Quantitative Detection of Platelet GPIIb-IIIa Receptor Antagonist Activity Using a Flow Cytometric Method

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Platelet-membrane surface receptors are important targets for pharmacologic intervention in cardiovascular disease. Among these, glycoprotein (GP) IIb-IIIa is dominant and integrally involved in platelet aggregation and thrombus formation. When activated, GPIIb-IIIa binds soluble fibrinogen (Fb) in a key, early step of this process. New drugs are under development that block Fb binding to GPIIb-IIIa and inhibit platelet aggregation. A thorough understanding of the relationship between circulating drug levels and the extent of GPIIb-IIIa receptor occupancy in humans is crucial for safe and efficacious use of these agents. Described here is the development of a new technique for measurement of GPIIb-IIIa receptor occupancy. In this

assay, activated human platelets are incubated with biotinylated fibrinogen (Fb-biotin) followed by antibiotin-FITC. The extent of Fb binding is determined using flow cytometric analysis. Our results indicate that Fb-biotin binds rapidly to activated platelets and its detection is dependent on incubation temperature. Platelets that were pre-incubated with the GPIIb-IIIa antagonist echistatin were inhibited from binding Fb-biotin in a concentration-dependent manner. The fluorescence of processed samples was stable for two weeks in the cold. The assay described here is simple, cost effective, and can be adapted for use in clinical evaluations of these new drugs. *J. Clin. Lab. Anal.* 12:191–196, 1998. © 1998 Wiley-Liss, Inc.

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INTRODUCTION

Platelet-surface receptors are key targets for pharmacologic intervention in thrombotic cardiovascular disease. The platelet glycoprotein (GP) IIb-IIIa is the most abundant platelet integrin with 50,000–70,000 sites per platelet (1). When platelets are activated, membrane-surface GPIIb-IIIa binds soluble fibrinogen (Fb) in a key, early step of platelet aggregation and thrombus formation (2). Currently, there is great interest in the clinical development of novel agents that can bind to platelet GPIIb-IIIa, block Fb binding, and be used in the prevention and management of thrombotic disease states.

One of the critical challenges in the clinical development of such platelet GPIIb-IIIa receptor antagonists is selection of doses that can provide effective antithrombotic activity without resulting in bleeding complications (3). Because the determination of safe and effective doses is so important, it is prudent to clearly establish the pharmacodynamic relationship between plasma drug levels and GPIIb-IIIa receptor occupancy in early clinical studies.

Methods for estimating the extent of GPIIb-IIIa recep-

tor occupancy have previously been described. Frequently, the extent of occupancy has been characterized functionally, by the inhibition of *in vitro* platelet aggregation (4,5). Alternatively, radiometric binding assays (6–9) and fluorescence- /flow-cytometric approaches have been used (10–15). For the latter, measurements of antiFb antibody binding (10–12) or that of fluorochrome conjugated Fb (14–16) were utilized. In the current study, we describe the development of a novel flow-cytometric assay that measures the binding of biotinylated-Fb (Fb-biotin) to platelets. The method described here provides a simple and inexpensive alternative approach for measurement of GPIIb-IIIa receptor occupancy. We used echistatin (ECH), a polypeptide GPIIb-IIIa antagonist derived from snake venom as a model agent for evaluating the system.

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MATERIALS AND METHODS

Working Buffer

The working buffer used throughout the experiments was a modified Krebs-Henseleit solution. Dehydrated base salts (#K-3753, Sigma Chemical Co., St. Louis, MO) were dissolved in 1 liter of sterile, nonpyrogenic water (Baxter Healthcare Corp., Deerfield, IL) and passed through a 0.22 μ m membrane filter unit. To 500 ml of this solution, 3.5 ml of a 7.5% (w/v) sodium bicarbonate (#S-8761, Sigma) solution and 1.75 g of fraction-V bovine albumin (#82-047-3, Bayer Corp., Kankakee, IL) were added and mixed well. This working buffer was freshly prepared for each experiment.

Chemicals and Antibody

Echistatin (# PFIB05B, Bachem, Feinchemikalien AG, Switzerland) was dissolved to 1 mM in dimethyl sulfoxide (DMSO), aliquotted, and stored at -70°C . Thrombin-receptor activating peptide (TRAP) was produced at Lilly Research Laboratories as previously described (17), dissolved in working buffer to 1 mM, aliquotted, and stored at -70°C . Fluorescein isothiocyanate (FITC) conjugated antibiotin antibody was purchased from Sigma (#F-6762). A paraformaldehyde (# 0380, Polysciences, Inc., Warrington, PA) solution (1% PF) was prepared by dissolving the powder to 1% in phosphate-buffered saline.

Biotinylated Fibrinogen

Preparation of Fb-biotin has been previously described (18). Briefly, human Fb was purchased from Enzyme Research Laboratories (South Bend, IN) and dialyzed overnight at 4°C in bicarbonate buffer (0.1M NaCl, 0.1 M NaHCO_3 , pH 8.2). The solution was cleared by centrifugation, and the Fb concentration in the supernatant was adjusted to 1mg/ml in the dialysis buffer. Sulfo-NHS-biotin ester (Pierce, Rockford, IL) was added (0.2 mg per ml of Fb solution) and reacted for 30 min at room temperature (RT) with gentle mixing. Unreacted biotin was removed by overnight dialysis at 4°C in Tris buffer (0.1 M NaCl + 50 mM Tris base + 0.05% NaN_3 , and concentrated HCl to achieve pH = 7.4). The protein content of the reagent was determined spectrophotometrically and its ability to bind to glycoprotein IIb-IIIa (GPIIb-IIIa) was confirmed in an ELISA assay. The biotinylated fibrinogen (Fb-biotin) reagent was aliquotted and stored (-70°C) for the ensuing studies.

Platelet Samples

Blood was collected by venipuncture from healthy human volunteers who had not consumed aspirin or ibuprofen for at least 2 weeks. The blood was drawn into syringes containing the anticoagulant citrate-phosphate-dextrose solution (# C-7165, Sigma) at the proportion of 1.4 ml anticoagulant/

8.6 ml blood. Anticoagulated blood samples were centrifuged at $400 \times g$ for 12 min at RT and the platelet rich plasma (PRP) was gently collected and adjusted to 5.6×10^7 platelets/ml in working buffer. All blood samples were processed within 1 hour of phlebotomy.

Binding of Fb-biotin to Platelets

Activated platelet suspensions were prepared by incubating platelet suspensions with TRAP (5 μM) for 15 min at 37°C . Nonactivated (resting) platelet suspensions were similarly diluted with the TRAP vehicle and held at RT. To 12×75 mm polypropylene tubes containing 10 μl of diluted ECH or buffer, 90 μl of activated- or resting-platelet suspensions were added and incubated for 15 min at RT. In whole blood experiments, ECH was added directly to the anticoagulated blood samples followed by a 15 min incubation at RT and centrifugation to prepare the PRP which was diluted and incubated with TRAP or TRAP vehicle as described above. Various dilutions of Fb-biotin in 10 μl volumes were added to the platelet-containing tubes and incubated for various times at RT. The resultant samples were then held at RT or 4°C and 10 μl of antibiotin-FITC (25 $\mu\text{g}/\text{ml}$ final concentration) were added to the tubes, gently mixed and incubated at RT or 4°C for 30 min. Samples were then fixed with 1.0 ml of 1% PF and held at 4°C in the dark until analyzed on the flow cytometer.

To examine the early phase of Fb-biotin binding to platelets, 90 μl of the platelet suspensions were added to a series of 12×75 mm tubes containing 10 μl Fb-biotin. At specific, short intervals, the platelet mixtures were fixed with the addition of 1.0 ml of 1% PF. The fixed samples were centrifuged at $1500 \times g$ for 10 min at RT and all but ~ 100 μl were then discarded. The resultant samples were reacted with antibiotin-FITC, fixed, and stored as described above.

Flow Cytometry

Reacted platelet samples were analyzed with an Epics XL flow cytometer (Coulter Corp., Miami, FL) is equipped with a 488 nm air-cooled argon ion laser. Forward- and right-angle light scatter signals along with green fluorescence through a 525 nm bandpass filter were collected from 10,000 platelets from each experimental sample. Data were acquired on a logarithmic scale and stored in listmode files. The light scattered mean fluorescence intensity (MFI) of each treated platelet sample was computed using WinListTM software (Verity Software House, Inc., Topsham, ME).

Data Analysis

Inhibition of Fb-biotin binding was expressed as the ratio of the MFI from each sample (minus the MFI of resting platelets) to the maximal MFI (minus MFI of resting platelets) for each donor's data set. Each ratio was then transformed into the percent inhibition as follows: % Inhibition

= $100 \times (1.0 - \text{Ratio})$. For each treatment set, an IC_{50} was determined by linear regression of the percent inhibition data versus the log of the ECH concentration over the linear range.

RESULTS

Flow-Cytometric Detection of Fb-Biotin Binding to Human Platelets

Flow-cytometric detection of Fb-biotin binding to TRAP-activated human platelets is depicted in Figure 1. Data from this representative experiment indicate that Fb-biotin selectively binds to activated platelets (in PRP) with an approximate 4-fold increase in MFI over that of either nonactivated platelets (MFI = 7.0) or those with no Fb-biotin incubation (MFI = 6.6). Fb-biotin binding was evident over the 100-fold range of Fb-biotin used. An MFI decrease was observed when concentrations below 0.1 mg/ml are used. From these data, we selected 0.1 mg/ml Fb-biotin for use in subsequent experiments. The effect of ECH preincubation on subsequent Fb-biotin binding to activated platelets is also shown in Figure 1. These results depict how ECH at 1 μM , fully inhibits Fb-biotin binding to activated platelets.

Early Time Course of Fb-Biotin Binding to Platelets

As shown in Figure 2, binding of Fb-biotin to TRAP-activated platelets (in PRP) occurred rapidly and appeared to be biphasic in nature. During the first 4 min of incubation, the MFI of TRAP-activated samples increased more than 3-fold while during the following 11 min, only an additional 1.5-fold increase was observed. No binding of Fb-biotin to resting platelets was detected during this incubation period.

Fig. 1. The effect of echistatin on the binding of biotinylated human fibrinogen to activated human platelets. Human platelet suspensions were activated with TRAP and incubated with echistatin (1 μM ; solid bars) or buffer (stippled bars) for 15 min at room temperature. The samples were then reacted with Fb-biotin, antibiotin-FITC, and fixed as described in the text. The amount of Fb-biotin binding was measured using flow cytometry and expressed as mean fluorescence intensity (MFI). The dotted horizontal line indicates the background MFI of similarly reacted, resting platelets.

Fig. 2. Early-phase binding of biotinylated fibrinogen to resting and activated human platelets. Human platelets were activated with TRAP (filled circles) or control buffer (open squares) and incubated with Fb-biotin at RT as described in the text. At the time points shown, the samples were fixed with 1% PF, washed, incubated with antibiotin-FITC and analyzed on the flow cytometer. Data for duplicate donors are expressed as the mean % of maximum MFI $\pm$ SE.

Extended Time Course of Fb-Biotin Binding to Platelets

When TRAP-activated platelets (in PRP) were incubated with Fb-biotin for periods longer than 15 min, Fb-biotin was dependent on the incubation temperature (Fig. 3). Incubation of platelets with Fb-biotin at 4°C resulted in increased binding of Fb-biotin (~1.5-fold increase) through the additional 75-min incubation. When the same mixture was held at RT, a time-dependent decrease (~2.6-fold) bound Fb was observed.

Fig. 3. Detection of biotinylated-fibrinogen binding to activated human platelets over an extended time course. Human platelets were activated with TRAP and incubated with Fb-biotin at RT for 15 min and then further incubated at 4°C (open circles) or at RT (filled squares). At the time points shown on the figure, the samples were incubated with antibiotin-FITC, fixed, and analyzed with a flow cytometer as described in the text. The data is expressed as the mean % of maximum MFI $\pm$ SE for 3 experiments.

The Effect of Echistatin on Fb-Biotin Binding to Platelets in PRP and Whole Blood

When platelets in diluted PRP were activated and treated with ECH for 15 min, the binding of Fb-biotin was inhibited in a concentration-dependent manner (Fig. 4, open squares). For 5 donors tested, the mean IC_{50} was 6.57 ± 0.48 nM. The inhibitory effect was linear ($r = 0.996$) between 3.1 and 12.5 nM. The inhibitory effect of ECH on Fb-biotin to platelets was also observed when the pretreatment occurred in whole blood (Fig. 4, filled circles). The mean IC_{50} for 5 volunteers was 29.1 ± 8.8 nM and the effect was linear ($r = 0.990$) between 10 and 100 nM. The IC_{50} of ECH in whole blood was approximately 4-fold higher than that determined in diluted PRP.

Stability of Fluorescence Measurements of Fixed Specimens

A group of processed and fixed samples was stored in the dark at 4°C and re-analyzed over a two-week period. As displayed in Figure 5, the fluorescence-intensity measurements were quite stable. No large variations or significant trends in fluorescence measurements were observed for these 5 representative samples. The average coefficient of variation for each sample over time was 8.7%.

DISCUSSION

Agents that selectively bind to platelet GPIIb-IIIa inhibit Fb binding and prevent platelet aggregation and thrombus formation. The therapeutic value of such agents in treating human cardiovascular disease requires a careful balance of antithrombotic effect versus the risk of excessive bleeding

Fig. 4. Echistatin blockade of Fb-biotin binding to activated human platelets in anticoagulated plasma and whole blood. Echistatin was added to anticoagulated, whole human blood (filled circles) or to PRP (open squares) for 15 min as described in the text. The treated PRP samples were activated with TRAP and incubated with Fb-biotin at RT for 15 min. The samples were then incubated with antibiotin-FITC, fixed, and analyzed with a flow cytometer as described in the text. The data is expressed as the mean % inhibition of maximum MFI $\pm$ SE for 5 subjects.

Fig. 5. Fluorescence stability of processed samples. Human platelet samples (in diluted PRP) were treated with ECH, Fb-biotin, antibiotin-FITC, and fixed as described in the text. The resultant samples were either analyzed immediately (0 days) or stored in the dark at 4°C for the time periods indicated and then analyzed. Five representative samples were tested and their MFI values are shown. The triangles and circles represent data from samples that were either not activated or blocked with ECH; while data represented by the squares were taken from TRAP-activated samples.

complications (3). Ideally, clinical evaluation of these new agents would include not only the measurement of plasma drug concentrations but also an estimate of platelet GPIIb-IIIa receptor occupancy. A careful titration of the dose of these agents may be required to avoid adverse events during early clinical trials. Several methods for estimating platelet GPIIb-IIIa receptor occupancy have been described. These include aggregometry (5,19,20), radiometric methods (6–9) and flow-cytometric approaches (13,15,16). While aggregometry may closely mimic pathological thrombus formation, it can be labor intensive, imprecise, and not amenable to the screening of large numbers of samples. Radiometric approaches provide a somewhat more simplified approach, but necessitate the handling issues of dealing with radioisotopes. A flow cytometric approach can provide the necessary precision and assay simplicity while avoiding the use of radioisotopes.

Flow cytometry has been shown to be a powerful tool for study of platelets. An excellent review of the use of flow cytometry for measuring platelet function has recently been published (21). Platelet activation can be detected by flow-cytometric measurements of platelet-shape change (22), surface-protein expression (22–24), and Fb binding (10–16). The binding of Fb to platelets has been measured flow-cytometrically with antifibrinogen antibodies (10–12) and FITC-labeled fibrinogen (14–16). Blockade of Fb binding to GPIIb-IIIa has been used to screen compounds for receptor antagonism (25) and is a logical choice for monitoring GPIIb-IIIa occupancy by candidate compounds in clinical trials. We believe this study is the first to report use of Fb-biotin binding to study platelet GPIIb-IIIa receptor occupancy.

In this article we described the development of a flow-

cytometric assay to quantitatively estimate the amount of Fb-biotin that has bound to activated platelets *in vitro*. In addition, we used echistatin, a polypeptide GPIIb-IIIa antagonist, as a model agent to validate the selectivity of the Fb-biotin binding and to serve as a surrogate for agents undergoing clinical evaluation. Toward this goal, we chose to develop an assay that would be suitable for use in a multicenter clinical trial. We considered issues such as processing time and ease, stability of processed samples, and cost effectiveness. As part of the assay's development, we also studied the binding kinetics of Fb-biotin to activated platelets.

The method we have developed is a homogeneous assay, i.e., no "washing" steps are involved. It employs the use of Fb-biotin and antibiotin-FITC that are added sequentially to diluted human PRP. The use of a homogeneous assay greatly reduces processing time and eliminates the variable level of platelet activation induced by high-speed centrifugation (26) which could confound clinical assay results. We determined the preferred concentration of Fb-biotin (Fig. 1) and selected the secondary reagent, antibiotin-FITC after streptavidin-FITC was deemed inappropriate due to high background binding (data not shown). We also determined that processed samples could be fixed with paraformaldehyde and stored without significant changes in fluorescence intensity (Fig. 5). The determination that the method described here performs well in the presence of native Fb adds to its utility. This procedure eliminates the isolation of platelets from PRP—a time-consuming and laborious step—and more closely reflects the intact physiological state. Furthermore, this method has other advantages over the radiometric procedure: stability of reagents and use of nonradioactive materials. The lot of Fb-biotin used in these studies has been stable (no detectable changes in ELISA binding to immobilized GPIIb-IIIa; data not shown) for 2 years. This implies that the same lot of reagent could be used for a large clinical trial, providing consistent results and eliminating the need for additional reagent-validation studies. Use of nonradioactive materials implies that clinical site selection could be simplified by the accompanying ease of waste disposal and shipment of processed samples to a central lab.

As part of our desire to understand the dynamic aspects of this assay, we studied the temporal effects of Fb-biotin binding to activated platelets. The data (Fig. 2) indicate that Fb-biotin binding to activated platelets occurs rapidly, with 66% of the binding occurring within the first 4 min of the assay. These results are consistent with published findings (12,14,15). Extended analysis of Fb-binding for intervals greater than 15 min (Fig. 3) suggests that Fb-biotin binding to platelets is a dynamic process. The ability to detect bound Fb-biotin is clearly dependent on the incubation temperature. A growing body of literature suggests that platelets internalize bound Fb and that this cycling of Fb may represent a fundamental regulatory mechanism of platelet function (27). This phenomenon was supported by our results. When antibiotin-FITC was added to platelet/Fb-biotin mixtures held at RT,

the fluorescence signal steadily declined over 90 min. This was not the case if the mixtures were placed on ice after the first 15 min. The data suggest that platelets held at RT internalize Fb-biotin over time and less Fb-biotin is available for detection with the antibiotin-FITC. Our data imply that use of the described assay for clinical specimens requires that incubation and storage temperatures be closely controlled for maximum precision.

Our study illustrates that flow cytometric measurement of Fb-biotin binding to activated platelets is a sensitive and reproducible method with which to measure the inhibitory activity of the polypeptide snake venom component ECH. We have determined (Fig. 4) that ECH has a mean IC_{50} (in PRP) of 6.57 ± 0.48 nM which is consistent with that obtained by other investigators who used platelet aggregation and radiometric approaches (19). In an effort to simulate the analysis of samples gathered from a clinical trial, ECH was added to whole blood and tested for its ability to block Fb-biotin binding in the resulting PRP. An approximate 4-fold decrease in inhibitory activity ($IC_{50} = 29.1 \pm 8.8$ nM) was observed. This decrease in potency could originate from an interaction of ECH with other formed elements in whole blood or from an increase in assay interference.

In conclusion, the measurement of platelet GPIIb-IIIa receptor occupancy in clinical evaluations of these novel, selective agents could in part help define the balance between efficacy and safety of these agents. The use of such an assay might be particularly suited for early safety and dose-ranging studies. The Fb-biotin binding assay described here meets many of the considerations pertinent to the assay needs of a clinical trial. As described here, sample processing is simple and rapid due to its homogeneous approach. The reagents are inexpensive and stable. The assay provides a reliable method for measuring the effects of a polypeptide platelet aggregation inhibitor and could easily be adapted for use with small-molecule platelet-receptor antagonists, typical of this therapeutic class. The stability of the fluorescence readouts allows for batch measurements and/or shipment of samples to a central site for analysis.

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