Evaluation of the platelet adhesiveness using a peptide-immobilized surface

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ABSTRACT

The adhesion of stimulated and unstimulated platelet to fibrinogen requires the receptor binding site of GPIIb/IIIa. These recognition sites are existed in the Aα chain(RGDS at positions 572-575 and RGDF at 95-98) and the carboxyterminal γ chain (HHLGGAKQAGDV at 400-411) of fibrinogen. The unstimulated platelet does not adhered on the fragment E-coated surface containing RGDF sequence. 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 petide(RGDF)-immobilized surface was observed by the fluorescence microscope using mepacrine.

INTRODUCTION

Glycoprotein IIb/IIIa (GPIIb/IIIa) is one of the specific platelet membrane receptors and has binding capability with a variety of adhesive protein including fibrinogen, von Willebrand factor, fibronectin, and vitronectin[1,2].

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 and is mediated platelet recognition sites of fibrinogen. These recognition sites are existed in the Aα chain(RGDS at positions 572-575 and RGDF at 95-98) and the carboxyterminal γ chain (HHLGGAKQAGDV at 400-411) of fibrinogen. Following different conformational states, platelet GPIIb/IIIa selectively interacts with one or more of the domains. Interestingly, unlike unstimulated platelet, stimulated platelets can adhere to surface-bounded fibrinogen derived fragment E which contains the Aα95-98 RGDF sequence[3]. In this study, we have developed synthetic peptide derivative (RGDF)-immobilized micro-patterened surface using photolithographic technology. We tried to detect activated platelets as a consequence of interaction of platelets with RGDF-immobilized surface. In order to evaluate the adhesivity of platelet according to the activation state, we used the fluorescence method using mepacrine.

MATERIALS and METHODS

preparation of RGDF-immobilized glass surface

Slide cover glass was spin-coated with a solution of 5% polystyrene(MW 125000) in xylene at 1000 rpm for 1 min to produce a polystyrene thin filim of 1 < μm thickness. A solution of 1% wt of N-hydroxysuccinimidyl azidobenzoate in nitromethane was spin-coated on the polystyrene film at 1000 rpm for 1 min. The film was baked in an oven at 60°C for 20 min. The polystyrene film was exposed at 254 nm UV light(3.3 mW/cm^2) for 5 min to react the azido group with C-H of polystyrene. To examine the

immobilization of primary amine-containing reagents in well defined patterns, a fluorescent dye was immobilized on the functionalized polystyrene film. The polystyrene film was immersed in a solution of 5-(aminoacetamido)fluorescein in ethanol at 25°C for 1 h. After washing it in pure nitromethane, the patterns were observed with the fluorescent microscope.The N-hydroxysuccinimidyl azidobenzoate coated polystyrene film was inoculated under 50 μM of GGGRGDF solution in carbonic acid buffer (pH 10) at 25°C for 3 h and was washed throughly with buffer followed by phosphate buffer(pH 7.4)

preparation of protein-coated glass surface

Fibrinogen, fragment E and BSA coated glass surface were prepared by overnight incubation at room temperature. The coating concentration of protein was 50 μg/ml in 20 mM phosphate buffer containing 150 mM NaCl(pH 8.0). BSA was used as a control protein.

PRP preparation and mepacrine staning

Platelet rich plasma(PRP) was prepared by centrifugation at 180 g for 10 min and platelet concentration was adjusted to $3.0x10^8$/ml.

For fluorescence labeling of platelet, PRP containing PPACK (D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone dihydrochloride; Calbiochem) were treated with the mepacrine(quinacrine dihydrochloride; Sigma) at a final concentration of 10 μM for 30 min.

platelet adhesion studies

Mepacrine labelled PRP were stimulated with ADP(20 μM) and epinephrine(20 μM) and inhibited with prostaglandin E1(10 μM) and apyrase(5 units/ml). 50 μl of stimulated and unstimulated PRP was dropped into the fibrinogen, fragment E, BSA coated surface and RGDF-immobilized surface.

Following incubation for 50 min at 22-25°C, nonattached platelets were removed and washed three times with PBS. The attached platelets were monitored with an inverted-stage microscope equipped with an epifluorescent illuminating source(IMT-2, Olympus Co.).

RESULTS

Figure 1 show the fluorescence images of adhered platelets on the fibrinogen, fragment E and BSA-coated surface. Three images in above line show platelets treated with PGE1 and apyrase to inhibit platelet activation. The images in bottom show platelets treated with ADP and epinephrine. On the fibrinogen-coated surface, stimulated and unstimulated platelets well attached and the fluorescence strengths of attached platelets were similar.

On the fragment E-coated and RGDF-immobilized surface, the fluorescence images show that unstimulated platelets pooly attached however when platelets become activated, they attached to the substrate. BSA-coated surface was used as the cotrol surface. These results indicate that RGDF sequence of fibrinogen fragment E mediate the adhesion of stimulated platelets to surface-bounded fibrinogen.

DISCUSSION

The adhesion of stimulated and unstimulated platelet to fibrinogen requires the receptor binding site of platelet membrane glycoprotein GPIIb/IIIa.

These sites are presented at the Aα chain(RGDS at positions 572-575 and RGDF at 95-98) and the carboxyterminal γ chain (HHLGGAKQAGDV at

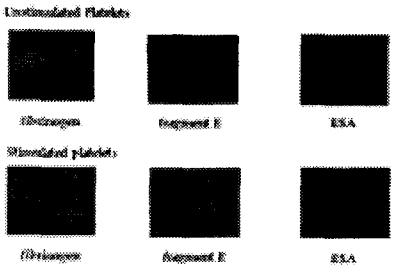

Figure 1. Fluorescence images of adhered platelets on the surface-coated fibrinogen, fragment E and BSA

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[4]. Following platelet activation , platelet membrane glycoprotein GPIIb/IIIa changes its conformation and becomes to interact with fibrinogen. Ruggeri et al[3] reported that unstimulated platelet do not attach to immobilized fibrinogen fragment E containing RGDF sequences. We observed that platelets treated with ADP and epinephrine (stimulated platelet) adhered to RGDF-immobilized surface but platelets treated with PGE1 and apyrase (unstimulated platelet) do not adhered to RGDF-immobilized surface. From these results, RGDF-immobilized micro-patterened surface using photolithographic technology can be used to detect platelet functional state.

ACKNOWLEDGMENTS

Authers acknowledge the Sambo Computer Co. on the their financial support for this research.This research was funded by Sambo Computer company.

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