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(57) Abstract

The present invention provides a method for the quantification and assessment of platelet activation in whole blood samples and monitoring of antiplatelet pharmacologic agents. The method for quantifying platelet activation includes exposing platelets to a physiological concentration of an agonist that activates some of the platelets, resulting in the formation of at least one binding site on the surface of the activated platelets, and measuring the activated platelets. The present invention provides a method of assessing specific components of platelet activation.

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SYSTEM FOR THE QUANTIFICATION OF PLATELET ACTIVATION AND FOR MONITORING AND ADJUSTING THE DOSAGE OF ANTIPLATELET PHARMACOLOGIC AGENTS

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to a method for quantifying and assessing platelet activation in whole blood samples and monitoring the dosage of antiplatelet pharmacologic agents. The method comprises exposing platelets to a physiological concentration of an agonist that activates some of the platelets, resulting in the formation of at least one binding site on the surface of the activated platelets, and measuring the activated platelets. The present invention permits the assessment of specific components of platelet activation.

2. Description of the Related Art

Platelet activation is a critical component of hemostasis, the process which prevents bleeding following vascular injury. During hemostasis, platelets adhere to the site of the vascular injury. The platelets undergo activation, which leads to a change in their shape, facilitating the formation of a hemostatic plug. Platelet activation also involves a process known as degranulation, which provides the following components: proteins (e.g., Factor V and

fibrinogen) and calcium necessary for the coagulation cascade; agonists (e.g., serotonin, thromboxane, and ADP) which activate and recruit additional platelets; and a negatively charged phospholipid surface on the platelet which promotes assembly and the activation of coagulation factors. An accurate assessment of platelet activation would allow clinicians to determine the risk of bleeding after invasive procedures in patients having impaired platelet function, both congenital and acquired (e.g., in association with renal failure or after ingestion of medications which alter platelet function).

Platelet activation is also critical in thrombosis, a pathologic process in which the activation of platelets and of the coagulation cascade leads to an occlusion of the blood vessel in response to atherosclerotic plaque rupture. Thrombosis complicating plaque rupture is responsible for the majority of myocardial infarctions (heart attacks) and cerebrovascular accidents (strokes). Therapy with antiplatelet agents such as aspirin, ticlopidine, and ReoPro has been shown to reduce the incidence of myocardial infarctions and cardiac death in patients having a known cardiac disease. Therapy with aspirin and ticlopidine has also been shown to reduce the

incidence of stroke in patients having a cerebrovascular disease.

The major complication of antiplatelet therapy is bleeding. An accurate assessment of platelet activation would facilitate determining an optimal therapy for each patient and help reduce the incidence of thrombosis, while maintaining the hemostatic response necessary to prevent bleeding.

Conventional assays of platelet function are performed in platelet-rich plasma rather than in whole blood. Preparation of platelets for such purposes can alter their functional properties and cause assay results to be spurious. For example, platelet aggregometry assesses the aggregation of platelets and reflects the activation of the surface glycoprotein IIb-IIIa and the degranulation of platelets. This method is based on an *in vitro* phenomenon which has little to do with platelet function *in vivo*.

U.S. Patent No. 5,529,902 discloses a fluorescence immunoassay for platelet function based on the determination of P-selectin expression. This assay suffers from the following drawbacks: (1) as in the conventional assays of platelet function, the method is performed using a platelet-rich plasma, and cannot be performed directly on whole blood samples; (2) the method is complicated by the fact that the

number of platelets in each sample must be quantitated independently prior to performing the assay; (3) the assay requires that each sample be split into two portions, one of which serves as a control; and (4) the assay permits the determination of only one marker of platelet activation.

Flow cytometry has been used in conjunction with platelet surface glycoprotein-specific antibodies to determine the extent of platelet activation [see, e.g., R.E. Scharf et al., *Arteriosclerosis and Thrombosis*, 12, 1475 (1992)]. Although whole blood samples can be tested using flow cytometry-based assays, the procedures are time consuming and require expensive instrumentation, and therefore are not practical for rapidly and economically testing large numbers of samples.

Another commonly used assay of overall platelet function is the measurement of bleeding time. This procedure assesses hemostasis and only indirectly reflects the contribution of platelets and the coagulation cascade. It is notoriously insensitive regarding actual platelet function.

In summary, the critical role of platelets in hemostasis and thrombosis and the development of new, more effective antiplatelet regimens, as well as the inadequacies associated with conventional assessments

of platelet function, have defined the need for an accurate assay of platelet activation. This assay would allow health care providers to define platelet reactivity and thus the risk associated with increased reactivity (thrombotic occlusion of vessels) and decreased reactivity (excess bleeding). Because each individual has a unique response to antiplatelet therapy, this assay would allow therapy to be tailored to the needs of that individual and facilitate needed adjustment of dosage over time.

SUMMARY OF THE INVENTION

To solve the above problems, it is an object of the present invention to provide an improved method for the assessment of platelet activation in whole blood samples. Unlike conventional assays of platelet function, such as platelet aggregrometry and bleeding times, the present invention permits the assessment of specific components of platelet activation. Thus, this assay facilitates a determination of increased platelet reactivity and thus the risk associated with increased reactivity (thrombotic occlusion of vessels) and decreased reactivity (excess bleeding). This assay also facilitates a determination of the adequacy of inhibition of reactivity secondary to treatment with antiplatelet regimens with regard to specific

components of platelet activation. Accordingly, because each individual subject has a unique response to antiplatelet therapy, this assay allows therapy to be tailored to the needs of each individual patient and facilitate appropriate dosage adjustment over time in each.

The specific components of platelet activation include the following: activation of the surface glycoprotein IIb-IIIa which reveals a binding site for fibrinogen and thus allows for the crosslinking of platelets by fibrinogen [see, e.g., S.J. Shattil et al., Blood, 70,307(1987), and C.S. Abrams et al., Blood, 75, 128 (1990)]; the release of the contents of the alpha granules, in which degranulation leads to the release of stored proteins and to the expression of the integrin P-selectin on the surface of the platelet [see, e.g., J.N. George et al., J. Clin. Invest., 78, 340 (1986)]; the release of the contents of the dense granules, in which degranulation leads to the release of nucleotides and calcium; the release of the contents of lysosomes, in which degranulation leads to the release of stored proteins; the procoagulant effect whereby platelets facilitate assembly and activity of coagulation factors; and shape change whereby platelets increase surface area thereby facilitating formation of a hemostatic plug.

In order to accurately reflect the reactivity of platelets, the present invention provides a method of determining the extent of platelet activation in response to physiologic concentrations of an agonist. After exposure to such an agonist, the platelets are exposed to solutions containing the following: fluorochrome-labeled ligands which bind to all platelets, both activated and quiescent; fluorochrome-labeled ligands which bind only to activated platelets; and biotin conjugated ligands which facilitate the binding of platelets to a solid phase. After exposing the platelets to a fixative (e.g., formaldehyde), the platelets are bound to a solid phase by exposure to a streptavidin or avidin coated surface.

The interaction of platelets with the coated surface is preferably facilitated by centrifugation. Unbound fluorochrome-labeled ligands are preferably removed by washing.

The specific components of platelet activation are preferably determined by measuring the fluorescent signal intensities emitted by each of the bound activation-dependent fluorochrome-labeled ligands, and emitted by the fluorochrome-labeled marker of all platelets.

BRIEF DESCRIPTION OF THE DRAWINGS

The present invention will be understood and appreciated more fully from the following detailed description taken in conjunction with the drawings in which:

FIG. 1 is a graphic illustration of the extent of platelet activation caused by exposure of a whole blood sample to selected concentrations of the agonist ADP (adenosine 5'-diphosphate), in which platelet activation is defined as the ratio between the fluorescent signal produced by fluorochrome-labeled antibodies bound to a protein, P-selectin, on the surface of activated platelets and the fluorescent signal produced by fluorochrome-labeled antibodies bound to all platelets in the reaction vessel.

FIG. 2 is a graphic illustration of the extent of platelet activation caused by exposure of a whole blood sample to selected concentrations of the agonist ADP, in which platelet activation is defined as the ratio between the fluorescent signal produced by fluorochrome-labeled fibrinogen bound to the surface of activated platelets and the fluorescent signal produced by fluorochrome-labeled antibodies bound to all platelets in the reaction vessel.

FIG. 3 is a graphic illustration of the decrease in platelet activation caused by the antiplatelet drug

ReoPro, where the extent of platelet activation, caused by exposure of a whole blood sample to selected concentrations of the agonist ADP, is defined as the ratio between the fluorescent signal produced by fluorochrome-labeled fibrinogen bound to the surface of activated platelets and the fluorescent signal produced by fluorochrome-labeled antibodies bound to all platelets in the reaction vessel.

FIG.4 is a graphic illustration of the decrease in platelet activation caused by the antiplatelet drugs aspirin and Ticlid, where the extent of platelet activation, caused by exposure of a whole blood sample to selected concentrations of the agonist ADP, is defined as the ratio between the fluorescent signal produced by fluorochrome-labeled antibodies bound to P-selectin on the surface of activated platelets and the fluorescent signal produced by fluorochrome-labeled antibodies bound to all platelets in the reaction vessel.

FIG. 5 is a graphic illustration of the decrease in platelet activation caused by the antiplatelet drugs aspirin and Ticlid, where the extent of platelet activation, caused by exposure of a whole blood sample to selected concentrations of the agonist ADP, is defined as the ratio between the fluorescent signal produced by fluorochrome-labeled fibrinogen bound to

the surface of activated platelets and the fluorescent signal produced by fluorochrome-labeled antibodies bound to all platelets in the reaction vessel.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method for the assessment of specific components of platelet activation in blood samples. In the preferred embodiment of this invention, whole blood samples are used because platelet reactivity may be altered by the methods used to obtain purified platelet preparations or by the removal of selected blood components.

In the preferred embodiment of the invention, the artificial activation of coagulation which occurs after blood is exposed to foreign surfaces must be prevented because this artificial activation alters platelet reactivity. Exposure of blood to a foreign surface activates a protein, Factor XII, which in turn initiates the coagulation cascade leading to the generation of thrombin; thrombin activates platelets and forms fibrin. On the other hand, the initiation of the coagulation cascade *in vivo* after vascular injury does not involve the activation of Factor XII. Vascular injury causes blood to be exposed to tissue factor, which complexes with Factor VIIa and initiates the coagulation cascade. Accordingly, in the

preferred embodiment of the invention, artificial activation of the coagulation cascade is prevented by inhibiting Factor XIIa, while the remainder of the coagulation cascade is left intact due to its interplay with the activation of platelets.

Thus, in the preferred embodiment of the invention, the assessment of platelet activation is carried out using whole blood samples preferably treated only with corn trypsin inhibitor, a specific inhibitor of Factor XIIa with no effect on other coagulation factors. Other anticoagulants, such as chelate calcium, would be avoided as they have been shown to cause artificial enhancement of ADP-induced platelet activation.

In the preferred embodiment of the invention, the extent of platelet activation is determined in response to physiologic concentrations of an agonist, such as, but not limited to, ADP (adenosine 5'-diphosphate) or TRAP (thrombin receptor agonist peptide). After exposure to such an agonist, the platelets are exposed to the following solutions (A-D), either simultaneously or sequentially:

(A) A solution containing fluorochrome-labeled ligands which bind to all platelets, both activated and quiescent; examples of such fluorochrome-labeled

ligands include, but are not limited to, fluorochrome-labeled antibodies which bind to all platelets.

(B) A solution containing fluorochrome-labeled ligands which bind only to activated platelets; examples of such fluorochrome-labeled ligands include, but are not limited to, fluorochrome-labeled antibodies which bind to P-selectin on the surface of activated platelets, or fluorochrome-labeled fibrinogen which binds to sites on the surface of activated platelets.

(C) A solution containing ligands which facilitate the binding of all platelets to a solid phase; examples of such ligands include, but are not limited to, biotin-labeled antibodies which bind to all platelets.

(D) A solution containing a fixative, which prevents further platelet activation; examples of such a fixative include, but are not limited to, 1.5% formaldehyde.

Interaction between platelets and the solid phase may be enhanced by centrifuging the samples after adding the samples to a vessel such as microtitration plates.

After binding the platelets to a solid phase including, but not limited to, at least one well of a microtitration plate, unbound fluorochrome-labeled

ligands are removed by washing, and the specific components of platelet activation are determined by measuring the fluorescent signal intensities emitted by each of the bound activation-dependent fluorochrome-labeled ligands, and emitted by the fluorochrome-labeled marker of all platelets.

The manner in which the assessment of specific components of platelet activation is performed can be understood more fully by reference to the following illustrative examples, which are not intended to limit the scope of the invention in any way.

Example 1

Capture of platelets in microtitration wells and determination of platelet activation by measurement of the extent of P-selectin expression on the platelet surface

P-selectin is an integral membrane protein in the alpha granules of platelets. During platelet activation, alpha granule exocytosis results in fusion of the granule membrane with the platelet membrane, bringing P-selectin to the platelet membrane surface. The expression of P-selectin can be measured by capturing platelets with an immobilized antibody in a microtitration well, and then using two fluorescent markers: CD42*PerCP (an IgG antibody to glycoprotein IX, with PerCP as the fluorescent label) which marks

all platelets, and CD62*PE (an IgG antibody to P-selectin, with phycoerythrin as the fluorescent label) which marks platelets expressing P-selectin and thus marks those platelets which are activated. Two concentrations of ADP, a weak agonist, are used to activate the platelets, and the dose response of platelets to ADP is characterized in whole blood. By using fixed doses of ADP, it is possible to determine the extent of platelet activation in each sample, and the efficacy of antiplatelet therapy can be determined by analyzing the slope of individual response curves or by the activation in response to a selected concentration of the agonist, as shown in FIG. 1.

Similar results were obtained with parallel experiments in which the association of labeled ligands with platelets was determined by using flow cytometry. When a flow cytometry determination of the association of ligands with platelets is made, in the absence of added agonists, minimal platelet activation is observed (i.e., less than 1 percent of platelets are activated in the absence of agonists when P-selectin is used as the marker for activation).

Example 1 Procedure

Phlebotomy was performed by using a two syringe technique whereby the first 3 ml of blood were discarded. Activation of platelets was delineated in

blood drawn directly into a syringe containing 32 μg/ml of corn trypsin inhibitor (CTI, 9:1 v/v), an agent which inhibits Factor XIIa without affecting other coagulation factors. The CTI was used as an anticoagulant to prevent activation of the contact factor pathway and permit analysis of activation of platelets dependent on addition of exogenous activators. Unlike CTI, other anticoagulants, particularly chelators of calcium such as citrate, alter the activation of platelets. 5 μl of blood were added to microcentrifuge tubes containing Hepes Tyrodes buffer (5 mM HEPES, 137 mM NaCl, 2.7 mM $NaHCO_3$, 0.36 mM NaH_2PO_4, 2 mM $CaCl_2$, 4 mM $MgCl_2$, and 5 mM dextrose, pH 7.4), a PerCP conjugated IgG directed against glycoprotein IX (CD 42a), a phycoerythrin (PE) conjugated IgG directed against P-selectin (CD 62), a biotin conjugated antibody directed against glycoprotein IIb (CD 41), and selected concentrations of ADP (0, 0.4, 0.8 μM). CD 42a binds to an activation independent epitope on glycoprotein IX and thus was used to tag and hence identify all platelets. The biotin conjugated CD 41 binds to an activation independent epitope on glycoprotein IIb and thus serves as the ligand to capture platelets. P-selectin is expressed on the surface of only activated platelets and thus serves to mark platelets which have

degranulated. After adding blood, the reaction solution was mixed gently and incubated at room temperature for 15 minutes. The platelets were fixed, and the red blood cells were lysed by adding Optilyse-C (1.5% formaldehyde) after a 15 minute incubation. The reaction mixture was then added to a streptavidin-coated well in a microtitration plate. The plate was centrifuged (1000 X g for 15 minutes) to optimize the interaction between the platelets and the solid phase. The wells were then washed three times with Hepes Tyrodes buffer. The intensity of fluorescence for each ligand was determined with a microplate fluorescent plate reader.

Example 2

Capture of platelets in microtitration wells and determination of platelet activation by measurement of the extent of fibrinogen binding on the platelet surface

Platelet activation is also reflected in the activation of the surface glycoprotein IIb-IIIa which reveals a binding site for fibrinogen. The extent of fibrinogen binding can be measured by capturing platelets with an immobilized antibody in a microtitration well, and then using two fluorescent markers: CD42*PerCP (an IgG antibody to glycoprotein IX, with PerCP as the fluorescent label) which marks

all platelets; and fibrinogen*FITC (fibrinogen conjugated with fluorescein isothiocyanate as the fluorescent label) which marks platelets in which activation of surface glycoprotein IIb-IIIa has occurred. Two concentrations of ADP are used to activate the platelets, and the dose response of platelets to ADP is characterized in whole blood. By using fixed doses of ADP, it is possible to determine the extent of platelet activation in each sample. The efficacy of antiplatelet therapy can be determined by analyzing the slope of individual response curves or by the activation in response to a selected concentration of the agonist, as shown in FIG. 2.

Example 2 Procedure

Phlebotomy was performed by using a two syringe technique whereby the first 3 ml of blood were discarded. Activation of platelets was delineated in blood drawn directly into a syringe containing 32 μg/ml of corn trypsin inhibitor (CTI, 9:1 v/v), an agent which inhibits Factor XIIa without affecting other coagulation factors. The CTI was used as an anticoagulant to prevent the activation of the contact factor pathway and permit an analysis of platelet activation dependent on the addition of exogenous activators. CTI was used rather than other anticoagulants because unlike CTI, other

anticoagulants, particularly chelators of calcium such as citrate, alter the activation of platelets. 5 μl of blood were added to microcentrifuge tubes containing Hepes Tyrodes buffer (5 mM HEPES, 137 mM NaCl, 2.7 mM $NaHCO_3$, 0.36 mM NaH_2PO_4, 2 mM $CaCl_2$, 4 mM $MgCl_2$, and 5 mM dextrose, pH 7.4), a PerCP conjugated IgG directed against glycoprotein IX (CD 42a), fibrinogen conjugated to fluorescein isothiocyanate (FITC), a biotin conjugated antibody directed against glycoprotein IIb (CD 41), and selected concentrations of ADP (0, 0.4, 0.8 μM). CD 42a binds to an activation independent epitope on glycoprotein IX and thus was used to tag and hence identify all platelets. The biotin conjugated CD 41 binds to an activation independent epitope on glycoprotein IIb and thus serves as the ligand to capture platelets. Fibrinogen binds to the surface of platelets only when the surface glycoprotein IIb-IIIa is activated and thus serves to identify activated platelets. After adding blood, the reaction solution was mixed gently and incubated at room temperature for 15 minutes. The platelets were fixed, and the red blood cells were lysed by adding Optilyse-C (1.5% formaldehyde) after the 15 minute incubation. The reaction mixture was then added to a streptavidin-coated well in a microtitration plate. The plate was centrifuged (1000

X g for 15 minutes) to optimize the interaction between the platelets and the solid phase. The wells were then washed three times with Hepes Tyrodes buffer. The intensity of fluorescence for each ligand was determined with a microplate fluorescent plate reader.

Example 3

Capture of platelets in microtitration wells and determination of inhibition of fibrinogen binding to platelets by ReoPro

Platelet activation is inhibited by the drug ReoPro, and is reflected by the inhibition of binding of fibrinogen to the activated conformer of the surface glycoprotein IIb-IIIa. The extent of fibrinogen binding can be measured by capturing platelets with an immobilized antibody in a microtitration well, and then using two fluorescent markers: CD42*PerCP (an IgG antibody to glycoprotein IX, with PerCP as the fluorescent label) which marks all platelets; and fibrinogen*FITC (fibrinogen conjugated with fluorescein isothiocyanate as the fluorescent label) which marks platelets in which an activation of surface glycoprotein IIb-IIIa has occurred. Two concentrations of ADP are used to activate the platelets, and the dose response of platelets to ADP is characterized in whole blood and

in whole blood spiked with ReoPro. The concentration of ReoPro used is that known to inhibit the aggregation of platelets as determined with the use of aggregometry. As shown in FIG. 3, pretreatment of blood with ReoPro inhibits binding of fibrinogen*FITC to platelets.

Example 3 Procedure

Phlebotomy was performed by using a two syringe technique whereby the first 3 ml of blood were discarded. Activation of platelets was delineated in blood drawn directly into a syringe containing 32 μg/ml of corn trypsin inhibitor (CTI, 9:1 v/v) and into a syringe containing CTI (32 μg/ml) plus ReoPro (3 μg/ml). 5 μl of blood were added to microcentrifuge tubes containing Hepes Tyrodes buffer (5 mM HEPES, 137 mM NaCl, 2.7 mM $NaHCO_3$, 0.36 mM NaH_2PO_4, 2 mM $CaCl_2$, 4 mM $MgCl_2$, and 5 mM dextrose, pH 7.4), a PerCP conjugated IgG directed against glycoprotein IX (CD 42a), fibrinogen conjugated to fluorescein isothiocyanate (FITC), a biotin conjugated antibody directed against glycoprotein IIb (CD 41), and selected concentrations of ADP (0, 0.4, 0.8 μM). CD 42a binds to an activation independent epitope on glycoprotein IX and thus was used to tag and hence identify all platelets. The biotin conjugated CD 41 binds to an activation independent epitope on

glycoprotein IIb and thus serves as the ligand to capture platelets. Fibrinogen binds to the surface of platelets only when the surface glycoprotein IIb-IIIa is activated and thus serves to identify activated platelets. After adding blood, the reaction solution was mixed gently and incubated at room temperature for 15 minutes. The platelets were fixed, and the red blood cells were lysed by adding Optilyse-C (1.5% formaldehyde) after the 15 minute incubation. The reaction mixture was then added to a streptavidin-coated well in a microtitration plate. The plate was centrifuged (1000 X g for 15 minutes) to optimize the interaction between the platelets and the solid phase. The wells were then washed three times with Hepes Tyrodes buffer. The intensity of fluorescence for each ligand was determined with a microplate fluorescent plate reader.

Example 4

Capture of platelets in microtitration wells and determination of inhibition of degranulation after ingestion of aspirin and Ticlid

Platelet activation is inhibited after ingestion of two antiplatelet agents, aspirin and Ticlid. Degranulation in response to ADP was determined before and after ingestion of aspirin and Ticlid by capturing platelets with an immobilized antibody in a

microtitration well, and then using two fluorescent markers: CD42*PerCP (an IgG antibody to glycoprotein IX, with PerCP as the fluorescent label) which marks all platelets; and CD62*PE (a phycoerythrin [PE] conjugated IgG directed against P-selectin). P-selectin is expressed on the surface of only activated platelets and thus serves to mark platelets which have degranulated. Two concentrations of ADP are used to activate the platelets, and the dose response of platelets to ADP is characterized in whole blood obtained from subjects before and after 4 days of ingestion of aspirin (325 mg/day) and Ticlid (250 mg BID). As shown in FIG. 4, ingestion of aspirin and Ticlid inhibits ADP-induced degranulation of platelets.

Example 4 Procedure

Phlebotomy was performed by using a two syringe technique whereby the first 3 ml of blood were discarded. Activation of platelets was delineated in blood drawn directly into a syringe containing 32 μg/ml of corn trypsin inhibitor (CTI, 9:1 v/v). 5 μl of blood were added to microcentrifuge tubes containing Hepes Tyrodes buffer (5 mM HEPES, 137 mM NaCl, 2.7 mM $NaHCO_3$, 0.36 mM NaH_2PO_4, 2 mM $CaCl_2$, 4 mM $MgCl_2$, and 5 mM dextrose, pH 7.4), a PerCP conjugated IgG directed against glycoprotein IX (CD 42a), CD 42

conjugated to phycoerythrin (PE), a biotin conjugated antibody directed against glycoprotein IIb (CD 41), and selected concentrations of ADP (0, 0.4, 0.8 μM). CD 42a binds to an activation independent epitope on glycoprotein IX and thus was used to tag and hence identify all platelets. The biotin conjugated CD 41 binds to an activation independent epitope on glycoprotein IIb and thus serves as the ligand to capture platelets. CD 42 binds to the surface of platelets only when degranulation has occurred and P-selectin is expressed on the surface of the platelet. After adding blood, the reaction solution was mixed gently and incubated at room temperature for 15 minutes. The platelets were fixed, and the red blood cells were lysed by adding Optilyse-C (1.5% formaldehyde) after the 15 minute incubation. The reaction mixture was then added to a streptavidin-coated well in a microtitration plate. The plate was centrifuged (1000 X g for 15 minutes) to optimize the interaction between the platelets and the solid phase. The wells were then washed three times with Hepes Tyrodes buffer. The intensity of fluorescence for each ligand was determined with a microplate fluorescent plate reader.

Example 5

Capture of platelets in microtitration wells and determination of inhibition of fibrinogen binding after ingestion of aspirin and Ticlid

Platelet activation is inhibited after ingestion of two antiplatelet agents, aspirin and Ticlid. Activation of glycoprotein IIb-IIIa in response to ADP was determined before and after ingestion of aspirin and Ticlid by capturing platelets with an immobilized antibody in a microtitration well, and then using two fluorescent markers: CD42*PerCP (an IgG antibody to glycoprotein IX, with PerCP as the fluorescent label) which marks all platelets; and fibrinogen*FITC (fibrinogen conjugated to fluorescein isothiocyanate [FITC]). Fibrinogen binds to the activated conformer of the surface glycoprotein IIb-IIIa. Two concentrations of ADP are used to activate the platelets, and the dose response of platelets to ADP is characterized in whole blood obtained from subjects before and after 4 days of ingestion of aspirin (325 mg/day) and Ticlid (250 mg BID). As shown in FIG. 5, the ingestion of aspirin and Ticlid inhibits ADP-induced binding of fibrinogen to platelets.

Example 5 Procedure

Phlebotomy was performed by using a two syringe technique whereby the first 3 ml of blood were

discarded. Activation of platelets was delineated in blood drawn directly into a syringe containing 32 μg/ml of corn trypsin inhibitor (CTI, 9:1 v/v). 5 μl of blood were added to microcentrifuge tubes containing Hepes Tyrodes buffer (5 mM HEPES, 137 mM NaCl, 2.7 mM $NaHCO_3$, 0.36 mM NaH_2PO_4, 2 mM $CaCl_2$, 4 mM $MgCl_2$, and 5 mM dextrose, pH 7.4), a PerCP conjugated IgG directed against glycoprotein IX (CD 42a), fibrinogen conjugated to fluorescein isothiocyanate (FITC), a biotin conjugated antibody directed against glycoprotein IIb (CD 41), and selected concentrations of ADP (0, 0.4,0.8 μM). CD 42a binds to an activation independent epitope on glycoprotein IX and thus was used to tag and hence identify all platelets. The biotin conjugated CD 41 binds to an activation independent epitope on glycoprotein IIb and thus serves as the ligand to capture platelets. Fibrinogen binds to the surface of platelets when the surface glycoprotein IIb-IIIa is activated. After adding blood, the reaction solution was mixed gently and incubated at room temperature for 15 minutes. The platelets were fixed, and the red blood cells were lysed by adding Optilyse-C (1.5% formaldehyde) after the 15 minute incubation. The reaction mixture was then added to a streptavidin-coated well in a microtitration plate. The plate was centrifuged (1000

X g for 15 minutes) to optimize the interaction between the platelets and the solid phase. The wells were then washed three times with Hepes Tyrodes buffer. The intensity of fluorescence for each ligand was determined with a microplate fluorescent plate reader.

While particular embodiments of the invention have been described, it will be understood, of course, that the invention is not limited thereto, and that many modifications and variations can be made by those skilled in the art, and that such modifications and variations are intended to fall within the scope of the appended claims.

CLAIMS

What is claimed is:

1. A method for measuring specific components of platelet activation in a whole blood sample, comprising

exposing platelets in a whole blood sample to a physiological concentration of an agonist that activates some of the platelets, resulting in the formation of at least one binding site on the surface of the activated platelets;

binding the activated and unactivated platelets to a solid surface; and

measuring the activated platelets.

2. The method of claim 1, wherein the at least one binding site is P-selectin.

3. The method of claim 2, further comprising exposing said platelets to fluorochrome-labeled antibodies which bind to the P-selectin.

4. The method of claim 1, wherein the at least one binding site selectively binds fibrinogen.

5. The method of claim 4, further comprising exposing said platelets to fluorochrome-labeled fibrinogen which binds to the activated platelets.

6. The method of claim 1, further comprising exposing said platelets to fluorochrome-labeled antibodies which bind to all platelets, both activated and unactivated.

7. The method of claim 1, wherein the agonist is ADP.

8. The method of claim 1, wherein the agonist is TRAP.

9. The method of claim 6, further comprising transferring the platelets to a vessel, wherein the vessel is at least one well of a microtitration plate, and wherein the antibodies which bind to all platelets capture the platelets onto a streptavidin-coated or avidin-coated solid surface of the vessel.

10. The method of claim 9, further comprising centrifuging the vessel to optimize interaction of the platelets with the solid surface.

11. An apparatus for determining specific components of platelet activation in a whole blood sample by the method set forth in claim 1, comprising

at least one vessel into which platelets are captured.

12. The apparatus of claim 11, wherein the vessel is at least one well of a microtitration plate.

13. A method for measuring specific components of platelet activation in a whole blood sample, comprising

exposing platelets in a whole blood sample to a physiological concentration of an agonist that activates some of the platelets, resulting in the formation of at least one binding site on the surface of the activated platelets;

simultaneously or sequentially exposing all platelets, both activated and unactivated, to each of the following solutions: a solution containing fluorochrome-labeled ligands which bind to all platelets, a solution containing fluorochrome-labeled ligands which bind only to activated platelets, a solution containing ligands which facilitate binding of all platelets to a solid phase, and a solution

containing a fixative, which prevents further platelet activation, to obtain a reaction mixture;

transferring the reaction mixture to a vessel having a solid surface onto which the platelets are captured; and

measuring the activated platelets captured in the vessel.

14. The method of claim 13, wherein the fluorochrome-labeled ligands which bind to all platelets are fluorochrome-labeled antibodies.

15. The method of claim 13, wherein the at least one binding site is P-selectin.

16. The method of claim 15, wherein the fluorochrome-labeled ligands which bind only to activated platelets are fluorochrome-labeled antibodies which bind to the P-selectin.

17. The method of claim 13, wherein the fluorochrome-labeled ligands which bind only to activated platelets are fluorochrome-labeled fibrinogen which binds to the activated platelets.

18. The method of claim 13, wherein the ligands which facilitate the binding of all platelets to a solid phase are biotin-labeled antibodies which bind to all platelets.

19. The method of claim 13, wherein the fixative is formaldehyde.

20. The method of claim 13, wherein the vessel is a microtitration plate having at least one well.

21. The method of claim 20, wherein the microtitration plate is streptavidin-coated.

22. The method of claim 20, wherein the microtitration plate is avidin-coated.

23. The method of claim 13, further comprising centrifuging the vessel prior to measuring the activated platelets, wherein the vessel is a microtitration plate having at least one well.

24. The method of claim 23, further comprising washing the wells to remove unbound fluorochrome-labeled ligands, after the centrifugation step and

prior to the step of measuring the activated platelets.

25. The method of claim 13, wherein the agonist to which the platelets are exposed is ADP.

26. The method of claim 13, wherein the agonist to which the platelets are exposed is TRAP, a peptide which binds to a thrombin receptor on the platelets.

27. The method of claim 3, wherein the fluorochrome-labeled antibodies emit a fluorescent signal having an intensity which is measured using a microplate fluorescence reader, to determine the number of activated platelets.

28. The method of claim 5, wherein the fluorochrome-labeled fibrinogen emits a fluorescent signal having an intensity which is measured using a microplate fluorescence reader, to determine the number of activated platelets.

29. The method of claim 9, wherein the fluorochrome-labeled antibodies emit a fluorescent signal having an intensity which is measured using a microplate

fluorescence reader, to determine the total number of platelets captured in the vessel.

30. The method of claim 16, wherein the fluorochrome-labeled antibodies emit a fluorescent signal having an intensity which is measured using a microplate fluorescence reader, to determine the number of activated platelets.

31. The method of claim 17, wherein the fluorochrome-labeled fibrinogen emits a fluorescent signal having an intensity which is measured using a microplate fluorescence reader, to determine the number of activated platelets.

32. The method of claim 14, wherein the fluorochrome-labeled antibodies emit a fluorescent signal having an intensity which is measured using a microplate fluorescence reader, to determine the total number of platelets captured in the vessel.

FIG. 1

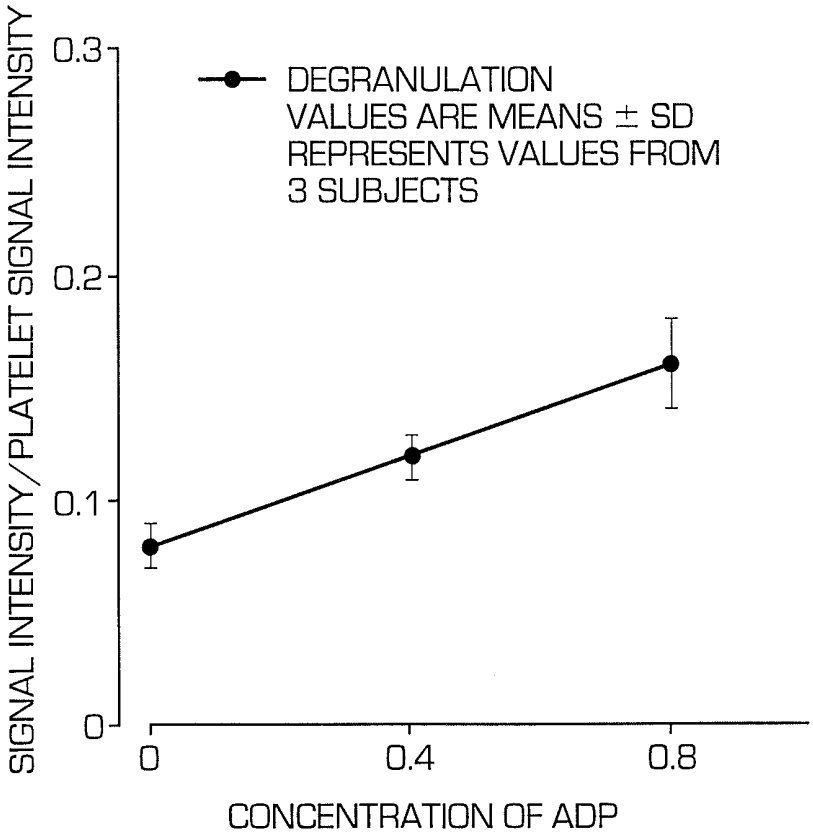

SIGNAL INTENSITY/PLATELET SIGNAL INTENSITY
0.3
0.2
0.1
0
DEGRANULATION
VALUES ARE MEANS ± SD
REPRESENTS VALUES FROM
3 SUBJECTS
0
0.4
0.8
CONCENTRATION OF ADP

FIG. 2

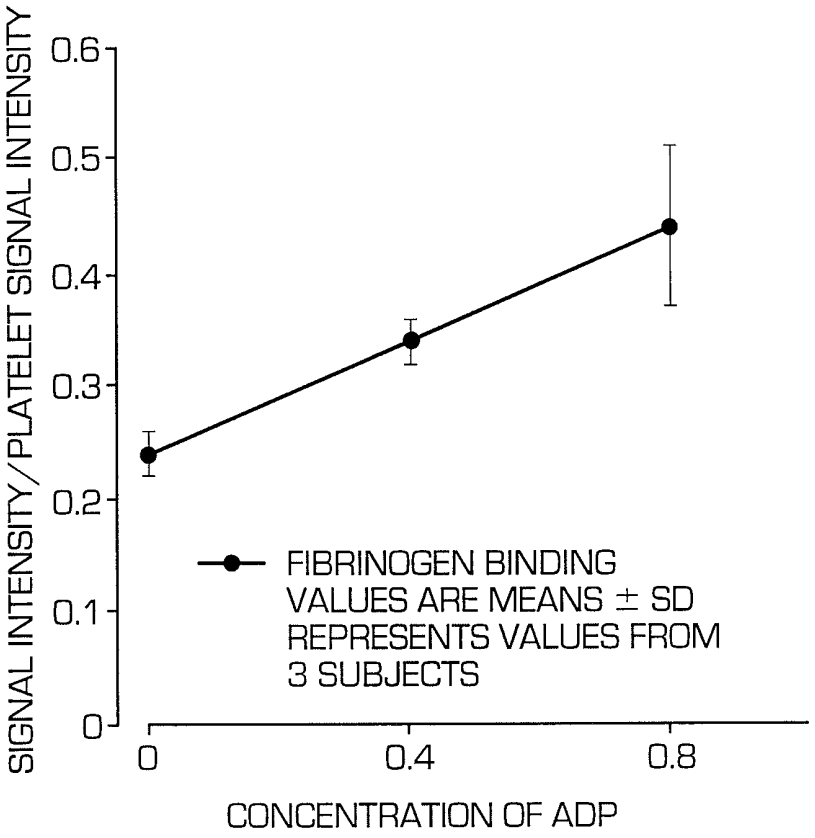

SIGNAL INTENSITY/PLATELET SIGNAL INTENSITY
0.6
0.5
0.4
0.3
0.2
0.1
0
FIBRINOGEN BINDING
VALUES ARE MEANS ± SD
REPRESENTS VALUES FROM
3 SUBJECTS
0
0.4
0.8
CONCENTRATION OF ADP

FIG. 3

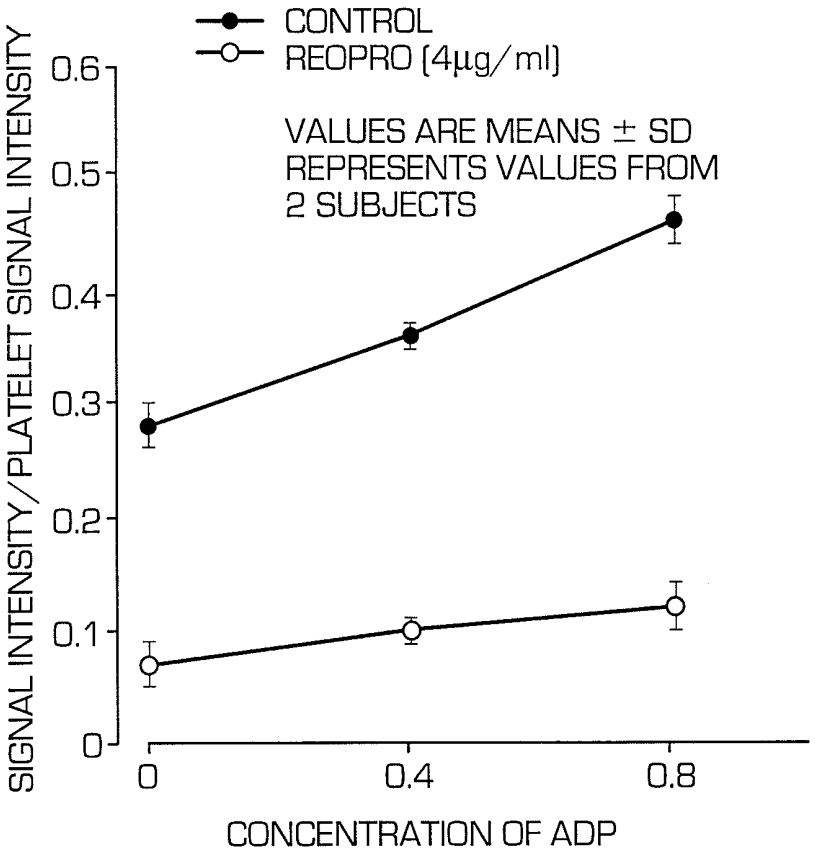

CONTROL
REOPRO (4μg/ml)
VALUES ARE MEANS ± SD
REPRESENTS VALUES FROM
2 SUBJECTS
SIGNAL INTENSITY/PLATELET SIGNAL INTENSITY
0.6
0.5
0.4
0.3
0.2
0.1
0
0
0.4
0.8
CONCENTRATION OF ADP

FIG. 4

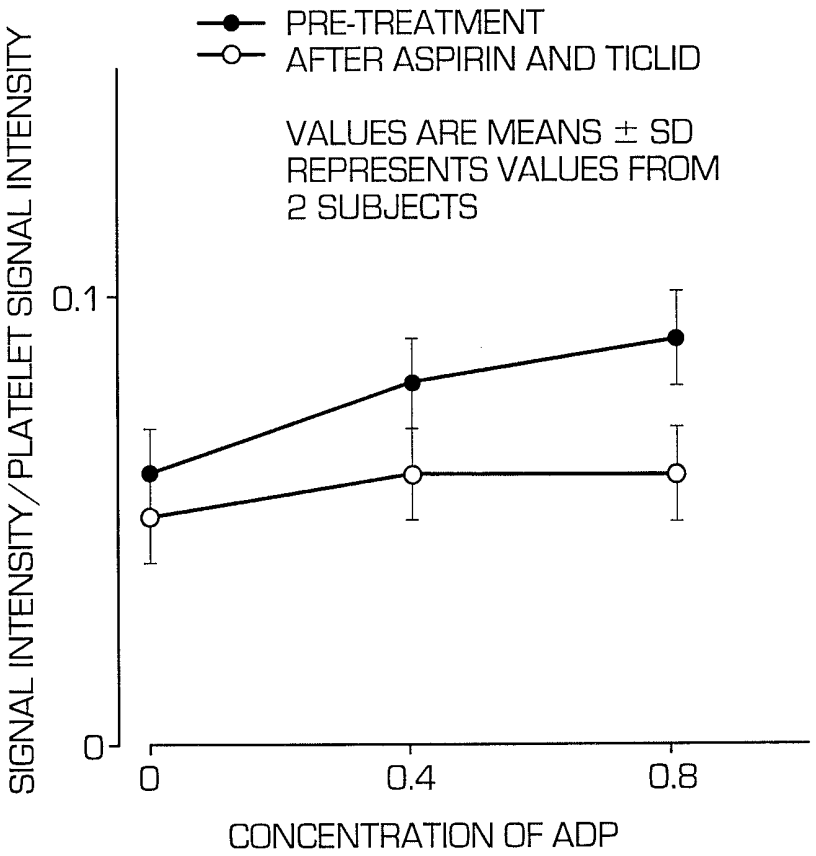
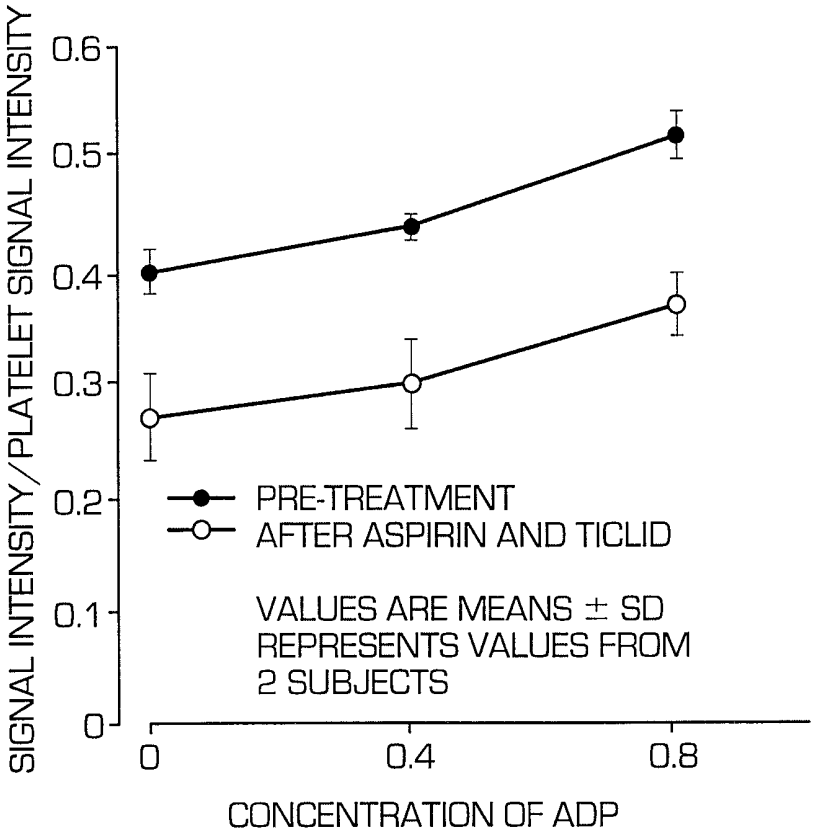

PRE-TREATMENT
AFTER ASPIRIN AND TICLID
VALUES ARE MEANS ± SD
REPRESENTS VALUES FROM
2 SUBJECTS
SIGNAL INTENSITY/PLATELET SIGNAL INTENSITY
0.1
0
0
0.4
0.8
CONCENTRATION OF ADP

FIG. 5


SIGNAL INTENSITY/PLATELET SIGNAL INTENSITY
0.6
0.5
0.4
0.3
0.2
0.1
0
PRE-TREATMENT
AFTER ASPIRIN AND TICLID
VALUES ARE MEANS ± SD
REPRESENTS VALUES FROM
2 SUBJECTS
0
0.4
0.8
CONCENTRATION OF ADP