Establishment of a Flow Cytometric Assay for Determination of Human Platelet Glycoprotein VI Based on a Mouse Polyclonal Antibody

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Glycoprotein VI (GPVI) is the major signaling receptor for collagen on platelets. Recent studies suggest that the surface density of GPVI is related to the activation of platelets by collagen. To measure the level of GPVI on platelets, a mouse polyclonal antibody BJ010 was prepared using an amplified fragment of extracellular domain in GPVI. The specific reactivity of BJ010 was identified by anti-GPVI specific monoclonal antibody 11a12 using immunoprecipitation, sandwich enzyme-linked immunosorbent assay (ELISA) and flow cytometry. The antibody BJ010 recognized both native and denatured human GPVI so that it was used to set up a flow cytometric assay to detect the level of GPVI in normal subjects. The relative level of GPVI on platelets was detected in 101 healthy

donors. The median geometric mean fluorescence intensity (GMFI) of platelet GPVI level was 57.7. The variation between minimum and maximum values of platelet GPVI and integrin $\alpha 2\beta 1$ were found to be 3.5- and 4.1-fold, respectively, in the normal subjects. There was a week correlation between the amount of GPVI and integrin $\alpha 2\beta 1$ on platelet surfaces. For the method, the intraassay and inter-assay coefficient of variation was 6.3% and 8.8%, respectively. The flow cytometric assay described here provides a simple, reliable, reproducible, and readily available means of quantitation of collagen receptor GPVI density on the platelet surface in a larger number of blood samples. *J. Clin. Lab. Anal.* 20:250–254, 2006. © 2006 Wiley-Liss, Inc.

Key words: platelet; glycoprotein VI; polyclonal antibody BJ010

INTRODUCTION

Platelet adhesion to collagen and collagen-induced platelet activation are initial links of hemostasis and acute artery embolism. Up to now, it is known that there are three receptors on the platelets' surface, namely glycoprotein Ib/IX/V complex, integrin $\alpha 2\beta 1$, and glycoprotein VI (GPVI), contributing to platelet adhesion to collagen. GPVI has attracted extensive attention due to its initial interaction with collagen in the platelets discovered through research on molecular cloning, chromosome location, structure, and function. GPVI molecule is a type I transmembrane glycoprotein, a member of the immunoglobulin superfamily with molecule weight of 62 kDa. The mature GPVI protein consists of 319 amino acids. Its extracellular domain contains 249 residues forming two immunoglobulin-like C-type folds and a highly glycosylated pericellular stem.

The 19-amino acid transmembrane domain contains an arginine residue, which is critical for the interaction with Fc receptor γ -chain with which GPVI forms a functional complex (1). The 51-amino acid cytoplasmic domain contains binding sites for calmodulin and Src homology 3 (SH3)-binding proteins (2,3). GPVI induced platelet activation is initiated by tyrosine phosphorylation of the immunoreceptor tyrosine-based activation motif (ITAM) in Fc receptor γ -chain, and the signal is transferred to many related proteins (1), finally resulting in platelet aggregation and platelet thrombus. The GPVI

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is widely recognized as a requisite factor for the formation of platelet aggregates on a collagen surface under blood flow.

Since GPVI has close relationship with both hemorrhagic diseases and thrombotic diseases, it is of great importance to determine GPVI density on platelet surface for understanding its significance in pathological situations. Furihata et al. (4) quantified the platelet GPVI by a ligand blotting assay using convulxin and reported the variation of platelet GPVI content in healthy donors. However, this method is rather complicated, time-consuming, and only semiquantitative, and it is impractical for use in clinical laboratories. Therefore, a simple method for the measurement of collagen receptor GPVI on platelet is required.

In the present study, we prepared a polyclonal antibody against extracellular domain of human GPVI and set up a method of flow cytometry for the measurement of GPVI on platelet surface using the antibody BJ010.

MATERIALS AND METHODS

Preparation of Polyclonal Antibody Against the Recombinant Fusion Protein

The recombinant fusion protein, a fragment of extracellular domain of GPVI (amino acid residues 103–248), was prepared and described in previous literature (5). A total of 50 µg of the recombinant fusion protein were emulsified with complete Freund's adjuvant and injected intraperitoneally into mice (Kunming strain). After a boosting with the immunogen on days 14, 21, 28, and 35, ascites were collected. Polyclonal antibody was purified from the ascites using a protein G Sepharose column (Amersham Pharmacia, Piscataway, NJ) and named as the antibody BJ010.

To examine the immunoreactivity of the antibody BJ010 to GPVI, immunoprecipitation of GPVI from platelet lysate by Western blot was performed. Platelet lysate was incubated with monoclonal antibody 11a12 against GPVI (provided by Dr. Peter J. Newman and Dr. Brian Boylan, Blood Research Institute, The Blood Center of Southeastern Wisconsin) (6). Protein G agarose was then added into the lysate and incubated. After washing, the protein G agarose pellet was mixed with 2× sample buffer and boiled for 5 min. The supernatant was loaded on 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a nitrocellulose membrane, and incubated with the antibody BJ010 conjugated with horseradish peroxidase. Blotted antibody was visualized by enhanced chemiluminescence.

Detection of GPVI by sandwich enzyme-linked immunosorbent assay (ELISA) was also carried out to

confirm whether the BJ010 recognizes the native GPVI. Monoclonal antibody 11a12 was coated in the 96-well plate to capture the GPVI from platelet lysate. The second antibody was the antibody BJ010 conjugated with horseradish peroxidase and the bound antibody BJ010 was detected by the hydrolysis of O-phenylenediamine (OPD) and read the absorbance at 490 nm. An irrelevant protein keyhole limpet hemocyanin and an irrelevant antibody horseradish peroxidase conjugated goat anti-rabbit immunoglobulin G (IgG) antibody were used as the negative controls in triplicate. The result was expressed as the difference of optical density values between the mean of tested samples and blanks. A positive color reaction was defined by the ratio of absorbance value from the tested wells to that from the negative control wells beyond 2.1.

Flow Cytometric Assay for the Quantification of GPVI and Integrin $\alpha 2\beta 1$ on Human Platelets

Before each measurement, the fluorescent sensitivity of the flow cytometry was always adjusted with calibration particles (Becton Dickinson, Franklin Lakes, NJ) for consistency of the quantification among samples. The reproducibility of the method was also examined by measuring platelet GPVI and integrin $\alpha 2\beta 1$ levels in triplicate from a male donor for consecutive 5 days. The saturated concentration of the antibody BJ010 was determined by different concentration 25, 50, 100, 250, and 500 µg/mL of the antibody.

Healthy volunteers aged 22–70 years (62 males and 39 females) were enrolled for the quantitation of GPVI after informed consent was obtained. None of the volunteers exhibited apparent episodes of bleeding and thrombosis. Whole blood was drawn at fasting stage and mixed with 1/10 volume of 3.2% sodium citrate.

Two microliters of platelet rich plasma (2.0×10^8 /mL) were incubated with 500 µg/mL of the antibody BJ010 or with 10 µg/mL of monoclonal antibody against integrin $\alpha 2$ subunit (CD49b, AK-7; BD Pharmingen, San Diego, CA). Platelets were then washed, resuspended in 10 µg/mL fluorescein isothiocyanate (FITC) conjugated goat anti-mouse IgG (Becton Dickinson) and stored at room temperature for 30 min, then directly loaded on a flow cytometer (BD FACSCalibur). A total of 10,000 events per sample were collected and analyzed by using CellQuest software (Becton Dickinson). Levels of GPVI and integrin $\alpha 2\beta 1$ were expressed as geometric mean fluorescence intensity (GMFI).

Statistical Analysis

Data were presented as median and range (minimum–maximum). The Spearman correlation coefficient was calculated and the Mann-Whitney U test was used

to evaluate statistical significance. A value of $P < 0.05$ was considered to be statistically significant.

RESULTS

Specificity of the Antibody BJ010

The specificity of the BJ010 was evaluated by immunoprecipitation and sandwich ELISA. As shown in Fig. 1, GPVI immunoprecipitated from platelet lysate by monoclonal antibody 11a12 could be recognized by the antibody BJ010. On sandwich ELISA, the ratio of optical density at 490 nm (OD_{490}) from the tested wells (0.54) to that from the control wells (0.045) was 12, indicating that the BJ010 can recognize the GPVI captured from platelet lysate by monoclonal antibody 11a12. The antibody BJ010 binding to GPVI on platelet surface was also investigated by indirect immunofluorescent staining and flow cytometry. As shown in Fig. 2, the peak from the antibody BJ010 moved to right, which is similar to the peak from the monoclonal antibody 11a12. In contrast, the peak from the antibody against glutathione S-transferase (GST) and the mouse pre-immunization serum did not move to right. Consequently, the antibody BJ010 recognizes the extracellular domain of human GPVI on platelets.

Fig. 1. GPVI immunoprecipitated from platelet lysate was recognized by the antibody BJ010. Human platelet lysate containing 20 μ g of proteins was incubated with monoclonal antibody 11a12 against GPVI. The antigen-antibody complex was bound on protein G beads and then separated by 12% SDS-PAGE. The immunoprecipitated GPVI on membrane was blotted by horseradish peroxidase conjugated antibody BJ010.

Fig. 2. Flow cytometric assay of GPVI on human platelets. Histogram of fluorescence intensity on platelet after incubation with various antibodies. Curve 1: preimmunized mouse serum; curve 2: monoclonal antibody against GST (10 μ g/mL); curve 3: the antibody BJ010 (500 μ g/mL); curve 4: monoclonal antibody 11a12 (5 μ g/mL).

Measurement of GPVI on Platelet

The antibody BJ010 was used for the measurement of GPVI by flow cytometry. Accuracy of the flow cytometry was also checked before the measurement. The coefficient of variation for interassays was 8.8% and 7.9% for GPVI and integrin $\alpha 2\beta 1$, respectively. The coefficient of variation for intraassays was 6.3% and 2.2% for GPVI and integrin $\alpha 2\beta 1$, respectively. Saturation concentration of the antibody BJ010 for flow cytometry was determined and 500 μ g/mL of the antibody was used for the measurement of platelet GPVI.

We then measured the levels of GPVI as well as integrin $\alpha 2\beta 1$ on the platelet surface in 101 healthy donors. In healthy donors, the median of platelet GPVI level was 57.7 and the variation between minimum and maximum levels of GPVI (28.0–98.0) was about 3.5-fold. The relative amount of integrin $\alpha 2\beta 1$ on platelets also varied to 4-fold (median 70.0; range: 28.5–117.0). No significant difference in GPVI density was found between males and females, but a weak correlation existed between the relative amount of GPVI and integrin $\alpha 2\beta 1$ on the platelet surface.

DISCUSSION

Recent studies have suggested that GPVI is placed in a central position in the complex processes of platelet tethering, activation, adhesion, aggregation, degranulation, and procoagulant activity on collagen (7). As a requisite factor for the firm adhesion and thrombosis growth on collagen surface, the quantity and quality of GPVI are critical in performing the platelet function, especially related to some hemorrhagic disorders and thrombotic diseases. A deficiency of GPVI may lead to abnormal bleeding tendency and hemorrhagic disorder. Up to now, lack of GPVI was found in a female patient with purpura and mild bleeding time prolongation (8) and a patient with gray platelet syndrome (9). Anti-GPVI antibody was detected in autoimmune diseases, including one patient with systemic lupus erythematosus (10) and four ITP patients (6,11–13). These reports suggested deficiency in GPVI expression or acquired GPVI destruction was closely related to the hemorrhagic disorders. On the other hand, higher GPVI levels correlates directly with the strength of platelet activation in response to collagen, collagen relative peptide and agonist (4,14). These studies support the concept that higher platelet collagen receptor levels on platelets may increase the risk of thrombotic diseases. However, there have been few studies highlighting the clinical value of GPVI measurement. Until now, there is still no clinical report of detecting GPVI amount directly. Therefore it

is necessary to establish a simple and rapid measurement method, which is suitable for clinical use.

Because preparation of polyclonal antibody is rather simple and quick, we chose this method to prepare the antibody and successfully obtained mouse polyclonal antibody BJ010 against the extracellular domain of human GPVI. The specific reactivity of BJ010 was demonstrated by anti-GPVI specific monoclonal antibody 11a12 using immunoprecipitation and sandwich ELISA. As shown in Fig. 1, the BJ010 antibody recognized not only denatured human GPVI but also native GPVI. Flow cytometric analysis proved that BJ010 antibody could identify native GPVI on platelet surface and was suitable for flow cytometric assay. In view of these characteristics, the antibody BJ010 was used for more experiments such as Western blot, immunoprecipitation, ELISA, and flow cytometry compared with monoclonal antibody 11a12. In addition, the antibody BJ010 would be combined with monoclonal antibody 11a12 to establish the sandwich ELISA to detect the soluble GPVI in plasma. Some GPVI specific polyclonal antibodies are provided commercially. Unfortunately, they are not suitable for flow cytometry because they only recognize denatured GPVI.

In this study, we use the BJ010 antibody to establish a method, which measures the relative amount of GPVI on platelets based on indirect immunofluorescence combined with flow cytometry. This method requires small amount of sample with simple operation process and is suitable for clinical routine use with good reproducibility (interassay coefficient of variation is 8.8%). During the detection, we strictly control the operation procedures to avoid platelet activation in vitro, which is the key point for getting reliable data. For example, drawing blood with 18-gauge needle, handling specimen gently, directly separating platelets without washing, and dealing with the sample in a timely manner by finishing the detection within 3 hr, which all merit attention. Despite these, the variation between minimum and maximum platelet GPVI values reached 3.5-fold among the 101 healthy subjects. Meanwhile, the integrin $\alpha 2\beta 1$ level also had a variation of 4-fold, which is similar to the results in the literature. There is also a weak correlation between integrin $\alpha 2\beta 1$ and GPVI. Among the reports about GPVI measurement on platelets in healthy donor, there is great disparity in GPVI level on platelet (0.4- to 5-fold) due to different markers and the small number of donors (4,14,15). In a report with a large number of donors, the variation of GPVI was lower. Best et al. (16) examined the level of GPVI in 102 healthy donors and found that the level of GPVI varied by only 1.5-fold. They also found a weak correlation between the GPVI and integrin $\alpha 2\beta 1$ level, similar to our result. It is not known

whether the variation in GPVI level is related to some physiological situations or genetic background exception to the differences caused by experimental technologies. It is known that GPVI expression level could be influenced by gene polymorphism (4,15) and the gene polymorphism in Chinese Han population differs from that in Western populations (17), which may lead to GPVI level variation in different races. Whether this variance is associated with gene polymorphism should be further studied.

In conclusion, we used self-made polyclonal antibody BJ010 to establish a method measuring the relative amount of the collagen receptor GPVI on platelet surface. This method requires a small amount of sample, the procedure is relatively simple, and the results are reproducible, providing a tool to probe the correlation of GPVI with various thrombotic diseases or bleeding disorders.

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REFERENCES

1. Watson SP, Asazuma N, Atkinson B, et al. The role of ITAM- and ITIM-coupled receptors in platelet activation by collagen. *Thromb Haemost* 2001;86:276–288.
2. Andrews RK, Suzuki-Inoue K, Shen Y, Tulasne D, Watson SP, Berndt MC. Interaction of calmodulin with the cytoplasmic domain of platelet glycoprotein VI. *Blood* 2002;99:4219–4221.
3. Suzuki-Inoue K, Tulasne D, Shen Y, et al. Association of Fyn and Lyn with the proline rich domain of glycoprotein VI regulates intracellular signaling. *J Biol Chem* 2002;277:21561–21566.
4. Furihata K, Clemetson KJ, Deguchi H, Kunicki TJ. Variation human platelet GPVI content modulates GPVI-specific prothrombinase activity. *Arterioscler Thromb Vasc Biol* 2001;21:1857–1863.
5. Qu Chen-xue, Li Chuan-bao, Wang Jian-zhong, Wu Shu-lan, Wan Wen-hui. Prokaryotic expression and polyclonal antibody preparation of human platelet glycoprotein VI extracellular domain. *Chin J Lab Med* 2005;28:1068–1071.
6. Boylan B, Chen H, Rathore V, et al. Anti-GPVI-associated ITP: an acquired platelet disorder caused by autoantibody-mediated clearance of the GPVI/FcR γ -chain complex from the human platelet surface. *Blood* 2004;104:1350–1355.
7. Nieswandt B, Watson SP. Platelet-collagen interaction: is GPVI the central receptor? *Blood* 2003;102:449–461.
8. Moroi M, Jung SM, Okuma M, et al. A patient with platelets deficient in glycoprotein VI that lack both collagen-induced aggregation and adhesion. *J Clin Invest* 1989;84:1440–1445.
9. Nurden P, Jandrot-Perrus M, Combrie R, et al. Severe deficiency of glycoprotein VI in a patient with gray platelet syndrome. *Blood* 2004;104:107–114.
10. Takahashi H, Moroi M. Antibody against platelet membrane glycoprotein VI in a patient with systemic lupus erythematosus. *Am J Hematol* 2001;67:262–267.

11. Arai M, Yamamoto N, Moroi M, et al. Platelets with 10% of the normal amount of glycoprotein VI have an impaired response to collagen that results in a mild bleeding tendency. *Br J Haematol* 1995;89:124–130.
12. Ryo R, Yoshida A, Sugano W, et al. Deficiency of P62, a putative collagen receptor, in platelets from a patient with defective collagen-induced platelet aggregation. *Am J Hematol* 1992;39:25–31.
13. Sugiyama T, Okuma M, Ushikubi F, et al. A novel platelet aggregating factor found in a patient with defective collagen-induced platelet aggregation and autoimmune thrombocytopenia. *Blood* 1987;69:1712–1720.
14. Joutsu-Korhonen L, Smethurst PA, Rankin A, et al. The low-frequency allele of the platelet collagen signaling receptor glycoprotein VI is associated with reduced functional responses and expression. *Blood* 2003;101:4372–4379.
15. Best D, Senis YA, Jarvis GE, et al. GPVI levels in platelets: relationship to platelet function at high shear. *Blood* 2003;102:2811–2818.
16. Yu Zi-qiang, Dong Ning-zheng, Gao Wei-qiang, Bai Xia, Ruan Chang-geng. Study on T13254C polymorphism of the platelet membrane glycoprotein VI in Chinese Han population. *Chin J Hematol* 2005;26:140–143.
17. Chen H, Locke D, Liu Y, Liu C, Kahn ML. The platelet receptor GPVI mediates both adhesion and signaling responses to collagen in a receptor density-dependent fashion. *J Biol Chem* 2002;277:3011–3019.