Morphological characterization of surface-induced platelet activation

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(Received 6 June 1989; revised 21 July 1989; accepted 24 July 1989)

Morphological changes of platelets activated on glass and dimethyldichlorosilane-treated glass were investigated using video microscopy. The platelet morphological changes were quantified by measuring the area and circularity of spreading platelets. In addition, re-organization of cytoskeletal structures of spread platelets was examined. The effects of precoated albumin and fibrinogen on the platelet spreading kinetics were examined as a function of surface protein concentrations. Results showed that platelet shape changes were very sensitive to the surface concentration of precoated proteins. In general, platelets on fibrinogen-precoated surfaces spread fully to a circular shape and developed an extensive inner filamentous zone. In the presence of albumin on the surface, however, platelets could not spread fully and the development of the inner filamentous zone was very poor. For both albumin and fibrinogen, the maximum effects of precoated proteins on platelet shape changes were observed when the surface protein concentration reached the monolayer concentration.

Keywords: Platelets, surface activation, albumin, fibrinogen

Although significant progress has been made recently in the application of biomaterials for substitutes for natural organs, the long-term success of blood-contacting prosthetic devices is still very limited. The occurrence of thrombosis on biomaterials presents major difficulties in the development of blood compatible materials. The mechanisms of surface-induced thrombosis are not completely understood and, as a result, scientific and engineering criteria necessary for the design of biocompatible materials have not yet been developed.

Platelet-surface interaction is a very complex and dynamic series of events. When a biomaterial is implanted *in vivo* or *ex vivo*, adhesion of individual platelets on the surface is invariably followed by the appearance of mural platelet aggregates or thrombi[1-4]. It is the activation of adherent platelets and subsequent thrombus formation that causes the potential danger of using artificial materials *in vivo*. Thus, platelet activation and thrombus formation should be considered to assess accurately the thrombogenicity of biomaterials. Although the clinical trial will provide the final verdict on the thrombogenicity of biomaterials, *in vitro* evaluation techniques need to be developed, since the number of biomaterials to be tested clinically is limited, due to the excessive time and cost involved[5].

Traditionally, the number of adherent platelets was measured as a parameter for surface thrombogenicity in most *in vitro* studies. The initial attachment of platelets, however, is known to be less surface specific than spreading and release[6]. Recently, it was suggested that the extent of platelet spreading was a better parameter than the number of contact-adherent platelets for estimating apparent blood compatibility of biomaterials[7]. The thrombogenicity of the upper surface of spread platelets has been well documented[8,9]. Even with blood of patients with von Willebrand's disease or the Bernard-Soulier syndrome, formation of platelet thrombi occurs normally once adhering platelets spread out[6]. The platelets of patients with acute myocardial infarction have an increased ability to undergo morphological changes after contact with activating surface such as glass[10,11]. Clearly, more understanding on the platelet spreading behaviour on biomaterials is necessary.

Morphological changes of platelets during spreading on surfaces have been usually determined by counting the number of platelets in various spreading stages, such as discoid, dendritic, partially spread, spread and fully spread forms[12-14]. It would be better if platelet spreading could be evaluated objectively using numeric values which describe the stage of platelet spreading. We have found that the area and circularity of spread platelets can be used as useful parameters for the accurate description of platelets at different spreading stages. These two parameters were used to examine platelet morphological changes as a function of experimental variables such as surface, protein type, and surface concentration of proteins.

MATERIALS AND METHODS

Platelet preparation

Blood was withdrawn from healthy adult volunteers by venous puncture after informed consent. All volunteers were

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free of aspirin. Twenty ml of blood was collected into Vacutainers (Becton Dickinson, Rutherford, NJ, USA) containing buffered sodium citrate and mixed by gentle inversion. Platelet-rich plasma (PRP) was separated from whole blood by centrifugation at 100**g** for 20 min at r.t.. Citrated PRP was then incubated in a 37°C water bath for 30 min. Such incubation helped the majority of platelets to recover a discoid shape and to retract pseudopods[15]. Platelets were isolated from plasma proteins by passage through a Sepharose CL-2B column (2.5 × 10 cm) which was pre-equilibrated with phosphate-buffered saline (PBS, pH 7.2)[16]. PBS used in our study was without divalent cations. The appearance of platelets was monitored by UV detector (Gilson, Middleton, WI, USA). The platelet peak was separated from the protein peak. The obtained platelet suspension was kept at r.t. and used in less than 30 min after separation.

Surfaces

Glass slides (Fisher, Chicago, IL, USA) and glass coverslips (1 × 3 inch, No. 1-1/2, Bellco, Vineland, NJ, USA) were cleaned using IsoClean (IsoLab, Akron, OH, USA). They were soaked in 2% IsoClean solution at 60°C for 3 h and washed extensively with distilled water. They were then soaked again in de-ionized distilled water at 60°C for 3 h. Finally, they were washed with plenty of de-ionized distilled water and dried at 80°C.

For the coating of glass coverslips with dimethyl-dichlorosilane (DDS, Aldrich, Milwaukee, WI, USA), clean glass coverslips were immersed in a 5% solution of DDS in chloroform for 30 min. The treated coverslips were rinsed twice with chloroform and ethanol in sequence. Finally, the DDS-treated coverslips were washed in de-ionized distilled water and dried at 80°C.

In addition to glass coverslips, glass tubes (0.1 inch inside diameter, Kimble, Vineland, NJ, USA) were cut into 6 inch lengths, cleaned and coated with DDS as described above. The glass tubes were used to measure the surface protein concentrations using radiolabelled proteins.

Perfusion chamber

A simple perfusion chamber was devised to coat the glass coverslips with proteins and to introduce platelets[17]. The chamber was assembled with a glass slide (1 × 3 inch), a glass coverslip (1 × 3 inch), and a silicone rubber gasket (0.005 inch thickness, Dow Corning, Midland, MI, USA) as a spacer. Two holes, 4 mm in diameter, were drilled in the glass slide, with their centres spaced apart by 4 cm. The silicone rubber gasket was treated lightly with silicone grease and placed between glass slide and coverslip. The three pieces were held together by pressing for several seconds. The perfusion chamber was made immediately before experiment. The PBS was added into the perfusion chamber by placing the end of a pipette tip to one end of the glass slide. The other end of the glass slide received a filter paper wick or a folded medicalwipe that drew the solution out from the perfusion chamber. All other solutions were added in this way.

Protein preparation

Commercially obtained human fibrinogen (Sigma, Type I, St Louis, MO, USA) was purified further by the Laki method[18] as described previously[19]. The purified fibrinogen showed clottability of at least 97%. The fibrinogen in PBS was frozen in aliquots at −70°C at a concentration of about 2.0 mg/ml. Human albumin (Sigma, 1X crystallized) was used as received. The concentrations of both proteins were monitored by absorbance at 280 nm using absorptivities of 1.506×10^3 cm^2/g and 5.8×10^2 cm^2/g for fibrinogen and albumin, respectively.

Protein adsorption

Protein solutions were added to the chamber by replacing PBS. The bulk protein concentration was varied from 0.1 to 50 mg/ml for albumin, and from 0.001 to 1 mg/ml for fibrinogen. After 1 h at r.t., the chamber was washed with PBS to remove unadsorbed protein.

The surface protein concentrations were measured using glass tubes. Fibrinogen and albumin were labelled with ^{125}I (Amersham, Arlington Heights, IL, USA), using Enzymobead reagents (Bio-Rad, Rockville Centre, NY, USA). The radioactivity was counted using a gamma counter (Gamma 5500B, Beckman, Arlington Heights, IL, USA).

Video microscopy

Platelets in PBS were introduced into the perfusion chamber and allowed to adhere to the surface at r.t.. Platelets were observed on an inverted light microscope (Diaphot, Nikon, Garden City, NY, USA) equipped with optics for differential interference contrast (DIC) and interference reflection microscopy (IRM). An oil immersion 100X plan achromatic objective (1.25 numerical aperture) was used throughout experiments. Microscope images were projected to a video camera (Newvicon, Model 65, Dage-MTI, Michigan City, IN, USA) using a 2X video-relay lens (Nikon). The image was optimized using a sync stripper (Model 302-2, Colorado Video, Boulder, CO, USA) and a video processor (Model 604, Colorado Video). The image was then directed to a computer with image processor (Imaging Technology, Woburn, MA, USA), where it was digitized, processed and analysed. Software for image analysis was obtained from PC Consulting (Madison, WI, USA). The monitor was photographed on Kodak Plus-X pan 125 film using a Nikon N2000 camera equipped with an AF Micro-nikkor 55 mm f/2.8 lens.

Observation of platelet cytoskeletons

Platelet spreading was continuously monitored by video-enhanced differential interference contrast microscopy or brightfield microscopy. After a predetermined time, platelets were treated with extraction buffer (0.1% Triton X-100 and 4% polyethylene glycol (mol wt 10 000) in PBS) for 1 min. The extraction buffer was replaced by PBS and 2% glutaraldehyde in PBS was added to the chamber. Fixation continued at least for 5 min before glutaraldehyde was replaced by PBS. The fixed cytoskeletal structures were further treated with a solution containing 1% acetic acid, 25% glycerol and 1% sodium chloride (acetic acid solution) and observed with video-enhanced interference reflection microscopy (VEIRM). This technique is useful to observe the cytoskeletal structures of spread platelets as described previously[17].

Circularity and area of spread platelets

The area and circularity of platelets were measured using an image analyser with software from PC Consulting. The glutaraldehyde-fixed platelets were stained with Coomassie Brilliant Blue (CBB) solution for at least 1 h. A 0.1% solution of CBB R-250 (Bio-Rad) was made in a mixture of acetic

acid, methanol and water in the ratio of 10:45:45[20,21]. The CBB-stained platelets were easily separated from the background by thresholding of the digitized image. The area and circularity were measured for platelets in 12 separate microscope fields for each time point. The total number of platelets examined per sample ranged from 100 to 200.

The circularity, C, is defined in the following equation.

$$C = P^2/4\pi A \quad (1)$$

where A and P are the area and the perimeter of a spread platelet, respectively. The circularity has a minimum value of 1.0 for a perfect circle and takes on larger values for more complex shapes.

RESULTS

Platelet spreading and circularity

The platelet transformation from spherical to fully spread shape on glass and DDS-coated glass was examined for 2 h. Platelet spreading was quantitated using spread area and circularity parameters. As a platelet spreads, its area increases. The circularity, however, can either increase or decrease depending on the shape of spread platelets. *Figure 1* shows platelets in different stages of spreading and their circularities. Whether platelets are dendritic or spread, the circularity depends largely on the number and length of pseudopods. The circularity of platelets with a few short pseudopods is usually around 2.0. As the number and length of the pseudopods increase, the circularity also increases. The top three rows show this trend. If platelets are spread fully and no distinct pseudopods are seen, then the circularity approaches the value of 1.0 (bottom row). In no circumstances, however, do platelets become perfectly circular.

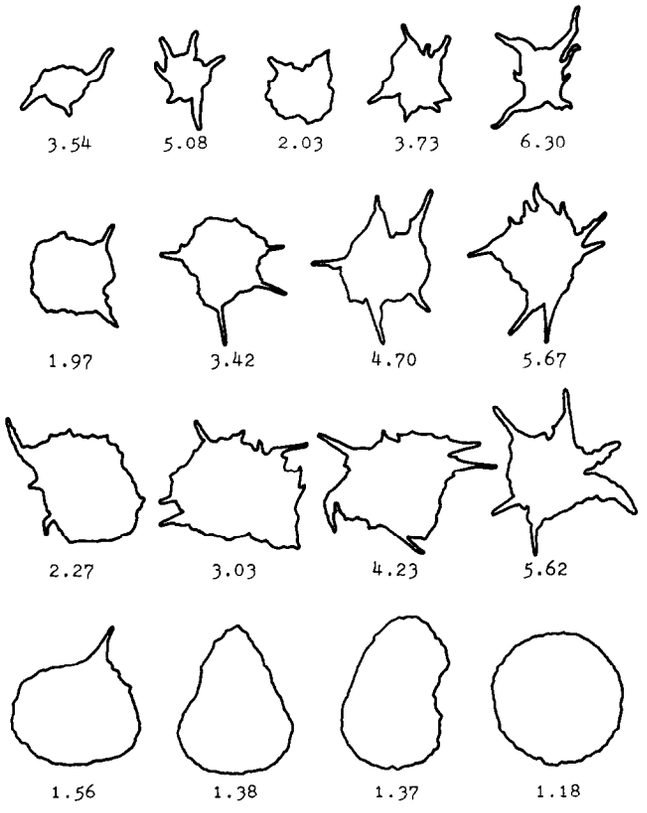

Figure 1 Circularity values of platelets in different stages of spreading.

When platelet suspensions are prepared and applied to a surface, not all platelets adhere to the surface at the same time and undergo shape changes at the same rate. This results in a distribution of platelets in different spreading stages. It is therefore important to examine a large number of platelets to characterize the status of platelet spreading on a given surface. In our study, the circularity and area of at least 100 platelets were measured. When the majority of platelets on the surface were fully spread, the average circularity value was smaller than 2.0. If the average value was higher than 2.0, the majority of platelets possessed distinct pseudopods. Therefore, the average circularity value of 2.0 was used as a reference value which indicates full spreading of the majority of platelets. It is recommended that the reference value of 2.0 should be used in conjunction with the area of spread platelets. Contact adherent or discoid platelets with the area of about 15 μm^2 cannot be described as fully spread, even though their circularity value may be below 2.0. The area of fully spread platelets is around 50 μm^2. The important point here is that platelet spreading can be numerically described using the circularity and the area.

Kinetics of platelet spreading

Figure 2 shows the kinetics of platelet spreading on glass. When platelets were allowed to adhere and spread on glass in the absence of exogenous proteins, the platelet area

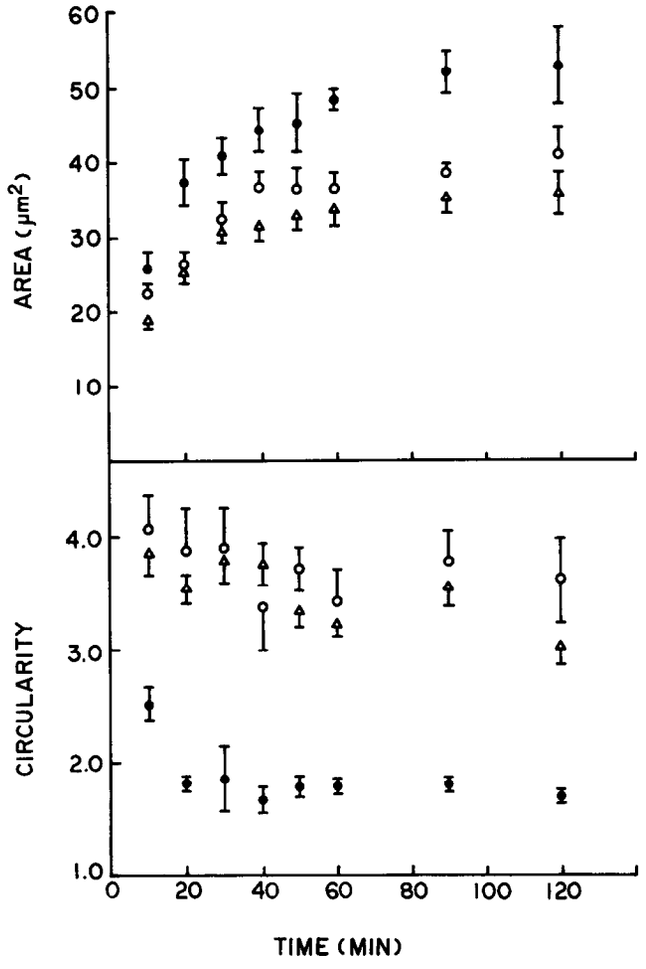

Figure 2 Kinetics of platelet shape changes on glass as measured by spread area and circularity (average ± SEM). Platelets were allowed to spread on glass (○), fibrinogen-coated glass (●) and albumin-coated glass (△). Glass was precoated with either fibrinogen (0.1 mg/ml) or albumin (1 mg/ml) for 1 h at r.t..

gradually increased as platelet spreading continued up to 2 h. The area of spread platelets reached about 40 μm^2 after 2 h of spreading. The circularity did not show any tendency of increase or decrease during platelet spreading and remained at relatively high values, usually above 3.0. The precoating of the glass surface with albumin at 1 mg/ml for 1 h at r.t. did not result in significant changes in the platelet spreading kinetics (*Figure 2*). As described below, the surface albumin concentration was only 0.05 $\mu g/cm^2$. This surface albumin concentration is well below a monolayer, making the slight difference in spreading behaviour relative to glass understandable. On the fibrinogen-precoated glass, however, platelets spread in such a way that the edges of spreading platelets were always smooth with only a few pseudopods. After 20 min spreading, the circularity became smaller than 2.0 and reached a plateau value of about 1.7. The area of spreading platelets on the fibrinogen-coated glass was about 50 μm^2 after 1 h spreading (*Figure 2*). The presence of fibrinogen on the surface accelerated the platelet spreading to a circular form.

The kinetics of platelet spreading on DDS-glass was also examined as shown in *Figure 3*. The area of spread platelets reached 50 μm^2 and the circularity was reduced below 2.0 after 40 min of spreading. As the circularity values in *Figure 3* indicate, most platelets spread fully to a circular shape on DDS-glass even in the absence of the precoated fibrinogen. The precoating of DDS-glass with fibrinogen did not increase the area of spread platelets to more than 50 μm^2, which appears to be the maximum area that platelets can spread under our experimental condition. The fibrinogen-precoating, however, reduced the circularity further down to 1.20. The effect of fibrinogen was noticeable only with the circularity values. Unlike on glass, the behaviour of platelets on DDS-glass was significantly influenced by precoating the surface with albumin. When DDS-glass was precoated with 1 mg/ml of albumin for 1 h at r.t., platelet spreading was markedly reduced. The area of spreading platelets was reduced to 30 μm^2 and the circularity became much larger than 2.0 (*Figure 3*). This pronounced effect of albumin on the platelet spreading behaviour may be explained by the surface albumin concentration of 0.22 $\mu g/cm^2$ which is the same as the monolayer concentration.

As shown in *Figures 2* and *3*, platelets continuously change their areas and circularities during spreading. Thus, it is important to control the time for platelet spreading, if platelet shapes on different surfaces or under different experimental conditions are to be compared. On both glass and DDS, the effects of precoated fibrinogen on the platelet area and the circularity were consistent: circularity decreased and the area increased. It should be noted that fibrinogen-precoating on DDS-glass did not increase the spread area, probably because platelets were able to spread to a maximum value (50 μm^2) even in the absence of fibrinogen. The decrease in circularity and the increase in area due to precoated fibrinogen will be called fibrinogen effects. When platelets were spread on the albumin-precoated DDS, the spread area was decreased and the circularity was increased (*Figure 3*). Thus, the increase in circularity and the decrease in area will be referred to as albumin effects.

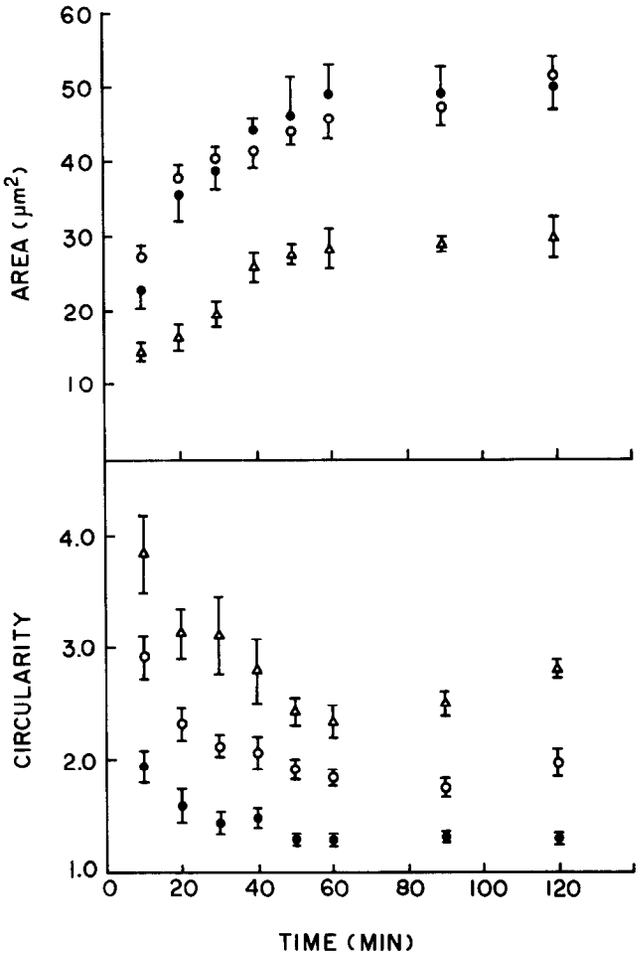

Figure 3 Kinetics of platelet shape changes on DDS-glass as measured by spread area and circularity (average ± SEM). Platelets were allowed to spread on DDS-glass (○), fibrinogen-coated DDS-glass (●) and albumin-coated DDS-glass (△). DDS-glass was precoated with either fibrinogen (0.1 mg/ml) or albumin (1 mg/ml) for 1 h at r.t..

Critical surface fibrinogen concentrations for platelet spreading

We examined relationships between the surface fibrinogen concentration and the extent of the fibrinogen effects. The surface fibrinogen concentration was varied by adjusting the bulk fibrinogen concentration for adsorption. The fibrinogen adsorption isotherms on glass and DDS-glass are shown in *Figure 4*. The initial part of the isotherms clearly indicates that fibrinogen adheres stronger to DDS-glass than to glass. *Figure 5* shows the changes in the area and circularity of platelets which were spread on glass and DDS-glass for 30 min as a function of the surface fibrinogen concentration.

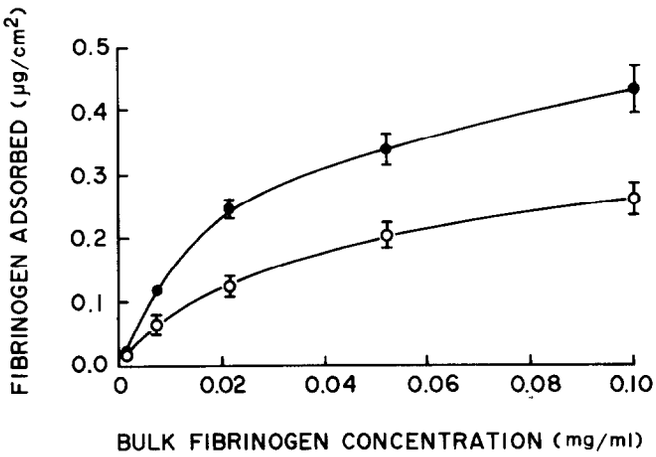

Figure 4 Adsorption isotherms of fibrinogen on glass (○) and DDS-glass (●). Fibrinogen was adsorbed for 1 h at r.t.. Average ± s.d, n = 4.

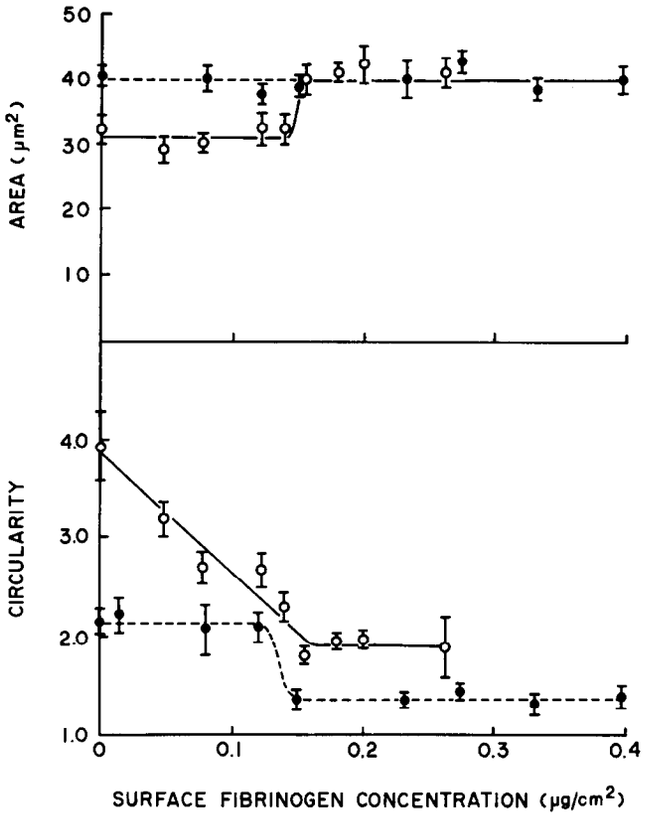

Figure 5 Changes in the area and circularity of platelets spread on glass (○) and DDS-glass (●) as a function of surface fibrinogen concentration. Platelets were allowed to spread for 30 min at r.t.. Average ± SEM.

Fibrinogen at different bulk concentrations was precoated at r.t. for 1 h and the surface fibrinogen concentration was calculated from the isotherms in *Figure 4*. The area of spread platelets on the fibrinogen-coated glass remained the same as that on the control glass until the surface fibrinogen concentration reached 0.16 $\mu g/cm^2$. The circularity, however, linearly decreased as the surface fibrinogen concentration increased up to 0.16 $\mu g/cm^2$. Above this concentration, area and circularity did not change further. Changes in the area and circularity on glass clearly show that the maximum fibrinogen effects appear when the surface fibrinogen concentration reaches 0.16 $\mu g/cm^2$. Therefore 0.16 $\mu g/cm^2$ appears to be the critical surface fibrinogen concentration above which the platelet response remains the same. It appears that the circularity is more sensitive than the area to changes in the surface fibrinogen concentration.

Figure 5 also shows the relationships between the platelet spreading behaviour on DDS-glass and the surface fibrinogen concentration. The area of platelets did not depend on the surface fibrinogen concentration as described above. The circularity, however, was changed as the surface fibrinogen concentration was varied. The drop in circularity values was observed at the surface fibrinogen concentration of 0.15 $\mu g/cm^2$. The critical surface fibrinogen concentration on DDS-glass is practically the same as that on glass. It appears that the maximum fibrinogen effects are observed as long as the surface fibrinogen concentration is 0.15 $\mu g/cm^2$ and higher. If we assume that fibrinogen[22, 23] is 45 nm long and 9 nm thick with a mol wt of 340 000 the surface fibrinogen concentrations required for side-on and end-on packed monomolecular layers are calculated to be 0.14 and 0.89 $\mu g/cm^2$, respectively. The critical surface fibrinogen concentration observed in this study is essentially the same as the concentration of side-on packed monolayer.

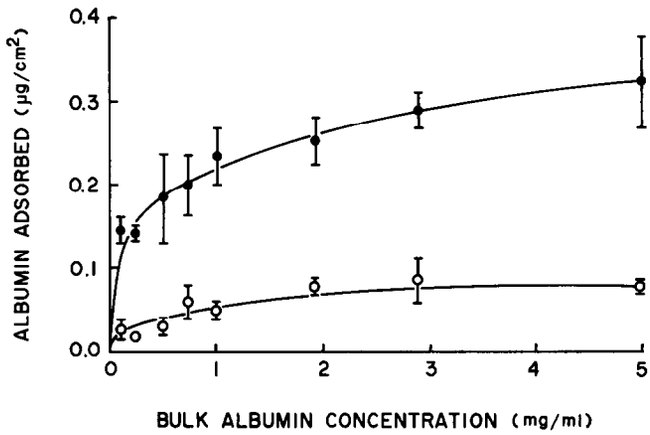

Figure 6 Adsorption isotherms of albumin on glass (○) and DDS-glass (●). Albumin was adsorbed for 1 h at r.t.. Average ± s.d., n = 4.

Albumin adsorption and platelet spreading

Albumin adsorption isotherms on glass and DDS-glass are shown in *Figure 6*. The amount of albumin adsorbed on glass was <0.1 $\mu g/cm^2$, even when the bulk albumin concentration was increased up to 5 mg/ml. The initial part of the isotherms shows that albumin adheres much stronger to DDS-glass than to glass. We examined the effects of surface albumin concentration on the area and circularity of spread platelets. Platelets were allowed to spread on glass and DDS-glass for 30 min at r.t.. The surface albumin concentrations were varied by precoating the surfaces with albumin at different bulk concentrations for 1 h at r.t.. On DDS-glass, the area and circularity remained the same as those on the control surface until the surface albumin concentration reached 0.16 $\mu g/cm^2$ (*Figure 7*). As the

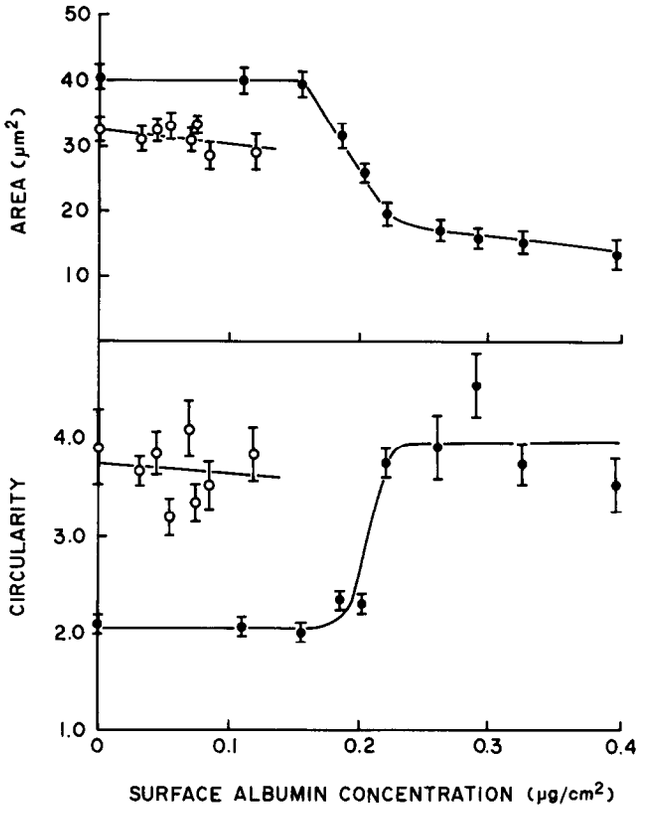

Figure 7 Changes in the area and circularity of platelets spread on glass (○) and DDS-glass (●) as a function of surface albumin concentration. Platelets were allowed to spread for 30 min at r.t.. Average ± SEM.

surface albumin concentration increased further, the area of spread platelets gradually decreased and reached a plateau value at the surface albumin concentration of 0.23 $\mu g/cm^2$. The circularity increased dramatically at the same surface albumin concentration. Thus, the critical albumin concentration appears to be 0.23 $\mu g/cm^2$.

It seems that the area is more sensitive than the circularity to changes in the surface albumin concentration on DDS-glass. On glass, no significant changes were apparent in the area and circularity due to the precoated albumin (*Figure 7*). Unlike on DDS-glass, the largest surface albumin concentration obtained on glass under our experimental conditions was slightly >0.1 $\mu g/cm^2$. Since this concentration is much smaller than the critical concentration, it is understandable that no apparent albumin effects were observed on glass.

If albumin can be depicted as an oblate ellipsoid[24, 25], with axes 4 × 14 nm and a mol wt of 66 500, the surface albumin concentrations required for side-on and end-on packed monomolecular layers are calculated to be 0.20 and 0.85 $\mu g/cm^2$, respectively. The critical albumin concentration observed in *Figure 7* is very close to the side-on monolayer concentration. Like fibrinogen, the maximum albumin effects were observed when the surface is covered with a monolayer of albumin.

Cytoskeletal re-organization during platelet spreading

Platelet spreading is accompanied by extensive re-organization of a three-dimensional cytoskeletal network. The platelet cytoskeletal reorganization during spreading was examined using VEIRM. As platelets spread fully on surfaces, they develop four distinct zones: peripheral web, outer filamentous zone, inner filamentous zone (IFZ) and central granulomere region[14, 26, 27]. *Figure 8a* shows platelets which were spread on the fibrinogen-precoated glass for 40 min. It is clearly seen that most of the spread hyaloplasm is filled with microfilament bundles. The extensive development of the IFZ (arrows in *Figure 8a*) is characteristic to the platelets spread on the fibrinogen-coated glass. Platelets developed such an extensive IFZ on DDS-glass even without precoated fibrinogen. In the presence of albumin on DDS-glass, however, platelets developed only a poor IFZ. Cytoskeletal structures of platelets spread on the albumin-precoated DDS-glass for 40 min are shown in *Figure 8b*. Not all platelets were able to fully spread on the surface. Even though some platelets spread to a circular shape, the development of the IFZ was very poor (arrows in *Figure 8b*). On DDS-glass, albumin precoating at the bulk concentration of 1 mg/ml for 1 h at r.t. was sufficient to inhibit formation of extensive IFZ. On glass such effects of albumin were noticeable only when the bulk concentration for precoating was 25 mg/ml and above. It was a consistent observation that, on the albumin surface, platelets could not spread well and the development of the IFZ was very poor, even in the spread platelets.

In addition to the poor development of IFZ, platelets on albumin-precoated glass and DDS-glass maintained a large central granulomere region and granule release was not active. Active degranulation was observed from platelets spreading on the fibrinogen-precoated surfaces. The differences between platelets spread on the albumin- and fibrinogen-coated surfaces are summarized in *Table 1*. It is clear that platelet spreading behaviour is markedly affected by the type of protein present on the surface.

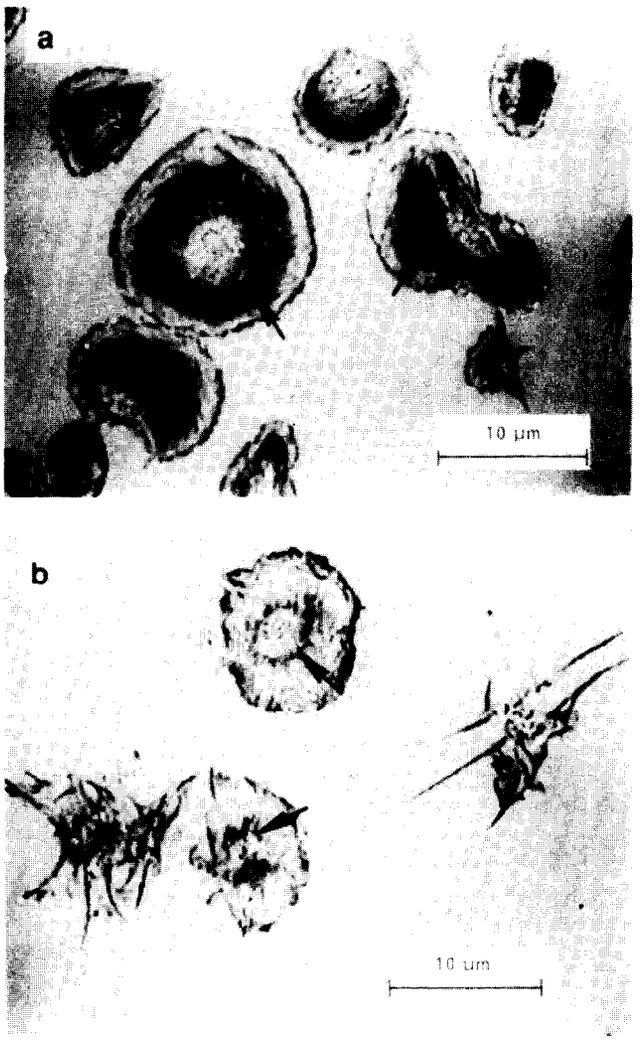

Figure 8 Cytoskeletal structures of platelets spread on glass precoated with fibrinogen (a) and DDS-glass precoated with albumin (b). Platelets were allowed to spread for 40 min before treated with Triton-X, glutaraldehyde and acetic acid solution in sequence and imaged with VEIRM. Glass and DDS-glass were precoated with fibrinogen (0.1 mg/ml) and albumin (1 mg/ml), respectively, for 1 h at r.t.

DISCUSSION

The ideal biomaterial would be the one which does not allow platelets to adhere at all. Unfortunately, however, complete prevention of platelet adhesion for an extended period of time is very difficult to achieve. Previous studies suggest that the transformation of contact-adherent platelets to the fully spread form on biomaterials is the first step toward the mural thrombus formation[4, 8, 9]. Platelets spread to increase the area over which the adhesive interaction occurs. Thus, the extent of spreading is expected to affect the total adhesive interaction[28]. The adhesion and desorption of individual platelets without significant transformation is not expected to cause any serious clinical problems. Therefore, it is

Table 1 Effects of precoated albumin and fibrinogen on platelet behaviour

Features	Albumin	Fibrinogen
Surface area	<40 μm^2	>50 μm^2
Circularity	>2.0	<2.0
Degranulation	Rare	Common
Development of IFZ	Poor	Extensive

reasonable to examine the ability of a surface to elicit platelet spreading for the evaluation of surface thrombogenicity. The kinetics and extent of platelet spreading were numerically described using the area and circularity. The two parameters are independent but complement each other in characterizing platelet spreading. Estimation of the numerical values of the two parameters allowed a rigorous approach to comparing platelet spreading kinetics under different experimental conditions.

The kinetics and extent of platelet spreading depend on the nature of a surface and the type of protein present on the surface. In the presence of fibrinogen on the surface above a certain concentration, platelets spread fully with the maximum spread area and the minimum circularity. The critical surface fibrinogen concentration on both glass and DDS surfaces was found to be 0.15 $\mu g/cm^2$. This surface concentration is the same as the monolayer concentration of side-on fibrinogen molecules. Platelet spreading was the same as long the surface fibrinogen concentration was above the critical value. It appears that a minimum or threshold surface density of fibrinogen is required for the full spreading of platelets. Since most of the surface is covered with fibrinogen molecules at the critical surface concentration, the binding of fibrinogen receptors of the platelet membrane to fibrinogen on the surface is ensured. In this way, platelets can make continuous, simultaneous contacts with the surface during spreading. Such contacts may be necessary for continued spreading and other platelet activities such as granule release or development of the extensive IFZ. It is common to observe the threshold surface concentration of cell-adhesive proteins for the maximum spreading of cells. Alpin *et al.*[29,30] observed that maximum spreading of trypsinized BHK cells required a certain concentration of fibronectin on the surface. Fibronectin is also known to promote platelet spreading significantly on a variety of surfaces[31,32]. It will be interesting to examine the critical surface concentration of fibronectin for maximum spreading of platelets.

The observation that platelets can spread fully and develop extensive IFZ on DDS-glass even in the absence of precoated fibrinogen deserves further consideration. It is possible that platelets release their own fibrinogen and other adhesive proteins to the surface before spreading. It is equally possible, however, that membrane proteins, including fibrinogen receptors, on platelet membranes directly adsorb to the surface. As shown in *Figures 4* and *6*, both albumin and fibrinogen have high affinity to DDS-glass. Thus, we can expect nonspecific, strong adsorption of membrane proteins onto DDS-glass. This may result in multiple platelet–surface interactions which generate signals for platelet activation. It is generally known that biocompatibility is improved by precoating surfaces with albumin[33]. It must be noticed, however, that platelets can still adhere and spread on the albumin-coated surfaces, although the number of adherent platelets and the extent of spreading is not as large as on the fibrinogen-coated surfaces.

Since it is believed that platelets do not have receptors for albumin, the adhesion and spreading of platelets in the presence of albumin suggests that platelets displace adsorbed albumin from the surface. Thus, platelets would have more difficulty in displacing albumin as the affinity of albumin to the surface becomes larger. This means that the albumin effects are expected to be more pronounced on surfaces which have higher affinity to albumin. This can explain why platelets could not spread well on DDS-glass when only a monolayer of albumin was present (*Figure 7*). We observed previously that platelets can spread fully on glass even in the presence of albumin in the bulk solution at the concentrations up to 50 mg/ml, although the kinetics of spreading was very slow[17]. As shown in *Figure 6*, albumin has a much higher affinity to DDS-glass than to glass.

The ability of glass and DDS-glass to elicit platelet spreading is strongly dependent on the nature and the amount of adsorbed proteins. Thus, when these surfaces are exposed to blood, the actual response will depend on the type of proteins adsorbed on the surface. Horbett[34] has suggested that the biocompatibility of polymers *in vivo* is determined by their ability to fractionate plasma proteins and concentrate them at their surface. The mechanisms of protein adsorption from a complex mixture such as plasma, however, are not yet understood. One of the factors which make the study of protein adsorption more difficult is the displacement of adsorbed proteins by other proteins[35,36]. A recent study by Slack and Horbett[37] suggests that the relative affinity of plasma proteins to a finite number of adsorption sites can explain various aspects of protein adsorption including displacement phenomenon. The study on protein adsorption has to be followed by the study on platelet activation. Whether the adsorbed proteins are biologically active or not can be determined by the ability of the adsorbed protein to elicit platelet activation. The quantitative comparison of platelet activation using numeric values such as the area and circularity should be useful in the study of various aspects of protein adsorption.

ACKNOWLEDGEMENT

This study was supported by the National Heart, Lung and Blood Institute of the National Institutes of Health through Grant HL 39081 and in part by the Petroleum Research Fund administered by the American Chemical Society.

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