procedures. Contrary to PF4 or β-thromboglobulin, GPV is released by surface proteolysis and hence should be less sensitive to platelet activation during sample processing. Another disadvantage of PF4 and β-thromboglobulin is their short half-lives. Soluble P-selectin was recently proposed as a novel marker of platelet activation. P-selectin is also present in endothelial cells and does not represent a specific platelet marker and moreover its exact mechanism of cleavage or secretion during thrombosis is still poorly defined. PAR-1 is another direct indicator of the action of thrombin on platelets. Ramachandran et al (31) have described an ELISA for soluble PAR-1 NH2-terminal peptide and were able to detect its release from concentrated washed platelets treated with thrombin but did not report plasma or serum values. Finally, glycocalicin, a soluble fragment of GPIbα, has also been proposed as a useful marker of platelet activation (16). GPIbα does not contain a thrombin cleavage site but is indirectly cleaved by calpain to generate glycocalicin, while shorter fragments can be produced by leukocyte elastase or cathepsin G. Although glycocalicin has proved of value to follow platelet activation during storage and in certain pathologies such as leukemia and cirrhosis, its elevation has not to date been observed in thrombotic disorders.

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