ADHESIVENESS EVALUATION OF ACTIVATED PLATELET USING Arg-Gly-Asp-Phe(RGDF)-IMMOBILIZED SURFACE

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ABSTRACT

The adhesion of activated and normal platelets to fibrinogen requires the receptor binding site of GPIIb/IIIa. These recognition sites exists in the A α chain(RGDS at 572-575 and RGDF at 95-98) and the carboxy-terminal of γ chain (HHLGGAKQAGDV at 400-411) of fibrinogen. In this study, we developed RGDF-immobilized surface to detect the functional state of platelet. RGDF-immobilized surface was prepared on the glass using photolithographic technology. Platelet adhesion to RGDF-immobilized surface was observed by staining platelets with mepacrine using a fluorescence microscope using mepacrine. Using the RGDF peptide of fragment E, we observed that the platelets pretreated with PGE1 interacted incompletely with RGDF-immobilized surface, whereas ADP activated platelets interacted with the surface extensively.

These results show that the distinct selectivity of RGDF-immobilized micro-patterned surface can be used to detect the functional state of platelets.

INTRODUCTION

Glycoprotein IIb/IIIa (GPIIb/IIIa) is one of the specific platelet membrane receptors and it has binding capability with a variety of adhesive protein including fibrinogen, von Willebrand factor (vWF), fibronectin, and vitronectin(1-3).

Fibrinogen participates in the platelet hemostatic function and is required for the rapid primary aggregation of platelets induced by agonists such as ADP, epinephrine, and thrombin.

The binding of fibrinogen to platelet depends on the functional state of platelet GPIIb/IIIa which is mediated by the platelet recognition sites of fibrinogen. These recognition sites exists in the A α chain (RGDS at 572-575 and RGDF at 95-98) and the carboxy-terminal of γ chain (HHLGGAKQAGDV at 400-411) of fibrinogen(4). Following different conformational states, platelet GPIIb/IIIa selectively interacts with one or more of the domains. Interestingly, unlike unstimulated platelet, stimulated platelets adhere to surface-bound fibrinogen derived fragment E which contains the A α 95-98 RGDF sequence(5).

In this study, we have developed synthetic peptide derivative (RGDF)-immobilized micro-patterned surface using a photolithographic technology. We tried to detect activated platelets as a consequence of interaction of platelets with RGDF-immobilized surface. In order to evaluate the adhesiveness of platelet according to the activation state, we used the fluorescence method by staining platelets with mepacrine.

MATERIALS and METHODS

Platelet preparation

Whole blood was obtained from a healthy volunteer and anticoagulated with one-ninth volume of acid-citrate-dextrose (ACD). To inhibit platelet activation, the thrombin inhibitor PPACK was added to a final concentration of 40 μM. Platelet rich plasma (PRP)

was prepared by centrifugation of whole blood at 180 g for 10 min at room temperature. Prostaglandin E1 (PGE1) (final concentration, 10 μM) and apyrase (5 units/ml) were added to PRP. For the preparation of activated platelets, PPACK, PGE1, and apyrase were not added to PRP. Platelet concentration was adjusted to 3.0 ~ 4.0 x 108 /ml using platelet poor plasma (PPP).

Peptide GGGRGDF synthesis

The peptide Gly-Gly-Gly-Arg-Gly-Asp-Phe (GGGRGDF) was synthesized using an automated peptide synthesizer (Milligen 9050, Millipore, Germany) in Korea Basic Science Institute(KBSI).

The sequence RGDF was chosen as a basic sequence at Aα 95-98 in fragment E. The GGG sequence was chosen as a structural probe. The synthesized peptide was cleaved from the resin in the trifluoroacetic acid (88%), phenol (5%), triisopropylsilane (2%), and H_2O (2%). After cleavage of the peptide from the resin, it was washed in cold ethyl ether, followed by precipitation and lyophilization. The peptide homogeneity was assessed by high performance liquid chromatography (HPLC). It was purified to greater than 99% homogeneity.

Preparation of micro-scale patterned RGDF array

Circular micro cover glass was spin-coated with a solution of 5 % polystyrene (MW 125,000) in xylene at 1,000 rpm for 1 min to produce a polystyrene(PS) thin film (below 1 μm in thickness). A solution of 0.5% N-hydroxysuccinimidyl phenyl azide(NHS-PA) in nitromethane was spin-coated on the PS film at 1,000 rpm for 1 min. The film was baked in an oven at 60 ℃ for 20 min. The PS film was exposed at 254 nm UV light (3.3 mW/cm2) for 5 min to react the azido-group with a C-H group of PS. The photomask was used stripped line with 250 μm width. After washing the film with nitromethane to remove unreacted NHS-PA, the NHS-PA coated PS film was incubated with 50 μM of GGGRGDF solution in 0.1 M $NaHCO_3$ buffer (pH 8.2) at 25 ℃ for 3 h. The GGGRGDF-immobilized surface was washed thoroughly with phosphate buffered saline (PBS, pH 7.4) and used to evaluate the platelet adhesion. Figure 1 shows the diagram of peptide immobilization by photoreaction. To examine the immobilization of NHS-PA in well defined patterns, a fluorescent dye was immobilized on the functionalized PS film. The PS film was immersed in a solution of 5-(aminoacetamido) fluorescein in ethanol at 25 ℃ for 1 h. After washing it in pure nitromethane, the patterns were observed with the fluorescent microscope(excitation wavelength 450-490 nm, emission wavelength > 510 nm).

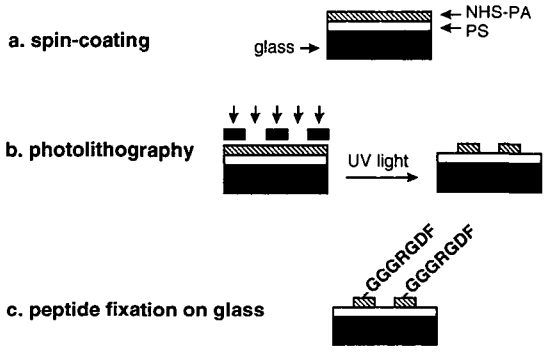

Figure 1. Schematic diagram for the photochemical microfixation of GGGRGDF peptide

Platelet adhesion assay

For fluorescence labeling of platelets, PRP was treated with the mepacrine(quinacrine dihydrochloride) at a final concentration of 10 μM for 30 min at 37 ℃. For the preparation of normal platelets, mepacrine was added to PRP containing prostaglandin E1 (10 μM), PPACK and apyrase (5 units/ml) to inhibit platelet activation. For the adhesion studies of activated platelets, mepacrine-labeled PRP was treated with ADP (20 μM). A 50 $\mu\ell$ of normal and activated PRP was dropped into the RGDF-immobilized surface.

Following incubation for 50 min at 22~25 ℃, non-adhered platelets were removed and washed three times with PBS (pH 7.4). The adhered platelets on the surface were fixed with 3.7% formaldehyde in PBS for 10 min. After replacing the formaldehyde solution with PBS, the fixed platelets were mounted with glycerol in PBS. The fluorescence of adhered platelets was monitored with an inverted-stage microscope equipped with an epifluorescent illuminating source (IMT-2, Olympus Co.) and the microscopic images were projected to a video camera (Sanyo Co.). The fluorescent image of adhered platelets was monitored in separate images by using 40 X objective lens. The microscopic images were directed to a computer for image analysis, and the fluorescent level of the adhered platelets was analyzed using a image processing software (BiPS, Biomedlab Co., Seoul, Korea).

Scanning electron microscopy (SEM) observation

The samples of scanning electron microscopy (SEM) observation were prepared by fixing platelets on the surface with 2.5% glutaraldehyde in PBS for 2 h, followed by dehydration in a series of ethanol, and critical point drying using liquid N_2 as a transition fluid. After coating with gold-palladium, the samples were observed with SEM at an accelerating voltage of 10 kV.

RESULTS

Platelet adhesion to RGDF-immobilized surface

The fluorescence images in Figure 2 show platelets adhered to RGDF-immobilized surface. It shows that the extent of platelet adhesion to the surface are different according to the platelet activation state. While activated platelets treated with ADP adhered to RGDF-immobilized surface extensively, normal platelets adhered to the surface much less than activated platelets. The results demonstrated that the adhesion of platelets to RGDF-immobilized surface depended on platelet activation.

SEM observation

The extent of platelet adhesion to various surfaces was observed using SEM. The SEM images of normal and activated platelets adhered to fibrinogen-coated and RGDF-immobilized surfaces were similar to the fluorescence images of adhered platelets. However it was different between SEM and fluorescence images on the fragment E-coated surfaces. Examination by SEM revealed that platelets adhered to the surface coated with fragment E appear to be weakly adhered compared to the other surfaces. During the preparation of the sample surface, weakly adhered platelets appeared to be detached.

Control platelet

Activated platelet

Figure 2. Fluorescence image of adhered platelets on RGDF peptide micropatterned surface

DISCUSSION

The adhesion of normal and activated platelets to fibrinogen requires the receptor binding site of platelet membrane glycoprotein GPIIb/IIIa. These sites are at the A α chain (RGDS at 572-575 and RGDF at 95-98) and the carboxy-terminal γ chain (HHLGGAKQAGDV at 400-411) of fibrinogen.

A normal platelet contains around 100,000 copies of GPIIb/IIIa complexes, 80% of which are randomly distributed and exposed on the platelet surface

according to the activation. GPIIb/IIIa functions as a promiscuous receptor for multiple RGD-containing proteins including fibrinogen, vWF, fibronectin and vitronectin.

On normal platelets, GPIIb/IIIa is maintained in an inactive conformation and serves as a low-affinity adhesion receptor for surface-coated fibrinogen.

Following platelet activation, GPIIb/IIIa changes its conformation and becomes to bind soluble plasma fibrinogen and some other macromolecules containing RGD sequence(6).

Ruggeri et al.(5) have shown that fibrinogen-derived fragment E, which contains the Aα 97-98 RGDF sequence, fails to support adhesion of normal platelet or less effective in this regard and that adhesion of activated platelets occurs equally well in the all fragments containing adhesive domains of fibrinogen. The RGD peptides blocked the binding of fibrinogen to GPIIb/IIIa of activated platelets and had the capacity to bind to platelets directly. The observations provide the evidence that an RGD sequence defines a recognition specificity involved in the interaction of fibrinogen with platelets.

Beer et al.(7) reported that the majority of RGD binding site on GPIIb/IIIa can be attached by peptide that extend out 11-32 Å from the surface of polyacrylonitrile beads and that peptides of intermediate size (GGGRGDF) showed the greatest sensitivity to the platelet activation state.

In the present study, we have used immobilized GGGRGDF peptides to detect the platelet functional state. The GGGRGDF peptide, which was the minimal sequence of RGDF in fragment E (GGG sequence as a structural probe)(5, 7), was covalently immobilized to the glass surface to produce biologically active, chemically well-defined substrates that support platelet adhesion.

The GGGRGDF-immobilized micro-patterned surface was prepared using a photolithographic technology(8). This technology has been recently used to immobilize peptides and oligonucleotides.

We observed that activated platelets treated with ADP and epinephrine adhered to RGDF-immobilized surface but normal platelets treated with PGE1 and apyrase do not adhered to RGDF-immobilized surface (Figure 2). The limited interaction of RGDF sequence to normal platelets indicated that the exposure of the RGDF sequence resulted in the conformational change of GPIIb/IIIa and a functional α chain is necessary to mediate the adhesion of activated platelets to immobilized fibrinogen.

In contrast to our results, others reported(9) that the fibrinogen γ chain is required to mediate the adhesion of activated platelets to immobilized fibrinogen. However, there are different possible mechanisms whereby GPIIb/IIIa on activated platelets recognize adhesive sites on immobilized fibrinogen. Further work is required to clarify these mechanisms.

In conclusion, the distinct selectivity of RGDF-immobilized micro- patterned surface can be used to detect platelet functional state.

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