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THE INFLUENCE OF PREADSORBED CANINE VON WILLEBRAND FACTOR, FIBRONECTIN AND FIBRINOGEN ON EX VIVO ARTIFICIAL SURFACE-INDUCED THROMBOSIS

L.K. Lambrecht[1], B.R. Young[1], R.E. Stafford[1], K. Park[1], R.M. Albrecht[2], D.F. Mosher[3] and S.L. Cooper[1]

Departments of Chemical Engineering[1], Pharmacy[2], and Medicine[3]
University of Wisconsin, Madison, WI, USA 53706

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ABSTRACT

We have examined the effects of preadsorption of several canine plasma proteins on surface-induced thrombogenesis in a canine ex vivo model. Our technique allowed determination of initial deposition and subsequent embolization of ^{51}Cr-labeled platelets and ^{125}I-fibrinogen onto and from polymeric arterio-venous shunts in non-anticoagulated canines. Segments of the tubing were removed at various time points between 2 and 120 minutes of blood contact for examination of the morphology of the thrombus by scanning electron microscopy. Thrombus deposition was measured on uncoated plasticized poly(vinyl chloride) (PVC) and PVC precoated with canine von Willebrand factor (vWF), fibronectin, partially purified fibrinogen (fibrinogen which contained vWF and fibronectin as impurities), or purified fibrinogen (fibrinogen which had been further purified to remove fibronectin and vWF). Preadsorption of all proteins studied enhanced the thrombogenic response relative to that of the uncoated surface. Precoating with vWF or partially purified fibrinogen resulted in the deposition of the greatest number of thrombi, and embolization was slower than on shunts precoated with canine fibronectin or purified fibrinogen. The deposition-embolization profiles for the fibronectin and purified fibrinogen-coated surfaces were similar. The amount and time sequence of initial adhesion and spreading of platelets was related to the extent and time sequence of peak thrombus formation. The partially purified fibrinogen-coated and vWF-coated surfaces had more adhered and spread platelets at the earliest time points and a greater number of larger thrombi at the peak deposition times. The slowest rate of platelet adhesion and spreading was seen on the purified fibrinogen-coated surface. White blood cells were present very early on surfaces precoated with vWF and partially purified fibrin-

Key Words: von Willebrand factor, fibronectin, fibrinogen, polymer, thrombosis

ogen, and were present prior to embolization on all surfaces.

Major conclusions from this work indicate that, although fibrinogen and fibronectin promote thrombogenesis when adsorbed to a surface, vWF is even more active in promoting platelet deposition and in anchoring thrombi to the surface of biomaterials. Thus, differences in vWF adsorption to biomaterials may be a determinant of surface-induced thrombogenesis.

INTRODUCTION

Upon contact with artificial surfaces, there is immediate adsorption of plasma proteins (1). Thus, the role of blood plasma proteins in initiation and promotion of artificial surface-induced thrombogenesis has been evaluated by a number of investigators. We have carried out experiments which allow for simultaneous ex vivo measurement of the rate of deposition and embolization of platelets and fibrin(ogen) onto and from protein precoated surfaces (2-4). We have shown that several proteins, including human fibronectin, von Willebrand factor (vWF), thrombospondin, fibrinogen, α_2-macroglobulin, and γ-globulins, enhance platelet and fibrin(ogen) deposition onto polymeric surfaces ex vivo (2-4). This enhancement has also been shown for several of these proteins in vitro (5-8). In contrast, preadsorption of albumin (3,8,9) or transferrin (unpublished data) passivates the surface. These previous studies have primarily involved the use of purified human proteins precoated onto polymeric surfaces implanted as arterio-venous (A-V) shunts in canines. Human proteins were used primarily because purification procedures for the human proteins are well developed. However, because of marked differences in surface-induced thrombogenicity among species and the indication that surface-adsorbed human proteins will support platelet deposition poorly in the dog and vice versa (10), studies using homologous proteins were required. In this report, we describe ex vivo platelet and fibrin(ogen) deposition on plasticized poly(vinyl chloride) precoated with canine fibronectin, vWF and fibrinogen and the appearance and morphology of platelets and leukocytes on these protein-coated surfaces following ex vivo exposure to flowing canine blood. We also compare the effects of preadsorbed canine proteins on thrombogenesis to our previous studies of human proteins in the canine shunt model.

METHODS

Preparation of canine plasma proteins Fibrinogen was prepared by the modified beta-alanine precipitation method of Jakobsen and Kierulf (11) using citrated canine plasma. Blood was obtained by venipuncture of the jugular vein of mongrel dogs, and plasma was prepared and fractionated immediately. Concentration of the protein solution was determined by absorbance at 280 nm (Abs of .1% solution = 1.506) (12), and aliquots were frozen in ethanol and dry ice, and stored at -70°C. Fibrinogen purified by this method was 90-96% clottable, and is referred to later as unpurified fibrinogen. Fibrinogen was then further purified by affinity chromatography on gelatin-sepharose and gel filtration to remove the fibronectin and vWF as described previously (2). This protein is referred to as purified fibrinogen.

Fibronectin was purified by affinity chromatography on a gelatin-sepharose column developed by Engvall and Ruoslahti (13). Ninety ml of frozen citrated canine plasma were usually processed. The fibronectin was elu-

ted from the column by application of 1 M sodium bromide, 0.2 M sodium acetate at pH 5.0, and then concentrated by precipitation with 35% saturated ammonium sulfate. The redissolved protein was quantified by absorbance at 280 nm (Abs of .1% solution = 1.28) (14). Yields averaged 50 mg of protein. Aliquots were frozen in dry-ice and ethanol, and stored at -70°C.

Von Willebrand factor was purified by a method developed by Johnson et al. (15). This method involves the formation of a cryoprecipitate from which vWF is separated by gel filtration. Frozen citrated canine plasma was used in each preparation to prepare the cryoprecipitate. This frozen plasma was thawed overnight at 4°C and 53% ethanol added to bring it to a 3% V/V solution. The cryoprecipitate formed was placed in a water-methanol ice bath at -2 to 5°C for 30-40 minutes, centrifuged at 0°C, and dissolved in 20 ml pH 7.4 Tris buffered saline. This solution was then centrifuged at room temp., and the supernatant applied to a 3 x 75 cm Sepharose CL-2B column with a flow rate of 30-40 ml/hr. The eluted protein fraction was then concentrated by ultrafiltration using a DiF10 PM-10 filter. Four to five milligrams of protein were recovered from 360 ml of plasma and concentrated to approximately 1 mg/ml.

The concentration of the vWF was determined by absorbance at 280 nm after correction for turbidity (Abs of .1% solution = 1.28) (15). Sodium dodecyl sulfate (SDS)/polyacrylamide slab gel electrophoresis of the final product after reduction revealed one band at a molecular weight of 190,000. Associated Factor VIII activity was quantified by a one stage clotting assay (16). In a typical preparation, the Factor VIII activities of the cryoprecipitate dissolved in 15 ml and the purified protein dissolved in 5 ml were 16-fold and 6-fold, respectively, greater than canine plasma.

Purity of the proteins was monitored by polyacrylamide gel electrophoresis in 0.1 per cent SDS by the slab gel system of Ames (17). The electropherograms of canine fibrinogen, purified fibrinogen, fibronectin, and von Willebrand factor are shown in Figure 1. Gels were stained with Coomassie brilliant blue. The following reduced size markers were analyzed alongside the protein in the slab gel: chymotrypsinogen, 24 Kd; phosphorylase b, 97.4 Kd; ovalbumin, 45 Kd; bovine albumin, 66 Kd; and fibronectin, 220 Kd. The purity of the protein fractions is summarized in Table I.

Preparation of surfaces and preadsorption of proteins Plasticized poly-(vinyl chloride) (PVC) (Tygon® R3603, Norton Plastics; 0.125 in. i.d.) was cut and used in 178 cm sections as shunts for implantation. Each tubing section was flushed with 200 ml of dilute Ivory detergent (0.125% solution) at room temperature and rinsed with 600 ml of distilled water. The tubing was then filled with Tyrode's solution (1.66 mM $CaCl_2$, 1 mM $MgCl_2$, 19 mM $NaHCO_3$, 2.7 mM KCl, 0.1% glucose and 137 mM NaCl in .36 mM sodium phosphate buffer, with osmolarity of 330 mOsm, pH 7.35) and left at 4°C overnight. Surface tension measurements of the Tyrode's solution and of the Tyrode's solution that was in contact with the tubing overnight were compared to insure that no soap remained in the tubing after rinsing. Contact angle measurements on the tubing after overnight equilibration with Tyrode's solution were performed to ensure that the surface properties were characteristic of previously used materials.

Shunts were filled with the protein solution of interest by displacing the Tyrode's solution from the shunt, thus preventing any protein-air interfacial contact. The protein solution remained in the tubing at room temperature for 2 hours before being rinsed from the tubing with 60 ml of Tyrode's solution. The tubing was implanted immediately in the test animal.

Bulk protein concentrations used for preadsorption were 0.33 mg/ml for purified fibrinogen, unpurified fibrinogen, and fibronectin, and 0.12 mg/ml

for von Willebrand factor. Separate studies using radiolabeled human proteins (3) indicated that proteins at these bulk concentrations adsorbed to the surface after 2 hr of contact with PVC resulted in surface concentrations of 0.74 μg/cm^2 for the fibrinogens and 1.2 μg/cm^2 for fibronectin. Young (18, and unpublished data) calculated that, based on the molecular dimensions of either fibrinogen or fibronectin, a surface concentration of 0.3 - 0.5 μg/cm^2 would be expected for side-on monolayer adsorption, while end-on adsorption would allow substantially higher monolayer surface concentrations. Therefore, based on the measured values of 0.74 μg/cm^2 and 1.2 μg/cm^2 for fibrinogen and fibronectin respectively, it appears that approximately a monolayer or bilayer of adsorbed protein layer was present. The surface concentration of adsorbed vWF was not measured. However, based on extensive adsorption studies using eleven different proteins (Young, unpublished data), vWF should cover the surface in at least a monolayer when adsorbed for 2 hours at a bulk concentration of 0.12 mg/ml.

Protein radiolabeling Purified canine fibrinogen was labeled with ^{125}I (New England Nuclear, Boston, MA), in 0.1M phosphate buffer (pH 7.35) using a modification of the lactoperoxidase method of Marchalonis (19) employing Enzymobeads® (Bio-Rad Lab., Richmond, CA) as recommended by the manufacturer. Ex vivo clottability of the ^{125}I-labeled protein was determined to be greater than 92%, and purity was checked by electrophoresis, followed by gel cutting and counting or by autoradiography of dried gels.

Autologous platelets were labeled with ^{51}Cr (New England Nuclear) by the method of Abrahamsen (20). The functional integrity of labeled platelets was proven by passage of whole blood, taken from the dog during the experiment, through a glass bead column using the technique developed by Salzman (21) and demonstration that the % ^{51}Cr and % platelets adhering to the column were equal.

Thrombus deposition measurements Thrombus deposition (platelets and fibrin(ogen)) from flowing blood in contact with femoral A-V shunts was measured in canine subjects as previously described (2,3). (The distinction between formed fibrin and adsorbed fibrinogen was not made in this study, and the term fibrin(ogen) is used to designate this uncertainty). The animal's blood contained ^{51}Cr-platelets (approximately 300 μCi in 2.5 x 10^{10} platelets) infused on the previous day and ^{125}I-fibrinogen (approximately 400 μCi in 0.5 mg) infused at least 30 minutes prior to the start of the experiment.

In order to detect fibrin(ogen) or platelets which deposited on the walls of the shunt, the whole blood contained within the shunt was removed. This was accomplished by clamping the artery with a vascular clamp and then flushing the blood in the shunt into the venous circulation via a small branch artery located proximal to the shunt entrance, using an aliquot (5 tubing volumes) of Tyrode's solution (22) (pH 7.35, 39°C). After flushing, radiolabeled fibrin(ogen) and platelets deposited on the shunt were detected with a sodium-iodide Tl-doped gamma detector and counted using a multichannel analyzer (Ino-Tech 5200). Following this, the blood flow was resumed. Sampling was done after 1, 2, 5, 10, 15, 30, 45, 60, 90 and 120 minutes of blood-polymer contact. During each sampling, the whole blood radioactivity (cpm/ml) was determined from a small aliquot of venous blood obtained via a jugular catheter. Although it is possible that the clamping of the artery may cause platelet activation or some thrombus formation, we feel that this produces minimal artifacts. The clamping procedure is necessary to quantitate the deposition of platelets and fibrin(ogen). Since various polymer surfaces showed significantly different profiles for platelet and fibrin(ogen) deposition, this method can confidently be used for comparison purposes.

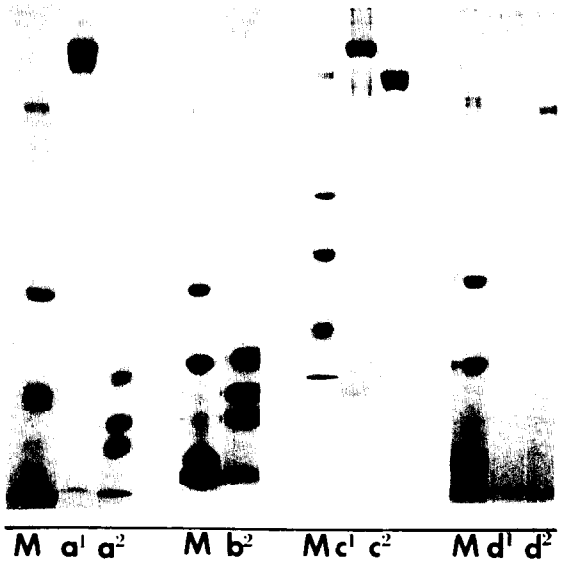

FIG. 1

Composite electropherogram of purified canine proteins. a. Partially purified fibrinogen (1-native, 2-reduced). b. Purified fibrinogen. c. Fibronectin. d. von Willebrand factor. M. molecular weight markers (fibronectin, 220 Kd; phosphorylase b. 97.4 Kd; bovine albumin, 66 Kd; ovalbumin, 45 Kd).

TABLE I

Cross Contamination of Various Canine Protein Preparations

	FGN+	FN+	vWF-VIII complex†
Purified Fibrinogen (FGN)	95%	<0.2%*	<0.01
Partially Purified Fibrinogen	90%	2.5%	20
Fibronectin (FN)	<4%*	>85%	<0.02
von Willebrand factor (vWF)	<3%*	<3%*	600

+ Determined by scanning densitometry. Estimated and presented as percent by weight.

† Associated Factor VIII activity (16) for protein of interest at a concentration of 1 mg/ml. Reported as percent of normal canine plasma activity (normal canine plasma activity is 25X normal human plasma activity).

* Less than the detectable limit.

The surface concentration of adherent platelets (number per 1000 μm^2) or fibrin(ogen) (μg per cm^2) was determined as reported previously (23). Averaged data for the same time point for different experiments were reported in platelets/1000 μm^2 or $\mu g/cm^2$ ± standard error from the mean (SEM).

Animal preparation and hematology Adult mongrel dogs weighing 20-35 kg were selected for this study following screening to be certain that the experimental animals had the following: Normal platelet aggregability (24) to ADP and epinephrine, platelet count of 150,000-400,000/µL, white blood cell (WBC) count of 8-17 x 10^3/µl, fibrinogen levels of 100-300 mg/dl, hematocrit of 35--50%, euglobulin lysis time greater than 1-1/2 hrs, and activated partial thromboplastin times (PTT) between 15 and 22 seconds. Each animal was fasted overnight prior to surgery, then anesthetized by intravenous thiamylal sodium (Surital®, Parke-Davis). Initial dosage was 20 mg/kg body weight followed by amounts appropriate to maintain anesthesia. During each sampling for radioactivity, a blood sample was taken from the jugular vein via a catheter and analyzed for platelet count, fibrinogen level, hematocrit, activated PTT, and euglobulin lysis time. Platelet aggregability to ADP and epinephrine was monitored at the start and end of each experiment.

Electron microscopy At each time point of each experiment, a segment of the shunt (1.5 cm) was removed distal to the section sampled for radioactivity, fixed for 24 hrs. in 2.0% glutaraldehyde, and then dehydrated in increasing concentrations of ethanol. Segments were dried by the critical point procedure using ethanol as the intermediate fluid and liquid CO_2 as the transitional fluid. Specimens were then evaporatively coated with carbon and either evaporatively or sputter-coated with gold palladium for scanning electron microscopy. The micrographs presented are each taken from a different animal experiment and are representative of the events as viewed in three fields per time point.

Statistical analysis Values for p, the probability of the difference between the normal distributions being due to chance, were obtained from comparison of the peak values for platelets (number per 1000 μm^2) or fibrin(ogen) ($\mu g/cm^2$) for canine or human (2,3) precoated proteins as evaluated by the Students t test. If $p < 0.05$, the difference between two samples should be regarded as significant. As p increases, the probability that two samples are different due to chance only increases.

RESULTS

Thrombus deposition on uncoated PVC and PVC precoated with canine fibronectin, fibrinogen and von Willebrand factor Platelets and fibrin(ogen) deposition profiles on uncoated PVC and PVC precoated with canine proteins are shown in Figure 2. On the uncoated surface, a distinct peak for both platelet and fibrin(ogen) deposition occurred at about 15 minutes of blood contact time, which was followed by a large decrease to near base-line values by 60 minutes of blood contact (23). In comparison, when albumin is precoated onto PVC, maximum platelet deposition is less than 60 platelets/1000 μm^2 and occurs 5 minutes after initial blood exposure (3).

The most active surface studied was PVC precoated with partially purified fibrinogen. The amounts of platelets and fibrin(ogen) deposited on this surface at the peak deposition times were 10-fold and 20-fold greater, respectively, than the amounts deposited on the uncoated surface. Platelet deposition on the surface coated with partiallly purified fibrinogen reached a

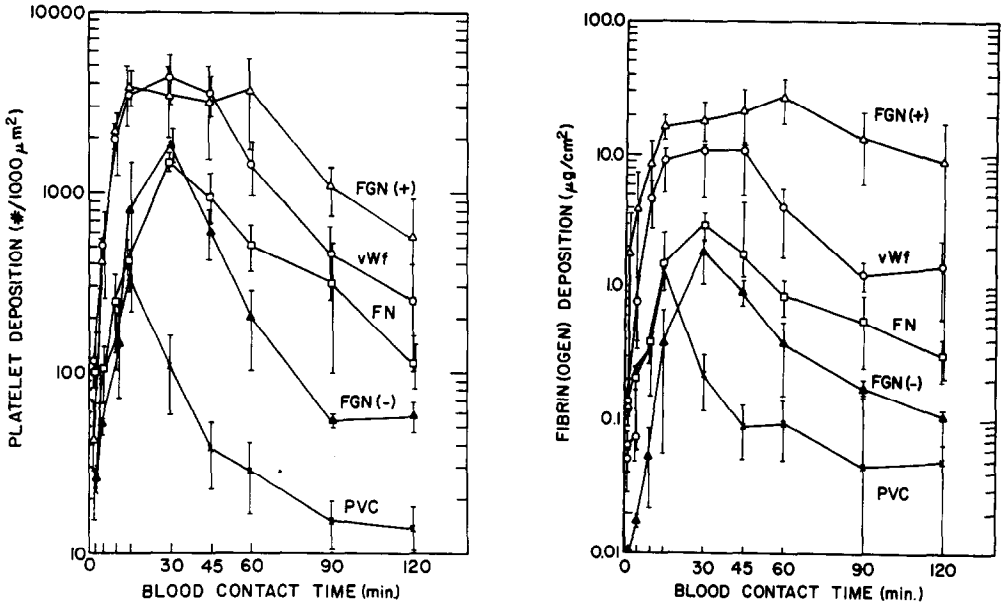

FIG. 2

Transient platelet and fibrin(ogen) deposition on PVC precoated with purified canine proteins. Platelet (a) and fibrin(ogen) (b) deposition (±SEM) on uncoated PVC, X (N=9) and PVC precoated with von Willebrand factor (vWF), O (N=4), fibronectin (FN), [] (N=5) and partially purified fibrinogen (fibrinogen containing vWF and FN) (FGN+), Δ (N=4), or purified fibrinogen (fibrinogen without vWF and FN) (FGN-), ▲ (N=2).

peak at 15 minutes of blood contact, and the level of platelet deposition was maintained until 60 minutes of blood contact.

The maximum platelet deposition on the vWF-coated PVC surface was about the same as that on the surface coated with partially purified fibrinogen, but embolization occurred faster. The platelet level at the peak deposition time of 30-45 minutes was over 10-fold greater than the peak level observed on the uncoated surface, three times greater than the peak level observed on fibronectin-coated PVC, and slightly greater than peak platelet deposition level observed on the partially purified fibrinogen-coated surface.

On the fibronectin-coated surface, platelet deposition reached a maximum later than on the uncoated PVC surface (30 minutes of blood contact as compared to 15 minutes), and the peak platelet deposition value was over five times greater. Platelet deposition on the surface coated with purified fibrinogen was similar to that on the fibronectin-coated surface. When the desorption of precoated proteins was monitored using radiolabeled protein, it was found that protein was released immediately after blood contact. The desorption continued until the maximum was reached in platelet deposition. This

desorption may be due to either displacement by plasma proteins or by removal assisted by protein attachment to platelet thrombi. It still remains to be seen which mechanism dominates protein desorption.

Fibrin(ogen) deposition on PVC and PVC precoated with various proteins paralleled the platelet deposition profiles. This result implies that fibrin(ogen) deposition may represent either plasma fibrin(ogen) molecules or fibrin strands nonspecifically trapped within the platelet thrombi or specifically adhering to platelet surfaces. However, both mechanisms may be important. The first possibility of purely nonspecifically trapped plasma fibrinogen may be questioned by the observation that the deposition of ^{125}I labeled canine serum albumin does not parallel that of platelet deposition (data not shown). This observation is significant because albumin does not have binding sites on platelet membranes. Deposition of albumin which parallels platelet deposition is expected if protein adsorption is due to nonspecific entrapment. Thus, it appears that at least part of the fibrin(ogen) adsorption measured in this study was due to specific adsorption. It is still not clear whether fibrin(ogen) deposited on the surface is a result of fibrin formation or specific fibrinogen adsorption to the platelet surfaces or both. This question might be resolved by measuring fibrinopeptide-A which is released within the *ex vivo* shunt.

Morphology of thrombi Scanning electron micrographs of uncoated PVC at 5 minutes (Fig. 3A) and 10 minutes (Fig. 3B) of blood exposure showed well-spread platelets and associated rounded platelets with pseudopods extended. By 15 minutes (Fig. 3C), platelet deposition increased substantially, forming a basal layer of completely spread platelets (arrows in the figure) which were sparsely covered with single rounded platelets showing initial stages of pseudopod formation. Figure 3C shows that at 15 minutes there were numerous moderately sized platelet thrombi (20 μm) and white blood cells (WBC, arrow heads in the figure) present on the PVC surface. By 60-120 minutes of blood exposure, large platelet aggregates were no longer present, and platelet density was similar to that at 2-5 minutes. By 120 minutes, only a uniform layer of single platelets and occasional white blood cells remained (Fig. 3D). Platelet pseudopod extension and the number of spread platelets were less at 60 or 120 minutes than that at 5 minutes.

Micrographs of the surfaces coated with partially purified fibrinogen (Fig. 4) and vWF (Fig. 5) showed similar sequences of thromboembolic events. Platelets adhered and spread, and this amorphous, spread layer served as sites for attachment of new platelets by 1-2 minutes of blood contact (Fig. 5A). Numerous white blood cells were also observed to adhere to this activated surface and were spreading by 5-10 minutes of blood contact (Fig. 4A, 5B). The resulting pattern of thrombus formation on these surfaces by 15 minutes was a lacy, interwoven network of deposited thrombi (Fig. 4B). At high magnification (Fig. 4C), it was evident that at the base of these thrombi were spread cells, above which platelets were uniformly deposited to form regular platelet aggregates.

By the peak deposition times of 60 minutes for the partially purified fibrinogen surface or 30 minutes of blood contact for the vWF surface, the clumps of thrombus material were extremely large, and extended out from the surface (Fig. 5C). In some areas, these larger thrombi were enmeshed in a fibrin-red blood cell (RBC) clot (Fig. 5D), and even more extensive WBC involvement is evident (Fig. 4D). By 90 minutes, most of this material had embolized from the surface, leaving behind a surface partially covered with platelets and a few WBC which was similar to the fibronectin-coated surface at this time (see below).

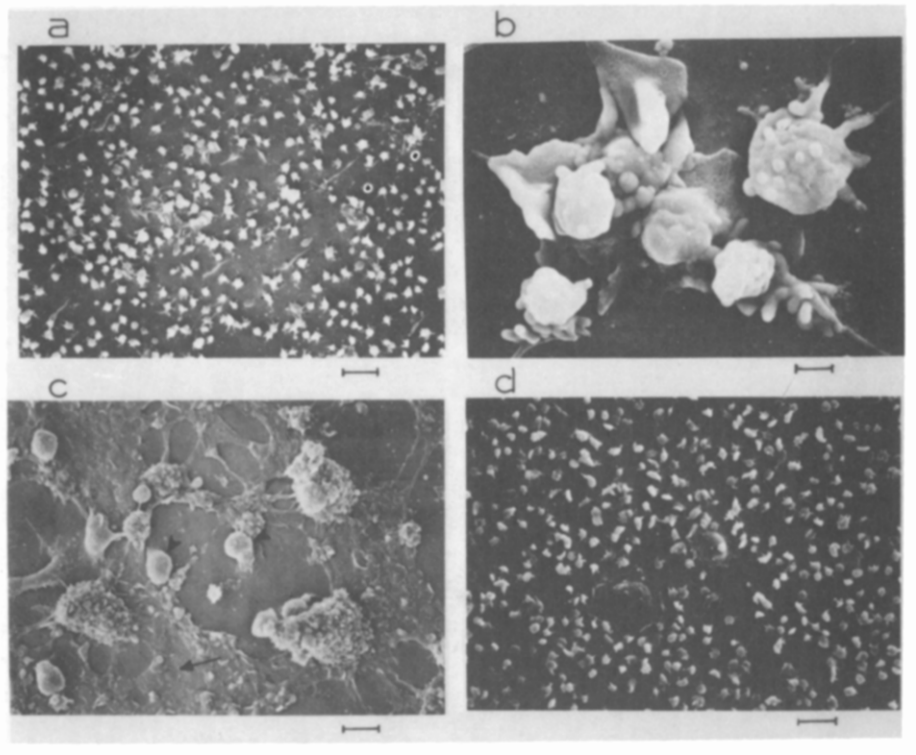

FIG. 3

Scanning electron micrographs of uncoated PVC surface. a. 5 min blood contact. Bar = 10 μm. b. 10 min blood contact, higher magnification. Bar = 1 μm. c. 15 min blood contact. Bar = 10 μm. d. 120 min blood contact. Bar = 10 μm.

Micrographs of the fibronectin-coated surfaces showed a layer of numerous well-spread and flattened platelets by 10 minutes of blood contact. Single platelets with extended pseudopods and small platelet aggregates deposited on top of these spread platelets were also observed. By 15 minutes, these spread platelets had formed a uniform amorphous mass which served as a base for newly deposited, more rounded platelets and fibrin strands (Fig. 6A). At higher magnification, platelets attached to the fibrin strands were observed (Fig. 6B). By 30 minutes, numerous large platelet thrombi and adhered WBC were noted. A typical massive platelet thrombus, with associated WBC, is seen in the process of embolizing from the surface (Fig. 6C) at 45 minutes of blood contact. Even at 90 minutes, there are still medium-sized platelet thrombi with numerous WBC (which also are in the process of spreading) associated with them (Fig. 6D).

Electron micrographs of the canine purified fibrinogen surface (not shown were similar to those for the uncoated surface, but the sequence of thrombogenic events was delayed. From 2 to 5 minutes of blood contact, there was deposition of a moderate number of platelets with extended dendritic pseudopods, but few fully spread platelets were observed until the 10 to 15 minute time points. The morphology of the medium-sized platelet thrombi

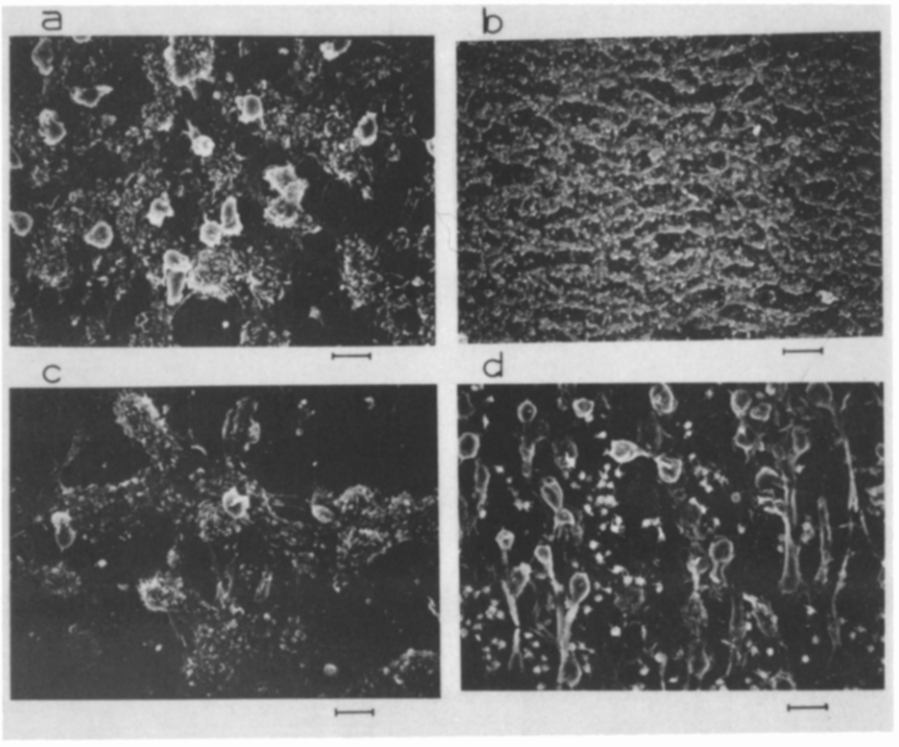

FIG. 4

Scanning electron micrographs of canine partially purified fibrinogen-coated PVC. a. 10 min blood contact. Bar = 10 μm. b. 15 min blood contact. Bar = 100 μm. c. Higher magnification of b. Bar = 10 μm. d. 60 min blood contact. Bar = 10 μm.

with associated WBC at the peak time of 30 minutes was very similar to the thrombi observed on the uncoated surface at 15 minutes of blood contact.

Thrombus deposition on PVC precoated with canine or human fibronectin, fibrinogen and von Willebrand factor Table II summarizes the peak fibrin(ogen) and platelet deposition values for uncoated PVC and PVC precoated with canine proteins and compares the results with similar precoated protein data for human proteins (2,3).

When compared to deposition onto PVC coated with canine protein (described above), platelet and fibrin(ogen) deposition on partially purified fibrinogen of human origin reached a lower peak at 30 minutes, decreased steadily out to 120 minutes of blood contact, and was not biphasic (3). The peak level of fibrin(ogen) deposition on a partially purified fibrinogen coating of canine origin was over 4 times greater than that observed on a partially purified fibrinogen coating of human origin ($p < 0.05$, Table II). Platelet and fibrin(ogen) deposition on canine vWF-coated PVC at the peak deposition time of 30 min blood contact were 2-fold and 3-fold greater, respectively, than on human vWF-coated PVC (3). The shapes of the two deposition-embolization profiles were similar. For surfaces precoated with human (2,3)

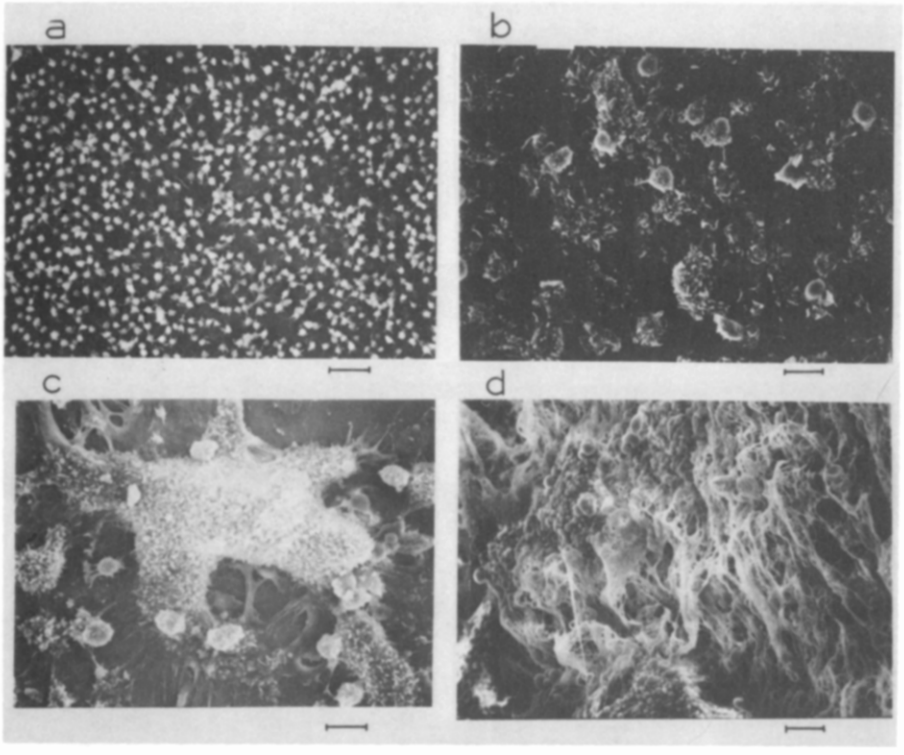

FIG. 5

Scanning electron micrographs of canine vWF-coated PVC surface. a. 2 min blood contact. Bar = 10 μm. b. 5 min blood contact. Bar = 10 μm. c and d. 30 min blood contact. Bar = 10 μm.

or canine fibronectin, platelet and fibrin(ogen) deposition were nearly identical. Platelet deposition was similarly increased over the uncoated surface for both human and canine precoated purified fibrinogen, but peak deposition occurred later on the surface precoated with purified fibrinogen of canine origin. Fibrin(ogen) deposition on the human purified fibrinogen surface was 5 times greater than on the canine protein-coated PVC surface ($p < .05$, Table II). Deposition values for both purified fibrinogen precoated surfaces were substantially lower than on surfaces precoated with partially purified fibrinogen.

Hematology In all experiments, plasma fibrinogen levels and packed cell volumes remained unchanged. In animals cannulated with the uncoated PVC shunt, platelet levels fell an average of 30%, and WBC levels increased by about 60% during the 4 hours of the experiment. In the fibronectin-coated or fibrinogen-coated (purified or partially purified) PVC shunts, platelet levels fell an average of 15%, and WBC levels increased by about 48%. The vWF/PVC surface showed a similar decrease in platelet levels (25%), but showed a marked increase in WBC levels, with average increases of 40% in the WBC counts by the 10 minute time point, and an increase of 150% by the end of the experiment. With all of the surfaces tested, the euglobulin lysis times, PTT

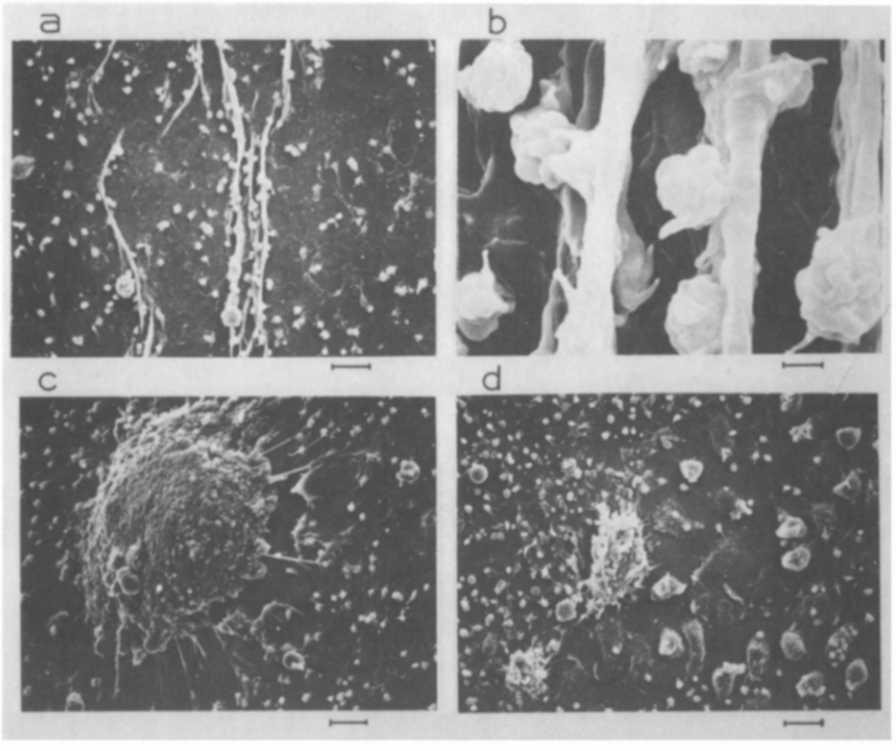

FIG. 6

Scanning electron micrographs of canine fibronectin-coated PVC surface. a. 15 min. blood contact. Bar = 10 μm. b. Higher magnification of a. Bar = 1 μm. c. 45 min blood contact. Bar = 10 μm. d. 90 min blood contact. Bar = 10 μm.

and prothrombin times remained unchanged during the entire time, indicating that there was no enhancement of systemic fibrinolytic activity, or large scale disruption of the intrinsic or extrinsic clotting systems. ADP-induced platelet aggregability also remained unchanged during each run, indicating that platelet aggregation remained normal throughout the course of the experiment.

DISCUSSION

Previous work in this laboratory using an ex vivo canine arterio-venous shunt model has shown that artificial surface-induced thrombogenesis is dependent upon the chemical nature of the surface being evaluated (23,25-27) and the composition of the initially adsorbed protein layer (2-4,28). In addition, ex vivo adsorption of plasma proteins was shown to be dependent on the chemical nature of the surface (29). Scanning electron microscopy studies (30-32) have correlated early changes in platelet morphology with the eventual rate and extent of thromboembolism and have also implicated possible procoagulant and fibrinolytic activities from the adhered WBC. In the pre-

TABLE II

Peak platelet and fibrin(ogen) deposition on PVC precoated with canine or human+ fibronectin, fibrinogen, or von Willebrand factor†.

Surface	Platelet Peak				Fibrin(ogen) Peak			
	n	time	Amt(#/1000 μm^2) (± SEM)	P*	n	time	Amt(ug/cm^2) (± SEM)	P
cFGN (partially purified)	4	60 min	3579 ± 1861		4	60 min	27.0 ± 9.73	
				<.4				<.05
hFGN (partially purified)	7	30 min	2070 ± 1104		7	30 min	6.45 ± 3.67	
cvWF	4	45 min	3470 ± 907		4	30 min	10.91 ± 7.2	
				<.3				<.1
hvWF	5	30 min	2100 ± 574		5	30 min	3.37 ± .54	
cFN	4	30 min	1754 ± 419		4	30 min	2.90 ± 6.64	
				<.9				<.6
hFN	4	30 min	1596 ± 443		4	30 min	3.94 ± .84	
cFGN (purified)	3	30 min	1814 ± 412		3	30 min	1.90 ± .54	
				<1.0				<.05
hFGN (purified)	4	15 min	1776 ± 1052		4	15 min	11.60 ± 4.71	
Uncoated PVC	9	15 min	332 ± 116		9	15 min	1.33 ± .998	

+ Data for human proteins were reported in Ref. #3, studies were performed as separate experiments.

† Abbreviations: c, canine; h, human; FGN, fibrinogen; FN, fibronectin.

* Values for p, probability of the normal distribution were obtained from analysis of human vs. canine peak platelet and fibrin(ogen) deposition values as evaluated by the Students t test.

sent study, the ex vivo thrombogenic response of plasma proteins preadsorbed to plasticized PVC tubing has been studied in depth in a completely homologous system.

Comparison of the thrombogenic response of canine and human vWF showed that platelet and fibrin(ogen) deposition on canine vWF-coated PVC were 2-3 times greater at the peak deposition time when compared to human vWF-coated PVC. In contrast, human and canine fibronectin had the same thrombogenic activity. Thus, the structures of canine and human vWF must be different enough to result in the functional difference detected in the ex vivo model described here. This finding agrees with the studies of Johnson and Dodds (33), and Johnson et al. (15), who found that purified human vWF did not support ristocetin-induced aggregation of fresh washed canine platelets, whereas canine vWF did. It has been found that the one-dimensional tryptic "maps" generated by sequential tryptic digestion of canine and human fibronectin are very similar (D. F. Mosher and R. B. Johnson, unpublished data). Thus, the functional domains (34) of the two fibronectins are probably well conserved. The reason for the remarkable differences observed in platelet and

fibrin(ogen) deposition on PVC precoated with purified fibrinogen from canine and human species is not clear. Although size differences in the Aα chains of human and canine fibrinogen were observed by polyacrylamide gel electrophoresis (35), further functional tests are needed. However, such tests are beyond the scope of the present investigation.

By scanning electron microscopy, the sequence of events observed on all surfaces studied involved initial adherence of platelets, pseudopod extension, and spreading of the platelet with extension of the hyalomere (36,37). Following spreading, activated platelets presumably release α-granule contents (including fibrinogen, fibronectin, vWF, thrombospondin and platelet factor 4) (34,38) and dense granule contents (including ADP and serotonin) (21), which together result in the enhanced adhesion and aggregation of more platelets and the formation of large thrombi. On all of the surfaces studied, it appeared that the initial amount and time sequence of platelet spreading was directly related to the extent and time sequence of peak thrombus formation. Variations in the time required for platelet adhesion and aggregation, and differences in the eventual size and morphology of formed thrombi were related to the protein which had been precoated on the surface. The canine vWF-coated surface had the greatest number of adhered and spread platelets at the earliest time points (1-2 minutes of blood contact) and a greater number of larger thrombi at the peak deposition time. Although the effects of precoated protein layers are clearly shown, caution is necessary to extrapolate this data to clinical situations. It is certain that when a polymer surface contacts blood, a complex mixture of plasma proteins competitively adsorbs in concentrations that likely change with time.

The presence of precoated vWF seemed to promote adherence of WBC as well as platelets. It is not known whether this resulted from adherence of WBC to adsorbed vWF or chemotactic and/or adhesive responses of the WBC to molecule(s) released from activated platelets. In a previous study, it was noted that early deposition of monocytes occurred on surfaces precoated with γ-globulin and we hypothesized that this resulted from adherence via Fc receptors (31). WBC were seen at later time points in thrombi which developed on uncoated PVC and PVC precoated with fibrinogen or fibronectin. The presence of WBC is potentially very significant because these cells are capable of initiating fibrin formation (39) and fibrinolysis (42) and thus may both amplify thrombus formation and cause embolization of thrombi.

The fact that preadsorbed vWF was thrombogenic is in accordance with extensive studies of the hemostatic defect in von Willebrand Disease. Platelets of these patients show disturbances in adhesion to glass beads (21) or deendothelialized blood vessels (41), indicating a role of vWF in platelet adhesion. In studies by Weiss et al. (41), platelet adhesion in five patients with von Willebrand disease was decreased in an *in vitro* test chamber, and the magnitude of the defect was strongly dependent on shear rate. At a shear rate of 1300 sec^{-1}, adhesion was 75% less than in normal subjects, while at 800 sec^{-1}, the reduction in adhesion was 29%. In a flat perfusion chamber, Houdijk et al. (42) showed that FVIII-vWF and fibronectin were both necessary for platelet deposition onto collagen, and that FVIII-vWF was more important at high shear rates. In studies with normal subjects, platelet adhesion and thrombus formation on artificial (43) and vascular (44) surfaces have been shown to increase significantly with increasing shear rates. The thrombi which formed at high flow rates consisted primarily of platelets, as was seen here *ex vivo*. The shear rates in the present study were in the range of 960 sec^{-1} to 1600 sec^{-1}, and thus the effect of vWF on thrombogenesis (here and in arterial flow conditions) may be expected to be large.

Fibronectin binds and cross-links to fibrin through the α-chain of fibrin (45). Interestingly, fibronectin was the only protein coating on which

we were able to observe clearly identifiable strands of fibrin. In studies by Young et al. (28) using the same canine model, fibronectin-coated PVC was shown to have an unusually high fibrin(ogen)/platelet ratio at early time points, suggesting that fibrinogen or fibrin is rapidly absorbing or binding to the precoated fibronectin-layer. Other investigators have demonstrated platelet receptor sites for fibronectin in solution (46,47), and fibronectin has been shown to enhance the spreading of platelets on collagen-coated and fibrin-coated surfaces (34). Studies by Houdijk et al. (42) suggest that fibronectin functions as the adhesive system for platelets at low shear rates, whereas vWf is the active adhesive protein at high shear rates. However, the present studies indicate that fibronectin also has an effect at high shear rates.

A surprising observation in this present study was the relatively minimal thrombogenic response observed when purified canine fibrinogen (which had been purified further to remove fibronectin and vWF) was preadsorbed onto the PVC surface, although fibrinogen has been found to mediate platelet adhesion to glass surfaces in static systems (5,8,48). The more active thrombogenic response of the partially purified fibrinogen is thought to result from contaminating FVIII-vWF (Table 1). Platelet receptors for fibrinogen are only exposed when platelets are stimulated (49-51). Fibrinogen is required for ADP-induced platelet aggregation, suggesting a role for fibrinogen in platelet aggregation. In studies in which ^{125}I fibrinogen deposition was measured on polymeric surfaces in an ex vivo canine series shunt model (25,32), it was noted that there was an initial high level of fibrinogen deposition after 30 seconds of blood contact, a decrease from 30 seconds to 2 minutes, and a secondary rise after 2 minutes. This secondary rise in fibrinogen deposition followed platelet deposition in all cases. It appears that initial adsorption of fibrinogen or fibrin is followed by desorption and exchange with other proteins, or possibly rapid dissolution due to the canine's active fibrinolytic mechanisms (52). The subsequent fibrinogen deposition is associated with platelet deposition and aggregation. In previous work by us, analysis of the ratio of fibrinogen to platelets under a variety of conditions suggests that much, if not all, of the fibrinogen may be bound to fibrinogen receptors of surface-activated platelets (28). Fibrinogen binding to surface activated platelets has been followed quite closely by Loftus and Albrecht, using scanning electron microscopy in conjunction with gold bead labels (53). As discussed above, only on fibronectin-coated surfaces were fibrin strands detected. However, other studies utilizing an arterio-venous shunt in rhesus monkeys showed early and substantial formation of fibrin strands (54).

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