Relationship between Platelet Aggregation and the Condition of GPIIb-IIIa Complex in Unstimulated Platelets

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Summary

We used a monoclonal antibody against GP IIb-IIIa complex (NNKY1-32) to study the relationship between platelet aggregation and the condition of GP IIb-IIIa complex in unstimulated platelets. The quantities of GP IIb-IIIa complex in Glanzmann's thrombasthenia (Type I, Type II), Heterozygote, EDTA-treated and cation-treated platelets were classified into three groups according to the binding of anti-GP IIb-IIIa complex antibody. Despite the similarity in binding of monoclonal anti-GP IIb-IIIa complex antibody within each groups, however, differences were found in platelet aggregability between samples. Ca^{2+} and Mg^{2+} promoted the reassociation of dissociated GP IIb and GP IIIa in intact cells, and restored aggregabilities of platelets. In particular, mixed materials ($\text{Ca}^{2+} + \text{Mg}^{2+} + \text{auto-plasma}$) significantly restored ADP-induced aggregabilities of platelets. These results suggest the following. The conformational change of GP IIb/IIIa after platelet activation also depends on the conditions of complex formation by GP IIb and GP IIIa and the microenvironment around GP IIb/IIIa in resting platelets. Complex formation alone is insufficient for ADP-induced aggregation, and the presence of Ca^{2+} , Mg^{2+} and some factors in plasma appears to be necessary condition.

Introduction

Platelet GP IIb and GP IIIa are said to be major constituents of the platelet membrane that are critically involved in the formation of the cell surface fibrinogen receptor on activated platelets^{1, 2)}. More recently, it has been reported that the GP IIb and GP IIIa exist predominantly

as the Ca^{2+} -dependent, heterodimer complex, and incubation of intact platelets with ethylenediamine tetraacetic acid (EDTA) at 37°C causes progressive dissociation of GP IIb/IIIa complexes³⁻⁵. The binding of GP IIb/IIIa to fibrinogen becomes apparent only after stimulation of platelets with an agonist such as ADP or thrombin^{6,7}. Agonists may induce a conformational change directly within GP IIb/IIIa so as to render it a competent receptor, or cause changes in the microenvironment so that a hidden receptor becomes accessible to macromolecular ligands^{8,9}. The conformational change of GP IIb/IIIa in stimulated platelets appears thus to be influenced not only by the quantity of GP IIb/IIIa complexes on the unstimulated platelets but also by the associated condition of the "complex" and its microenvironment. In this study, we investigated the relationship between platelet aggregability and GP IIb/IIIa complex in the platelets with various GP IIb/IIIa complexes quantitatively and qualitatively using flow cytometry^{10,11}.

Materials and Methods

Monoclonal antibodies : Monoclonal anti-GP IIb/IIIa complex antibody (NNKY1-32) was used. This antibody was described previously^{4,5,11-13}.

Glanzmann's thrombasthenia (GT)^{14,15} and Heterozygote : Type I GT ; 39-year-old woman. Platelet count was normal, bleeding time prolonged, and clot retraction abnormal. Platelet aggregation induced by ADP, collagen, epinephrine and thrombin were defective or declined extremely. Type II GT ; 44-year-old woman. Platelet count was normal, bleeding time prolonged, but clot retraction normal. Platelet aggregation was the same as Type I GT. Heterozygote ; 33-year-old woman, younger sister of the Type I GT patient. Platelet count, bleeding time and platelet aggregation were almost normal.

Incubation of intact platelets with EDTA : Washed platelets were resuspended to a platelet count of $3 \times 10^8/\text{ml}$, and incubated with EDTA-phosphate buffered saline (PBS) (-) (containing 2 mM EDTA) for 5 to 30 min at 37°C . Platelets were washed by centrifugation and treated in 1% paraformaldehyde (PFA).

Preparation of cation-treated platelets : Washed platelets were incubated with EDTA at 37°C for 10 min, washed by centrifugation and resuspended in Tyrode's buffer modified to give a final concentration of 0.1 mM EDTA and 2 mM cation (Ca^{2+} or Mg^{2+}). Platelets resuspended in modified Tyrode's buffer were incubated for 60 min at 37°C . In order to investigate the effect of auto-plasma for GP IIb/IIIa complex, EDTA-treated (2 mM, 37°C , 10 min) platelets were incubated with Ca^{2+} (2 mM) and Mg^{2+} (2 mM) for 60 min at 37°C , then incubated with auto-plasma for 30 min at 37°C . After incubation, platelets were washed by centrifugation and treated with 1% PFA.

Fluorescence flow cytometry : Details have been published previously^{4, 5, 11)}. In brief, PFA-treated platelets were incubated with monoclonal antibody $10 \mu\text{g}/\text{ml}$ for 30 min at room temperature, then washed 3 times by centrifugation (1400 g for 10 min at room temperature) and resuspended in the same buffer. The treated platelets were incubated with the fluorescein-labeled goat anti-mouse antibody (Kirkegard & Perry Laboratories, Inc., U.S.A.) for 30 min at room temperature, washed 3 times by centrifugation. The labeled platelets were analyzed on a FACS analyzer (Becton Dickinson & Co., U.S.A.). Instrument settings were as follows : volume gates were set at channel 10 and 255, FL1 markers were set at channel 0 and 255. One negative control (autofluorescence), for which unstained platelets were used, was set at 0 and 50. The fluorescence-positive rate was calculated from the total platelet count above channel 51. Another negative control, for which platelets were incubated with fluorescein-labeled goat anti-mouse antibody, was set at the condition that the fluorescence-positive rate is infinitely close to 1%. To quantify fluorescence, SIGMA was defined as

$$\text{SIGMA} = [\text{Mean channel of positive region} \times \text{total platelet counts of positive region}] / 10^3$$
 The change of binding percentage of monoclonal antibodies was expressed as the S.R. (RATIO to SIGMA of normal control). 10,000 events were analyzed in the one-parameter mode. For the two-parameter mode analysis, volume was set at Gate 1 and FL1 was Gate 2. 3,000 events were analyzed in the two-parameter mode.

Change of platelet aggregation : An aggregometer (NKK HEMATRACER 1, Niko Bioscience, Japan) was used for aggregation studies. Platelet samples (Control, GT, Heterozygote, EDTA-treated or cation-treated) were washed with EDTA-PBS(-), then preincubated with Tyrode's buffer containing $0.2 \text{ mM CaCl}_2 + 400 \mu\text{g}/\text{ml}$ fibrinogen before the addition of platelet aggregating agent. Final concentrations of platelet agonists were : ADP (Sigma Chemical Co., U.S.A.) $10 \mu\text{M}$; thrombin (Sigma), $0.5 \text{ U}/\text{ml}$.

Results

Reaction of monoclonal anti-human platelet antibodies with GT platelets : Fig. 1 shows two-parameter mode histograms obtained for platelets from a healthy adult, patients with GT (Type I and Type II) and Heterozygote with NNKY1-32 (anti-GP IIb/IIIa complex). In comparison with the healthy control, Type I GT showed a marked decline in fluorescence intensity, indicating the quantitative absence of GP IIb/IIIa ; but in Type II GT and Heterozygote, slight or considerable binding of monoclonal antibody was found, suggesting that GP IIb and GP IIIa are partially present.

Dissociation and reassociation of GP IIb-IIIa complexes : The S.R. of NNKY1-32 bound to platelets after incubation for 5, 10, 15, and 30 min at 37°C in the presence of 2 mM EDTA

Fig. 1 The two-parameter mode histogram of GT and Heterozygote platelets using monoclonal anti-GP IIb-IIIa complex antibody (NNKY1-32).

Fig. 2 Dissociation of GP IIb-IIIa complex by EDTA treatment and the effect of cations on the reassociation of GP IIb-IIIa complex using NNKY1-32. The change of binding percentage of NNKY1-32 is expressed as the SIGMA RATIO. Results shown are the mean $\pm$ SD of three experiments. *1 EDTA-treated platelets (2 mM, 37°C, 10 min) were incubated with Ca²⁺ and Mg²⁺ for 90 min at 37°C. *2 EDTA-treated platelets were incubated with Ca²⁺ and Mg²⁺ for 60 min at 37°C, then incubated with auto-plasma for 30 min at 37°C.

was reduced remarkably and progressively, as reported previously^{4, 5)}. In the experiment on the reassociation of GP II b-III a complex after EDTA treatment, treated platelets incubated with cation-free Tyrode's buffer showed progressive dissociation of GP II b-III a complex, while those incubated with the Tyrode's buffer containing Ca^{2+} or Mg^{2+} showed reassociation of GP II b-III a complex, as reported previously¹⁶⁾. Fig. 2 shows the changes of GP II b-III a complex on EDTA or cation treatment in terms of the S.R. of NNKY1-32.

Change of platelet aggregation : Samples were classified into three groups by the fluorescence

Table 1 Platelet aggregation in GT, Heterozygote, EDTA-treated and cation-treated platelets.

Samples	Platelet Aggregation(%)	
	ADP	Thrombin
I. Type I GT	0	9
Type II GT	0	12
Cation free ^{*1}	0	20 ± 2
II. EDTA 37°C 10min	0	68 ± 2
Auto-Plasma ^{*2}	8 ± 3	71 ± 3
III. Heterozygote	95	98
EDTA 37°C 5min	31 ± 2	78 ± 4
Ca^{2+} (2mM) ^{*3}	45 ± 3	72 ± 3
Mg^{2+} (2mM) ^{*3}	48 ± 3	76 ± 2
$\text{Ca}^{2+} + \text{Mg}^{2+}$ ^{*4}	47 ± 4	77 ± 3
Mixed Materials ^{*5}	70 ± 2	85 ± 4

Platelet aggregation is expressed as the ratio (mean±SD) of the maximum aggregation of the sample platelets to that of the control platelets. *1 : EDTA-treated platelets (2 mM, 37°C, 10 min) were incubated with cation-free Tyrode's buffer for 60 min at 37°C. *2 : EDTA-treated platelets were incubated with auto-plasma for 60 min at 37°C. *3 : EDTA-treated platelets were incubated with cation plus Tyrode's buffer for 60 min at 37°C. *4 : EDTA-treated platelets were incubated with Ca^{2+} and Mg^{2+} for 90 min at 37°C. *5 : EDTA-treated platelets were incubated with Ca^{2+} and Mg^{2+} for 60 min at 37°C, then incubated with auto-plasma for 30 min at 37°C. Relative quantity of GPIIb-IIIa complex by fluorescence intensity of NNKY1-32 : I (<15%), II (50~60%), III (80~90%). Results shown are one experiment with Type I GT, Type II GT and Heterozygote, and are the mean±SD of three experiments with the other samples.

intensity of NNKY1-32, and their aggregabilities are shown in **Table 1**. ADP- and thrombin-induced aggregation were defective or declined markedly in GT (Type I, Type II), but retained in Heterozygote platelets. On the other hand, in intact platelets incubated with EDTA at 37°C, ADP-aggregability declined more rapidly than the quantity of GP IIb/IIIa complex, while thrombin-aggregability was much better maintained. ADP- and thrombin-induced aggregations were observed in Ca^{2+} , Mg^{2+} , auto-plasma or mixed materials ($\text{Mg}^{2+} + \text{Ca}^{2+} + \text{auto-plasma}$) treated platelets. But differences were found in ADP-aggregability among these samples.

Discussion

Platelet GP IIb and GP IIIa are major constituents of the platelet membrane, and autoantibodies against GP IIb/IIIa are recently found in patients with chronic ITP^{13, 17, 18}. The fibrinogen receptor, which appears to be composed of GP IIb/IIIa, is expressed only in activated platelets, so that surface conformational change does appear to be required for the induction of the fibrinogen receptor that mediates platelet-platelet contact interactions¹⁹. And, it has been reported that but GP IIb and GP IIIa exist predominantly as the heterodimer complex in unstimulated platelets²⁰. On the other hand, it has also been reported the incubation of intact platelets with EDTA at 37°C causes progressive dissociation of GP IIb-IIIa complexes and leads to the decline or loss of ADP-induced aggregation^{3-5, 20}. It appears to that the GP IIb-IIIa complex could be reversibly dissociated by brief incubation at 37°C with EDTA and could be reassociated by Ca^{2+} treatment, while longer time was required for polymerization of GP IIb and GP IIIa^{3, 5, 20}. Moreover, GP IIb is the subunit of the complex that binds Ca^{2+} , and putative Ca^{2+} -binding sequences have been identified in GP IIb^{21, 22}. In this study, we investigated the relationship between platelet aggregation and the quantity of GP IIb-IIIa complex in GT, EDTA-treated and cation-treated platelets.

The various unstimulated platelets were classified into three groups by the fluorescence intensity of anti-GP IIb-IIIa complex antibody bound to them and investigated their ADP- and thrombin-induced aggregation. When washed platelets were incubated with EDTA at 37°C for 10 min and with cation free Tyrode's buffer at 37°C for 60 min, the fluorescence intensity of anti-GP IIb-IIIa complex antibody decreased to about 10% of that of control platelets. In these platelets the quantities of GP IIb and GP IIIa were normal, while the quantity of "complex" was markedly decreased, as in Type I and Type II GT. These platelets, classified as group I, underwent the slight thrombin-induced aggregation but did not undergo ADP-induced aggregation. It was speculated that the quantity of GP IIb-IIIa complex in the unstimulated platelets was below the level needed for fibrinogen-mediated aggregation to be induced when platelets are stimulated.

When EDTA-treated platelets were incubated with auto-plasma, the fluorescence intensity of anti-GP IIb-IIIa complex antibody was about 60% of that of control platelets, about the same as that of platelets incubated with EDTA at 37°C for 10 min. This is the interesting result. Because it is speculated that the concentration of cations in auto-plasma is insufficient for the reassociation of GP IIb-IIIa complex. In the previous⁵⁾ and this studies, we reported that Ca^{2+} (2 mM) and Mg^{2+} (2 mM) promoted the reassociation of dissociated GP IIb and GP IIIa in intact cells. Moreover, recently, we confirmed the reassociation of GP IIb-IIIa complex by cation using immunoelectron microscopy¹⁶⁾. It appears to need the same concentration of cations as in vivo to promote the reassociation of GP IIb-IIIa complex.

When EDTA-treated platelets were incubated with Tyrode's buffer containing Ca^{2+} , Mg^{2+} or mixed materials ($\text{Ca}^{2+} + \text{Mg}^{2+} + \text{auto-plasma}$), the fluorescence intensity of anti-GP IIb-IIIa complex antibody was about 80% that of control platelets. This quantity of GP IIb-IIIa complex was almost as same as that of platelets incubated with EDTA at 37°C for 5 min and of the Heterozygote platelets which used this study. ADP- and thrombin-induced aggregation was observed under these conditions, the latter stronger than the former. Differences in both aggregabilities were also observed between samples (EDTA treatment, cation treatment and Heterozygote). Particularly, there existed the marked discrepancy in ADP-induced aggregation. ADP-aggregability of Ca^{2+} or Mg^{2+} treated platelets was stronger than that of platelets incubated with EDTA at 37°C for 5 min. And ADP-aggregability of the mixed materials treated platelets and the Heterozygote platelets were stronger than that of Ca^{2+} or Mg^{2+} treated platelets. This result explains the "quantitative" as well as "qualitative" importance of GP IIb-IIIa complex in unstimulated platelets. It was speculated that these quantities of GP IIb-IIIa complexes could be aggregated by ADP, but that if GP IIb and GP IIIa were merely associated, the conformational change of GP IIb/IIIa was difficult of induction by ADP. Perhaps it is important that quality of GP IIb-IIIa complex, the microenvironment around "complex" or some factor in plasma.

In this study, the discrepancy was observed between thrombin-induced aggregation and the quantity of GP IIb-IIIa complex in unstimulated platelets. It has been reported that fibrinogen, von Willebrand factor (vWF), fibronectin and thrombospondin (TSP), which are members of a platelet-adhesive protein family including Arg-Gly-Asp (RGD) peptide²³⁾, associated with activated platelets, and that the three adhesive proteins other than TSP can directly bind to or near the GP IIb-IIIa complex when platelets are stimulated with thrombin²⁴⁾. Moreover, recently, it is reported that the quantity of GP IIb-IIIa complex increased in the expression of GP IIb/IIIa-related epitopes on the platelet surface following activation with strong agonists^{25, 26)}. It may be that thrombin-induced aggregation involves aggregating mechanisms different

from ADP-induced aggregation²⁷⁾.

The qualitative importance of GP IIb-IIIa complex has been reported previously. A variant of GT²⁸⁾, acquired GT²⁹⁾ and functional GT³⁰⁾ appeared to be caused by abnormality of GP IIb/IIIa itself, autoanti-platelet antibody and drug metabolites, respectively. In all these cases, GP IIb-IIIa complex was normal quantitatively. Therefore, it is speculated that both quantitative and qualitative factors concerning the GP IIb-IIIa complex of unstimulated platelets are important for the conformational change of GP IIb/IIIa in the activated platelets by ADP.

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