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THE EFFECT OF FIBRINOGEN SIALIC ACID RESIDUES ON EX VIVO PLATELET DEPOSITION ON BIOMATERIALS

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ABSTRACT

The effect of fibrinogen sialic acid residues on platelet deposition onto polymer surfaces was examined using a canine ex vivo shunt model. To test the hypothesis that desialylated fibrinogen may enhance platelet deposition when precoated on biomaterials, canine fibrinogen was desialylated and precoated on polyvinyl chloride (PVC) shunts. When protein-coated PVC shunts were exposed to flowing whole blood, both the native and the desialylated fibrinogen elicited the same profile of platelet deposition. This study indicates that platelet deposition and thrombus formation on biomaterial surfaces is not mediated by a mechanism which involves the sialic acid residues of fibrinogen.

INTRODUCTION

The occurrence of surface-induced thrombosis presents major difficulties in the development of blood-contacting prosthetic devices. Although the exact mechanism of platelet adhesion onto polymeric biomaterials at the molecular level is not understood, platelet adhesion is known to be mediated by the protein layer adsorbed on the polymer surface before platelet arrival. Since Roseman (1) proposed that the interaction of glycosyl transferases with various monosaccharide receptors might mediate various types of cell-cell recognition and adhesion, numerous reports have appeared suggesting the possible involvement of carbohydrate residues in platelet function. Platelet surfaces are known to contain sialyltransferase (2) which is stimulated by collagen, thrombin and, to a lesser degree, by ADP (3). Scialla et al. (4) found that sialyltransferase activity in human platelet membranes was increased in a group of cancer patients resulting in increased thrombus formation and embolization. These studies have suggested a possible functional significance for the sialyltransferase and the terminal

Key Words: sialic acid, fibrinogen, platelet, biomaterial, thrombosis

carbohydrate residues in platelet function.

Kim and Lee (5) reported a slight enhancement of *in vitro* platelet adhesion to various substrates which were precoated with desialylated fibrinogen, and suggested the possible involvement of sialyltransferase in the platelet-protein interactions. In contrast, other investigators observed that native and desialylated fibrinogen were equally effective in inducing platelet aggregation (6) and platelet adhesion to protein precoated glass *in vitro* (7). We were interested in the role that the sialic acid residues of fibrinogen play in artificial surface-induced thrombosis. The results of *in vivo* or *ex vivo* experiments are different from those of *in vitro* experiments in that platelet spreading and thrombus formation is seldom observed in *in vitro* experiments (8). In the present study, native and desialylated canine fibrinogen were precoated on PVC shunts and platelet adhesion, changes in platelet morphology, and subsequent thrombus formation were compared using the canine *ex vivo* model developed in this laboratory.

METHODS

Fibrinogen Preparation Human and canine fibrinogen was prepared from fresh citrated plasma by a β-alanine precipitation method (9). Fibrinogen prepared by the β-alanine method was further purified to remove fibronectin using an Affi-Gel gelatin (Bio-Rad) column (1.5x15 cm). Fibronectin-free fibrinogen was precipitated with saturated ammonium sulfate and reconstituted in the desialylation buffer (0.13 M NaCl, 0.02 M sodium acetate, and 0.02% sodium azide, pH 6.2) and dialyzed against the same buffer. Fibrinogen prepared by this method showed a clottability greater than 97%. Fibrinogen concentrations were determined by absorbance at 280 nm (Abs of 0.1% solution = 1.506) (10) for both species.

Sialic Acid Assay To measure the total sialic acid content, fibrinogen (2 mg/ml) was rapidly mixed with an equal volume of 0.1 N H_2SO_4 solution and incubated at 80°C for one hour. If the fibrinogen concentration was greater than 2 mg/ml, precipitation occurred which resulted in underestimation of the number of sialic acid residues per fibrinogen molecule. A standard solution of N-acetylneuraminic acid (Sigma, Type IV) treated in the same manner was the control. Enzymatic desialylation was carried out at 37°C by incubating fibrinogen in the desialylation buffer with neuraminidase (Clostridium perfringens) obtained from either Worthington or Sigma (Type X). No difference was observed in the activity of the enzyme obtained from the two sources. The neuraminidase concentration was adjusted to 0.01-0.05 unit/mg fibrinogen. Enzymatic desialylation was also carried out with insoluble neuraminidase (Clostridium perfringens attached to agarose beads, Sigma, Type X-A). In the latter method, the insolubilized neuraminidase was easily separated from fibrinogen by centrifugation after cooling in an ice bath for a few minutes. Free sialic acid was assayed by the thiobarbituric acid method (11). Neuraminidase was devoid of esterase or protease activity at the concentration used. Esterase activity was detected using tosylarginine methyl ester (Sigma). When ^{125}I-labeled fibrinogen (see below) was incubated with neuraminidase for 3 hr at 37°C and precipitated with an equal volume of 20% trichloroacetic acid, the radioactivity remaining in the supernatant was the same as in the control.

Clottability and Clotting Time Measurement Clottability was measured as

described by Coller (7). Clotting time was measured using a Fibrometer (Baltimore Biological Laboratory). Fibrinogen clotting was initiated by adding 0.1 ml of thrombin (10 unit/ml, Parke-Davis) to 0.2 ml of fibrinogen solution (0.3 mg/ml) at 37°C.

Radiolabeling of Fibrinogen and Fibrinopeptide-B Release Canine native and desialylated fibrinogen was radiolabeled by ^{125}I (New England Nuclear) using the Chloramine-T method (Iodo-Beads, Pierce Chemical). Fibrinopeptide-B of canine fibrinogen contains tyrosine residues which can be iodinated (12). The release of fibrinopeptide-B after thrombin treatment was measured. 0.1 ml of thrombin (1.0 unit/ml) was added to 1 ml of fibrinogen (0.3 mg/ml) and, at each time point, 0.3 ml of 50% trichloroacetic acid was added. The precipitated fibrinogen was centrifuged and the radioactivity remaining in the supernate was counted. For a control, buffer was added instead of thrombin and the fibrinogen precipitated in the same manner. Atroxine (Sigma) which releases only fibrinopeptide-A released no radioactivity. This served as a further control to prove that the radioactivity released was due to fibrinopeptide-B cleaved by thrombin.

Gel Electrophoresis SDS-polyacrylamide gel electrophoresis was carried out using the method of Laemmli (13) with 3% stacking and 7.5% separating gels. Fibrinogen was reduced by heating in boiling water for 5 min in stacking buffer containing 10% glycerol, 1% SDS and 5% 2-mercaptoethanol (pH 6.8). Protein was stained with Coomassie blue.

Plasmin degradation kinetics were measured by SDS-PAGE. The native and the desialylated fibrinogen in acetate buffer (pH 6.2) were mixed with plasmin (0.05 unit/mg fibrinogen, Sigma) and incubated at 37°C. Samples were collected at time intervals and a comparison was made between the native and desialylated fibrinogen degradation products.

Observation of Fibrin Fibrils by Electron Microscopy Fibrin fibrils of native and desialylated fibrinogen on Formvar-coated grids were prepared by placing a drop of fibrinogen and thrombin mixture in the desialylation buffer on the grid. The mixture was composed of 200 µl of fibrinogen (0.3 mg/ml) and 50 µl of thrombin (5 unit/ml). After a given time period, the grid was washed in the buffer and fixed in 2% glutaraldehyde in the same buffer. Samples were dehydrated in a graded ethanol series and were dried by the critical point method. Samples were sputter-coated with gold and examined on a JEOL 35C scanning electron microscope (SEM) at 12 kV accelerating voltage. When micrographs of different samples were taken, the height of specimen was adjusted to the same focal point, while fixing the objective lens focus.

Platelet Deposition in a Canine Ex Vivo Model A polyvinyl chloride (PVC) shunt (0.125 inch I.D., Norton) was precoated with native or desialylated canine fibrinogen at a bulk concentration of 0.3 mg/ml for 2 hours at room temperature. The surface concentration was 0.79 µg/cm^2 for both proteins as measured using ^{125}I-labelled fibrinogen. The protein solution was displaced with divalent cation-free Tyrode's solution and the shunt implanted into the femoral artery and vein of a canine subject. During cannulation, blood flow was interrupted by clamping the artery with a vascular clamp. Blood was allowed to flow through the shunt by removing the clamp. A 24-inch segment of the shunt was in contact with a shielded solid crystal detector. This method allowed measurement of thrombus deposition over a large surface area. At selected time points, the blood flow was stopped by clamping the artery, and

the shunt was flushed with 50 ml of Tyrode's solution. After flushing, the number of platelets deposited on the surface was measured by counting the radioactivity. The blood flow was resumed following counting. Autologous platelets labeled with ^{51}Cr were reinfused into the animal 18 hours prior to the experiment and the number of platelets deposited on the shunt surface was measured using a multichannel analyzer (Ino-Tech 5100). Platelet morphology was examined by scanning electron microscopy. The details of this procedure have been published elsewhere (14).

RESULTS AND DISCUSSION

Desialylation of Fibrinogen The total number of sialic acid residues on fibrinogen was measured after acid hydrolysis, and 9.4±0.5 (mean ± S.D.) and 7.4±0.3 sialic acid groups per fibrinogen molecule were found for canine and human species, respectively. It is noteworthy that canine fibrinogen contains more than 9 sialic acid residues. Assuming that the canine fibrinogen possesses 4 bi-antennary oligosaccharides that are fully sialylated on the Bβ and γ chains, the total number of sialic acids is expected to be 8. It is possible that the Aα chain of canine fibrinogen is sialylated, at least partially, as in chicken fibrinogen (15). The possible glycosylation of the Aα chain of canine fibrinogen requires further investigation. Fibrinogen from each species showed a distinct migration pattern by SDS-PAGE after reduction. However, differences in electrophoretic mobilities between the native and the desialylated fibrinogen were negligible for all of the fibrinogens tested.

In this study, it was necessary to use neuraminidase to desialylate the fibrinogen for functional studies, because fibrinogen desialylated by acid hydrolysis lost its ability to clot. Fig 1 shows the release kinetics of sialic acid residues from canine and human fibrinogen by neuraminidase. It appears that both types of fibrinogen are equally susceptible to neuraminidase attack. When neuraminidase that had been immobilized onto agarose beads was used (0.15 unit/mg fibrinogen), both canine and human fibrinogen released all of their sialic acid residues in 4 hours. Since platelet deposition on fibrinogen precoated surfaces was measured using a canine ex vivo model, canine fibrinogen was desialylated using the immobilized neuraminidase and the properties of the fibrinogen were compared to those of human desialylated fibrinogen which has been relatively well characterized.

Characterization of Canine Desialylated Fibrinogen When increasing amounts of sialic acid residues were removed from canine fibrinogen, the clotting time was shortened, as observed with human fibrinogen (7, 16-18). At the same protein concentration, desialylated fibrinogen formed a clot which was more turbid and thicker than the clot formed by native fibrinogen as measured by the absorbance ratio at 350 nm and 600 nm (19) and as observed by scanning electron microscope (Fig 2). The fibrils formed with desialylated fibrinogen were approximately 50% thicker compared to those formed with native fibrinogen (170 nm vs. 120 nm). The same result was obtained with human fibrinogen. The formation of thicker fibrils with desialylated fibrinogen is consistent with the finding that hypersialylated fetal fibrinogen (20) forms thinner fibrils than fibrinogen from normal adults. The fetal fibers were found to be nearly half as thick as the adult fibers (21). The extent of plasmin degradation of the fibrinogen monomer and the fibrin clot were the same for both native and desialylated fibrinogen as measured by SDS-PAGE. The release of fibrinopeptide-B from canine fibrinogen was easily quantified, since

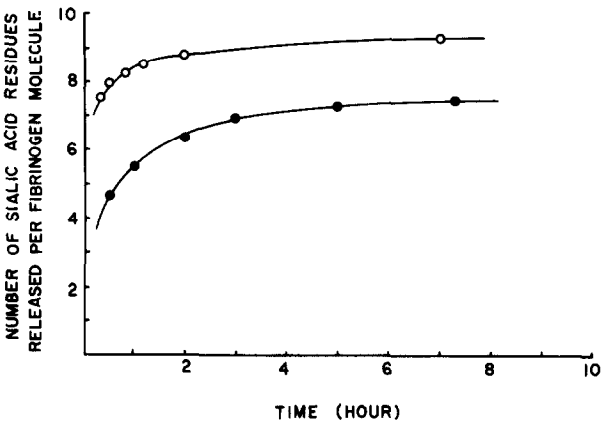

FIGURE 1

The number of sialic acid residues released from canine (o) and human (•) fibrinogen. Fibrinogen was incubated with soluble C. perfringens neuraminidase (0.05 unit/mg fibrinogen) at 37°C in acetate buffer (pH 6.2).

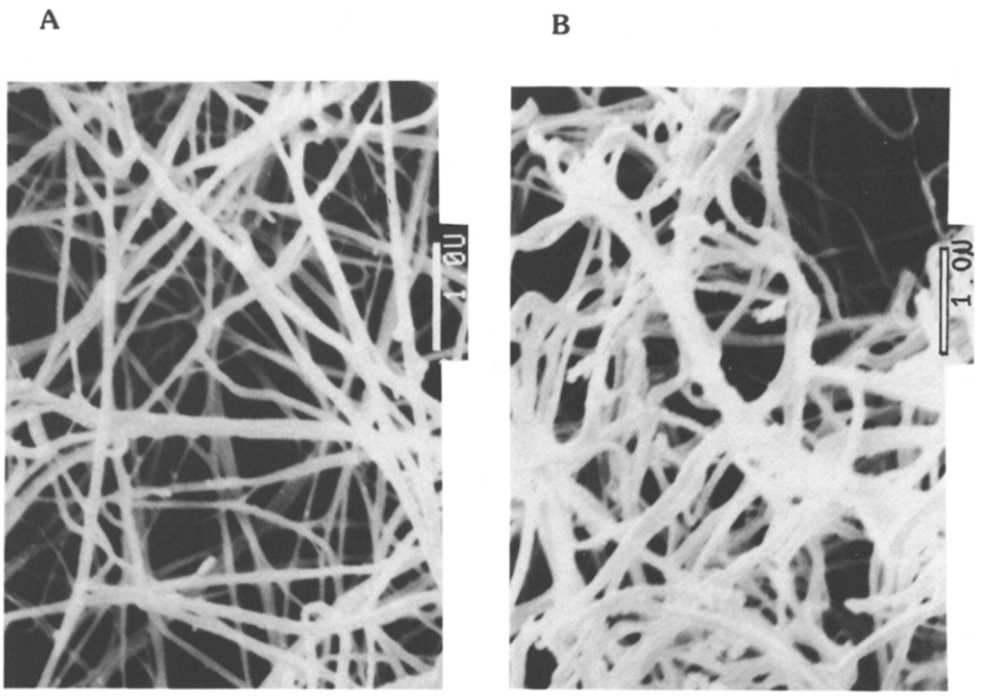

FIGURE 2

Scanning electron micrographs of native (A) and desialylated (B) canine fibrin fibrils.

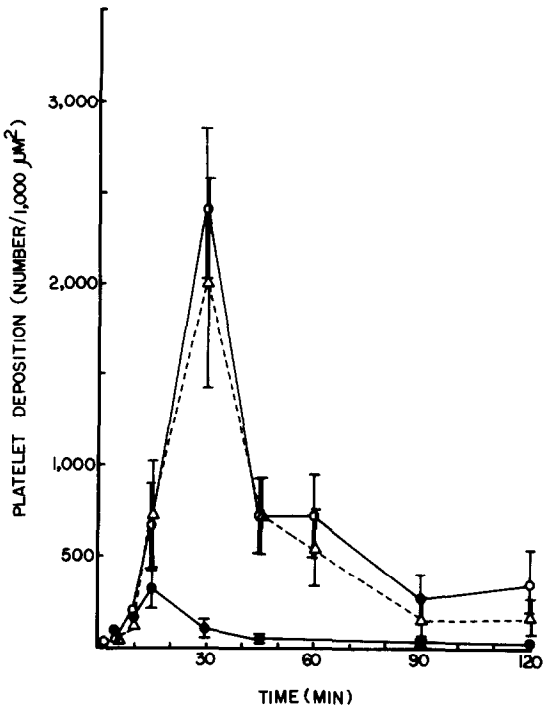

FIGURE 3

Transient platelet deposition (mean ± SEM) on PVC surfaces coated with native (Δ) (n=4) and desialylated (o) (n=9) fibrinogen. Platelet deposition on the bare polymer surface (•) (n=4) is shown for comparison.

fibrinopeptide-B of canine fibrinogen contains tyrosine residues which can be iodinated (12). Using this technique, no difference in the rate of fibrinopeptide-B release between the native and the desialylated canine fibrinogen was observed. Martinez (16) showed that the release of the fibrinopeptides A and B from native and desialylated human fibrinogen was the same, and it appears that the release of fibrinopeptide-A from canine desialylated fibrinogen is the same as that from native fibrinogen. The only differences observed between the native and desialylated fibrinogen were the shorter clotting time and the thicker fibril formation with desialylated fibrinogen.

Thrombus Formation on PVC Precoated with Desialylated Fibrinogen The ability of native and desialylated fibrinogen to aid in the attachment of platelets onto a polymer surface was evaluated using a canine ex vivo shunt experiment. When fibrinogen was precoated on the surface of PVC shunts, the same desorption profiles of both proteins after exposure to flowing blood were observed as measured by the decrease in the radioactivity of ^{125}I labeled fibrinogen (8). The transient platelet deposition on fibrinogen precoated PVC surfaces was increased by an order of magnitude over that on the bare PVC surface (Fig 3). No significant difference was observed, using Kruskal-Wallis rank test, in comparing the platelet deposition profiles on PVC precoated with native and desialylated fibrinogen. From previous studies (8), it was found that the spreading of adherent platelets on polymer surfaces is an essential step for the formation of thrombi. Thus, an examination of the change in platelet morphology, in addition to the number of platelets deposited on the surface, was necessary to accurately determine the effects of native and

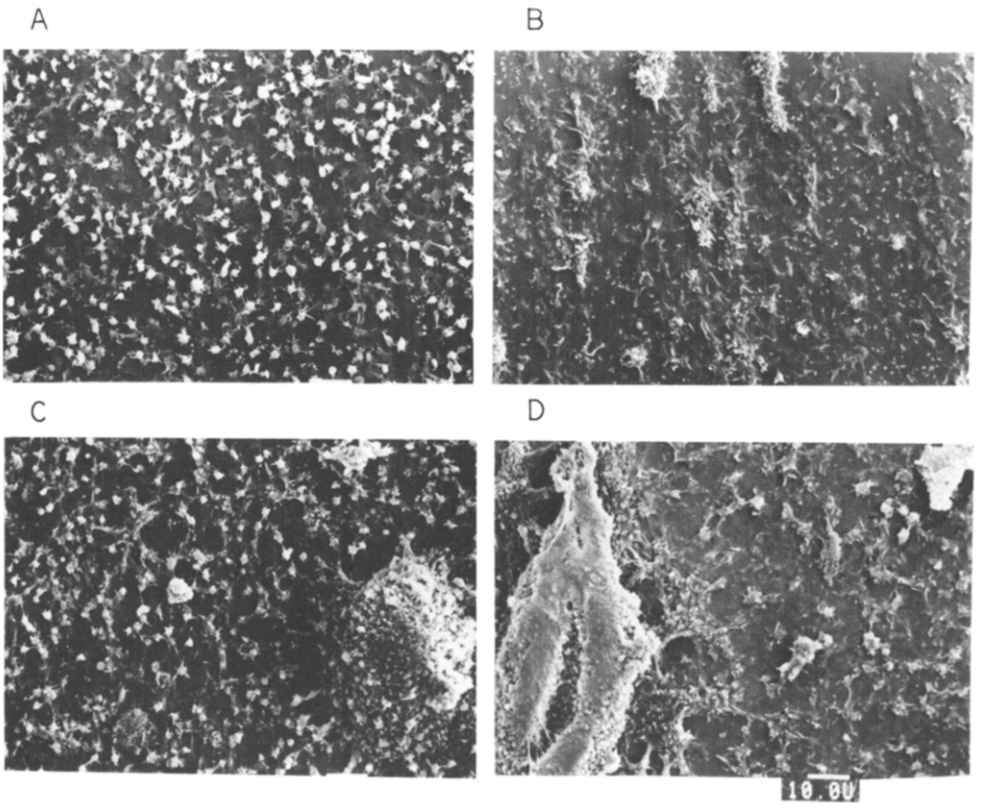

FIGURE 4

Electron micrographs of ex vivo canine platelet deposition on PVC surfaces precoated with native and desialylated canine fibrinogen. Platelet deposition after 5 min of blood exposure on PVC coated with native (A) and desialylated (B) fibrinogen. Thrombus deposition after 30 min of blood exposure on PVC coated with native (C) and desialylated (D) fibrinogen.

desialylated fibrinogen on artificial surface-induced thrombosis. At early blood exposure times (less than 10 min), the first layer of platelets on the native fibrinogen precoated surface were fully activated (Fig 4-A). On top of these spread platelets accumulated round platelets with pseudopods which were eventually activated and formed thrombi as shown in Fig 4-C. Comparison of platelet morphological changes on the surfaces coated with native (Fig 4-A) and desialylated (Fig 4-B) fibrinogen suggests that the platelet shape change occurred faster on the desialylated fibrinogen precoated surface. However, at the peak platelet deposition time (30 min of blood exposure), the native and desialylated fibrinogen supported comparable levels of thrombus on the surface. The important observations are that platelets can undergo shape change to the fully spread form on both native and desialylated fibrinogen coated surfaces and that maximum platelet deposition is statistically the same on both surfaces.

From this study, it appears that platelet adhesion is not likely to be mediated by a mechanism which involves the sialic acid residues of fibrinogen. This result is expected considering the known mechanisms of the platelet fibrinogen interaction. Among the three chains of human fibrinogen, the γ chain (γ 385-411) bears the main site recognizing the human platelet receptor induced by ADP (22). The platelet receptors are also recognized, although less avidly, by a site associated with the Aα chain (Aα 250-610) (23). Since carbohydrate is linked to Asn 52 on the γ chain (24) and Asn 364 on the Bβ chain of human fibrinogen (25), the effect of sialic acid residues on the interaction with platelets should be indirect, i.e., through conformational changes, if any, of the fibrinogen upon desialylation.

The adhesion of platelets to fibrinogen molecules which are precoated on the PVC surface occurs even in the presence of plasma fibrinogen. The binding of fibrinogen to polymer surfaces may induce conformational changes which result in a higher affinity for platelets while there is little or no interaction between platelets and fibrinogen molecules in the bulk blood phase (26). The same extent of platelet shape change and thrombus formation induced by the native and desialylated fibrinogen implies that a change in fibrinogen properties at the polymer-solution interface does not occur, or occurs to a negligible extent, upon desialylation. This study suggests that sialic acid residues of fibrinogen do not specifically influence the platelet-fibrinogen interaction.

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