A New Binding Assay of von Willebrand Factor and Glycoprotein Ib Using Solid-Phase Biotinylated Platelets

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Abstract. To obtain compounds that inhibit the interaction of von Willebrand factor (vWF) and glycoprotein (GP) Ib, a novel binding assay was established. The binding of fixed platelets to vWF-R497 mutant was quantified by a solid phase assay. In this assay, fixed platelets bound to the vWF-R497 mutant, carrying the deletion of Glu497-Tyr508 and the missense mutation of Arg545 to Ala, without binding modulators such as ristocetin. The K_d value of the binding was 2.8 nM, which was consistent with the result from liquid binding assay. The binding was inhibited by aurin tricarboxylic acid (ATA) and an anti GPIb antibody, AK2. Using this binding assay, we screened our library compounds and obtained D74-3736. This compound also inhibited ristocetin-induced platelet aggregation in the human platelet-rich plasma.

Keywords: biotinylation of fixed platelets, binding assay of von Willebrand factor (vWF) to glycoprotein Ib (GPIb)

The interaction of plasma von Willebrand factor (vWF) with denuded subendothelial tissues is thought to be the crucial event that leads to the exposure of the platelet glycoprotein (GP) Ib binding domain of vWF that is cryptic in normal circulation (1, 2). Although no in vivo modulators for vWF-GPIb interaction have yet been identified, ristocetin (3) and snake venom botrocetin (4, 5) have been used in experiments in vitro as artificial modulators to induce the binding of vWF to GPIb. Ristocetin apparently binds to both platelets and vWF (6), whereas botrocetin binds to the vWF A1 domain in the GPIb binding site on vWF but not to GPIb (7). A conformational change of vWF to a higher affinity state appears to be required for the binding to GPIb. vWF weakly interacts with platelets without the modulators. Some mutants of the vWF A1 domain acquired high affinities to GPIb and could bind to GPIb without the modulators (8). In particular, a vWF-R497 mutant carrying the deletion of Glu497-Tyr508 and the missense mutation of Arg545 to Ala, showed spontaneous binding to GPIb with the highest affinity among the various vWF mutants (8).

In spite of the clinical importance of the interaction between vWF and GPIb, its inhibitors have not been extensively investigated. Conventional receptor binding assays using ¹²⁵I-vWF require specific facilities for radioactive work (9, 10). Although an ELISA-based binding assay using vWF conjugated with biotin has been reported (11), it was difficult to apply it to high throughput screening because of the complicated procedures involved. We have now established a simple and highly sensitive binding assay based on Dissociation Enhanced Lanthanide Fluoroimmunoassay (DELFIA) using purified recombinant vWF-R497 mutant immobilized on 96-well plates without modulators. This assay should provide a convenient method for the screening of new developmental antithrombotic agents that block the interaction of vWF with GPIb.

The fixed platelets were purchased from HELENA Laboratories (Beaumont, TX, USA). The biotinylation reagent, Sulfo-NHS-LC-biotin, was purchased from PIERCE (Rockford, IL, USA). Aurin tricarboxylic acid (ATA) was obtained from WAKO Pure Chemicals (Osaka). The anti-GP Ib antibody AK2 (FUMCA740) was obtained from Funakoshi (Tokyo). Fluorescein isothiocyanate (FITC)-labeled streptavidin and goat
anti-mouse Ig conjugated with FITC were obtained from BD Biosciences (Franklin Lakes, NJ, USA). Anti-vWF antibodies (A0082 and P0226) were purchased from Dako (Glostrup, Denmark). Ristocetin was purchased from Sigma (St. Louis, MO, USA). Europium-labeled streptavidin was purchased from Perkin Elmer (Waltham, MA, USA). All other chemicals were commercial products of reagent grade.

Hybridoma producing an anti-GPⅡb/Ⅲa antibody, 7E3 (HB-8832) and HEK293 (CRL-1573), were purchased from ATCC (Manassas, VA, USA). HB-8832 was cultured at 37°C in 5% CO₂ humidified air in RPMI (Invitrogen, Carlsbad, CA, USA) containing 10% fetal calf serum (FCS, Invitrogen), and HEK293 was cultured at 37°C in 5% CO₂ humidified air in DMEM with high glucose (Invitrogen) containing 10% fetal calf serum.

The recombinant vWF-R497 protein was purified by anion-exchange chromatography from the culture supernatants of HEK293 cells stably overexpressing the mutant. The anti-glycoprotein IIb/Ⅲa antibody, 7E3 was purified from the culture supernatants of HB-8832 using prosep A (Millipore, Billerica, MA, USA).

Lyophilized fixed platelets were reconstituted with TBS. The reconstituted platelets were washed twice with PBS. The biotinylation reagents (0.54 mg per 2 × 10⁸ cells) were added into the fixed platelets suspended in PBS. After 1-h reaction, the biotinylated platelets were washed three times with PBS. After the washing, the biotinylated platelets were resuspended in TBS containing 0.05% NaN₃. The platelets were aliquoted and stored at −80°C.

One-color cell surface immunostaining was performed to test the efficiency of the biotinylation of the fixed platelets and the antigenesity of GPⅡb. FITC-labeled streptavidin was used to determine the efficiency of the biotinylation of the fixed platelets. The anti-GPⅡb antibody AK2 and goat anti-mouse Ig conjugated with FITC were used to test GPⅡb antigenesity. The labeled cells were analyzed by a FACSCalibur flow cytometer and CellQuest software (BD Biosciences). An isotype control Ig or bovine serum albumin (BSA) was used to determine the positive and negative cell populations.

The vWF binding assays in the liquid phase were described previously (12). In brief, vWF (wild-type or R497 mutant) were mixed with fixed platelets or biotinylated platelets. In the case of wild-type vWF, ristocetin (1.2 mg/ml) was added to the reaction mixture. After 1-h incubation at room temperature, the samples were centrifuged at 15,000 × g at 4°C. The unbound vWF in the supernatants was measured by a sandwich ELISA using anti-vWF antibodies. The bound vWF was calculated from the amounts of unbound vWF.

The recombinant vWF (0.15 – 15 µg/well) was immobilized on 96-well plates at 4°C overnight. The plates were blocked by TBS containing 3% BSA at room temperature for 1 h. After washing the plates with wash buffer (TBS containing 0.05% Tween 20), biotinylated platelets (2 × 10⁶ cells/well) were added. For the library screening, compounds were added simultaneously with biotinylated platelets. After 2-h incubation, the plates were washed four times with wash buffer, and the europium-labeled streptavidin (25 ng/ml) was added. After incubation for 1 h, the plates were washed four times and enhancement solution (Perkin Elmer) was added. The fluorescence of the europium was measured using a time-resolved fluorometer (Perkin Elmer).

Human blood was collected from healthy volunteers with 3.8% citrate. The blood was centrifuged at 180 × g for 10 min at room temperature. The platelet-rich plasma was collected in new tubes and stored at 4°C. The platelet-rich plasma was dispensed into glass cuvettes and the compounds were added to test their inhibitory effects. After incubation for 2 min at 37°C, ristocetin was added to the cuvettes and the transmission of visible light was measured for 7 min using HEMATRACER 313M (MC Medical, Tokyo).

For the establishment of a reliable solid-phase vWF binding assay between platelet GPIb/IX, biotinylated platelets were selected as a source of GPIb. Fixed platelets were used for biotinylation because large amounts of the material are commercially available for the implementation to high-throughput screening. Although biotinylation of the platelets from fresh human platelets was reported, the biotinylated platelets showed less sensitivity to various agonists (13). Therefore, the procedure was modified for fixed platelets. Platelets were efficiently labeled under the improved conditions, and most of the treated platelets were stained with streptavidin conjugated with FITC by a flow cytometric analysis (Fig. 1A). The fixed platelets conjugated with biotin had the same antigenesity of GPIb as non-labeled platelets (Fig. 1B), suggesting that GPIb still maintained its functions after the biotinylation. The affinity of GPIb in biotinylated- or non-labeled platelets to wild-type vWF was determined using a liquid phase binding assay. Similar amounts of vWF bound to both biotinylated and non-labeled platelets (Fig. 1C). These observations suggested that the botinylated platelets are applicable to vWF binding assays. In our experimental conditions, 0.54 mg of Sulfo-NHS-LC-biotin was mixed with 2 × 10⁶ platelets, and this ratio gave the best balance of biotinylation and vWF reactivity. When more than 0.54 mg of biotin reagents were used, the affinity of GPIb to vWF was reduced (data not shown).

Among various vWF mutants, vWF-R497 spontaneously binds to GPIb with the highest affinity. Re-com-
binant wild-type and the mutant vWF proteins were purified from HEK293 cells, and their binding to GPIb was characterized by the liquid phase binding assay. Non specific binding of wild-type vWF was determined in the absence of ristocetin. The binding of wild-type vWF to the platelets increased in a dose-dependent manner. The $K_d$ and $B_{max}$ values were 3.7 nM and 21,000 sites per platelet, respectively. This $K_d$ value was consistent with previous reports (11). The platelets binds to the vWF-R497 mutant with a higher affinity even in the absence of ristocetin. The $K_d$ value and $B_{max}$ were 2.7 nM and 21,000 site per a platelet, respectively (Fig. 2A).

Next, a solid-phase binding assay was developed using vWF-R497 and fixed platelets conjugated with biotin (Fig. 2: B – D). In this assay system, purified vWF-R497 protein was immobilized on a microtiter plate, and the binding of the biotinylated platelets was detected using DELFIA technology. The binding of the biotinylated platelets depended on the amounts of immobilized vWF-R497. The $K_d$ value of vWF-R497 to the biotinylated platelets was 2.8 nM, and this value was consistent with the results from a liquid binding assay. As shown in Fig. 2C, ATA, an inhibitor for the binding of vWF to platelet GPIb/IX (14), suppressed the binding of biotinylated platelets to immobilized vWF-R497 with an IC$_{50}$ value of 1.4 µM. This value was comparable to the previous results from other binding assays (15). The binding of the biotinylated platelets to immobilized vWF-R497 was specifically mediated by GPIb as the anti-GPIb inhibitory antibody AK2 (16) suppressed the binding (Fig. 2D). The inhibitory effect of AK2 on the binding between biotinylated platelets and vWF-R497 was incomplete because the affinity of vWF-R497 to GPIb was so strong that the amount of AK2 added to the reaction was not sufficient to fully perturb the binding. Taken together, the specific interaction between vWF and GPIb was detectable by solid phase binding assay. The anti-GPIIb/IIIa antibody 7E3 did not alter the binding of the platelets. It should be noted that no interaction between vWF and GPIIb/IIIa was observed under our experimental conditions using fixed platelets conjugated with biotin. The activation of GPIIb/IIIa is necessary for binding to vWF (1, 2). The loss of the effects of the anti-GPIIb/IIIa antibody 7E3 can be explained by the fact that GPIIb/IIIa activation did not occur in the fixed platelets.
A screening program was carried out using this solid-phase binding assay, and D74-3736 was obtained as a hit compound (Fig. 3A). The IC$_{50}$ value of this compound was 15.4 µM (Fig. 3B). For further evaluation of the effects of D74-3736 on the platelet activation induced by the interaction between vWF and GPIb, a ristocetin-induced platelet aggregation assay was performed using the human platelet-rich plasma (Fig. 3C). The ristocetin-induced platelet aggregation was inhibited by 100 µM D74-3736, and the IC$_{50}$ value for the inhibition was 68 µM.

In this study, a new binding assay for the detection of the interaction between vWF and GPIb was established using the vWF-R497 mutant and fixed platelets conjugated with biotin. The fixed platelets conjugated with biotin were added to the purified recombinant vWF-R497 protein immobilized on 96-well plates, and the bound platelets were quantified by a DELFIA system using europium-labeled streptavidin. This assay did not require the artificial activation of vWF by stimulators and the results from the binding assay were consistent with the previous results from other binding assays. As a screening system, our method consisting of biotinylation of fixed platelets and usage of mutant vWF is simpler and more tractable compared with the method by Miura et al. (11) and Federici et al. (15), which includes a more complicated purification step for vWF and requires ristocetin for binding to occur. Moreover, our binding assay may be applicable to diagnostic research. The competition assay with our method would more readily determine the level of vWF than the conventional assay, although our assay, excluding ristocetin as a modulator, should be limited to a gain-of-function vWF protein generated from a particular type of von Willebrand disease (vWD) such as type 2B vWD.

D74-3736 was obtained from a chemical library screening and was shown to inhibit ristocetin-induced platelet aggregation in the human platelet-rich plasma. D74-3736 might have potential for the development of new antithrombotic agents mediated by the inhibition of the interaction between vWF and GPIb.
New Binding Assay Between vWF and GPIb/IX

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References