Cells and Materials

Volume 1 | Number 1 Article 8

1991

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CELLS & MATERIALS, Vol. 1, No. 1, 1991 (Pages 73-82) 1051-6794/91$3.00+.00
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ANALYSIS OF PROTEIN ADSORPTION ON RETRIEVED HUMAN VASCULAR GRAFTS USING IMMUNOGOLD LABELLING WITH SILVER ENHANCEMENT

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(Received for publication March 9, 1990, and in revised form September 28, 1990)

Abstract

The adsorption of proteins on biomedical materials such as vascular grafts is important in modulating thrombosis, one eventual cause of vascular graft failure. We have identified proteins expressed on the surfaces of retrieved vascular grafts to determine if certain proteins were present at the end stage of vascular failure. Scanning electron microscopic analysis of protein adsorption on the surfaces of retrieved vascular prostheses was determined using antibodies to human blood proteins fibrinogen, fibronectin, Hageman factor (factor XII) and factor VIII/von Willebrand Factor. The detection of these proteins on the blood contacting surface was evaluated by immunogold labelling with protein A-gold beads followed by silver enhancement. Fibronectin was the most abundant protein detected on retrieved expanded polytetrafluoroethylene or umbilical vein grafts. Protein adsorption was disperse for all proteins and one protein, fibronectin, was found in great amounts on all surfaces. Proteins such as fibronectin may be important in the adhesion and activation of platelets and leukocytes and thus contribute to the development of thrombosis. These analyses of human vascular grafts indicate that surface proteins can be readily detected by our methods and thus may be useful in determination of specific proteins important in the thrombogenicity and/or biocompatibility of cardiovascular devices.

KEY WORDS: Biomaterials, Biocompatibility, Protein Adsorption, Vascular Grafts, Thrombosis, Immunogold Labelling, Silver Enhancement.

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Introduction

Replacement of diseased arteries with prosthetic vascular grafts of biological origin or synthetic substitutes is common in vascular reconstructive surgery. However, when small diameter vessels (< 6 mm) are replaced with prosthetic devices such as femoral-popliteal arteries, success rates are variable [34]. The most common problems associated with these prosthetic devices are thrombosis, thromboembolism and infection [13, 32]. Although exact mechanisms for failure are uncertain, it is clear that when prosthetic materials are implanted and exposed to circulating blood, one of the initial events to occur is the adsorption of proteins from the blood onto the surface of the graft [2, 3, 7, 35, 36]. Following initial protein adsorption, the success or failure of the implant is determined by continued deposition of cells and matrix molecules. This deposition eventually constitutes the pseudointima (neointima in animals); a layer of tissue extending from the luminal surface of the graft material to the blood contacting surface [32, 37].

Previous studies performed in our laboratory have determined the presence of various blood and tissue components on retrieved Dacron and expanded polytetrafluoroethylene (ePTFE) human vascular grafts [1]. In other studies, we showed, using an in-vitro artificial recirculation system, that protein adsorption occurs on biomaterials after exposure to flowing blood as early as one minute and that the composition and characteristics of the protein layer at the blood contacting surfaces changed with time [39]. Therefore, the goal of this study was to determine the surface protein composition of vascular grafts retrieved from patients after prolonged implantation. We hypothesize that proteins involved in blood coagulation and cell adhesion may be present not only early but also at later stages of graft failure.

Many methods to determine protein adsorption have been described and include direct protein iodination, gold labelling of purified proteins with visualization of gold beads using scanning electron microscopy

(SEM), antibody iodination, or enzyme linked immunoassays (ELISA) [4, 6-10, 11, 12, 17, 19, 20, 24, 26-27, 29, 31, 35]. We have demonstrated that other methods may be applicable to the determination of protein adsorption such as radioimmunoassay or immunogold labelling with silver enhancement [25, 39]. The advantages of our methods are first, the ability to detect proteins adsorbed from a complex fluid (such as blood) to the contacting surface and second, the detection of virtually any protein provided an antibody to the protein of interest is available. Pasquinelli et al. [28] have used immunogold labelling with silver enhancement on tissue sections of deendothelialized blood vessels but have used light microscopy to evaluate protein deposition.

Materials and Methods

Human, Explanted, Prosthetic Vascular Grafts

Prosthetic vascular grafts were retrieved from patients subsequent to graft failure and frozen at 0°C. The grafts were shipped to the Institute of Pathology, Case Western Reserve University, on dry ice and stored at -70°C until use. The grafts were composed of ePTFE or human umbilical vein (HUV). Our choice of materials was limited to the availability of grafts. The grafts were cut into 0.5 cm segments and processed for protein labelling.

Protein Labelling Procedure

Our methods for protein labelling have been described elsewhere [25] and are a modification of previously described techniques [14-16, 24, 27-29, 33]. Each graft was cut into 0.5 cm segments, bisected longitudinally, and fixed for 7 minutes in a solution containing 0.1% glutaraldehyde (Grade 1, Sigma Chemicals, St. Louis, MO) and 2% formaldehyde. The segments were rinsed three times in phosphate buffered saline (PBS) followed by neutralization of residual glutaraldehyde with 50 mM glycine for 20 minutes at room temperature. The samples were then rinsed again in PBS. Non-specific protein binding sites on the polymers were blocked using 15% nonfat dry milk, in PBS, containing 2 mM sodium EDTA. Background adsorption was further reduced by blocking endogenous Fc receptors or adsorbed plasma immunoglobulins with 0.02 mg/ml of unlabeled protein A from S. aureus, Cowan strain (Sigma, St. Louis, MO) added to the milk solution above. The samples were incubated in this solution for 1 hour at 37°C. The segments were then rinsed in PBS (three times) and incubated with one of the following primary polyclonal antibodies (1:500 dilution in PBS containing 1% ovalbumin) for 1 hour at 37°C: Rabbit antihuman fibronectin (IgG fraction, Organon Technika, Cochranville, PA) and fibrinogen, Hageman factor (factor XII), and factor VIII/vWF antibodies (IgG fractions) prepared from crude antisera by octanoic acid precipitation [39].

Control studies were performed in which the primary antibody was substituted with 1% ovalbumin (in PBS), or antiherpes virus, or anti-alpha-fetoprotein

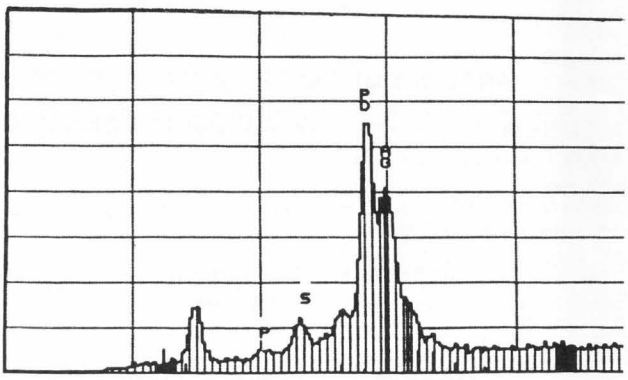


Figure 1. EDX analysis of the surface of a retrieved ePTFE graft stained for fibrinogen using immunogold labelling with silver enhancement at 0.02 keV. AG = silver, PD = palladium.

(Dako Corp., Santa Barbara, CA). Herpes virus and alpha-fetoprotein would not be expected to be found in normal adult blood. The initial (stock) concentration of antibodies was 10mg/ml and the specificity of each antibody was tested by a "checkerboard" analysis in which each antibody was reacted with polymers adsorbed with protein from pure solutions of each protein of interest at 37°C for 1 hour. Antifibrinogen had low cross reactivity with fibronectin but the other antibodies had no significant cross-reaction. The samples were again rinsed in PBS (three times) and then fixed overnight in 2.5% glutaraldehyde at 4°C. The next day, the samples were placed in clean glass test tubes, rinsed in PBS, and then treated with 50 mM glycine as above to neutralize glutaraldehyde. The samples were incubated with 1:100 dilution of protein A-gold complex using 10 nm diameter gold beads (Janssen, Piscataway, NJ) for 1 hour at 37°C. The samples were rinsed in PBS, and then in distilled water, followed by silver enhancement (IntenSE BL, Janssen, Piscataway, NJ) according to the manufacturer's directions for light microscopy. The samples were rinsed in distilled water, then dehydrated in increasing ethanol concentrations (30, 70, and 100%) for 5 minutes each. This was followed by critical point drying, using CO_2 (Balzers Union, Hudson, NH), after which each sample was mounted on aluminum stubs and sputter coated with palladium or gold-palladium. All specimens were examined in the secondary electron mode using a JEOL model 840A scanning electron microscope set at 15-25 keV accelerating voltage. The average working distance used was approximately 22 mm. Silver enhanced gold beads were easily identified at magnifications greater than x4000 with the bead size near 100 nm in diameter. The composition of the observed beads on the surfaces of the specimens was confirmed to be silver by energy dispersive x-ray analysis (EDX). Photomicrographs from these explanted grafts were analyzed for bead distribution.

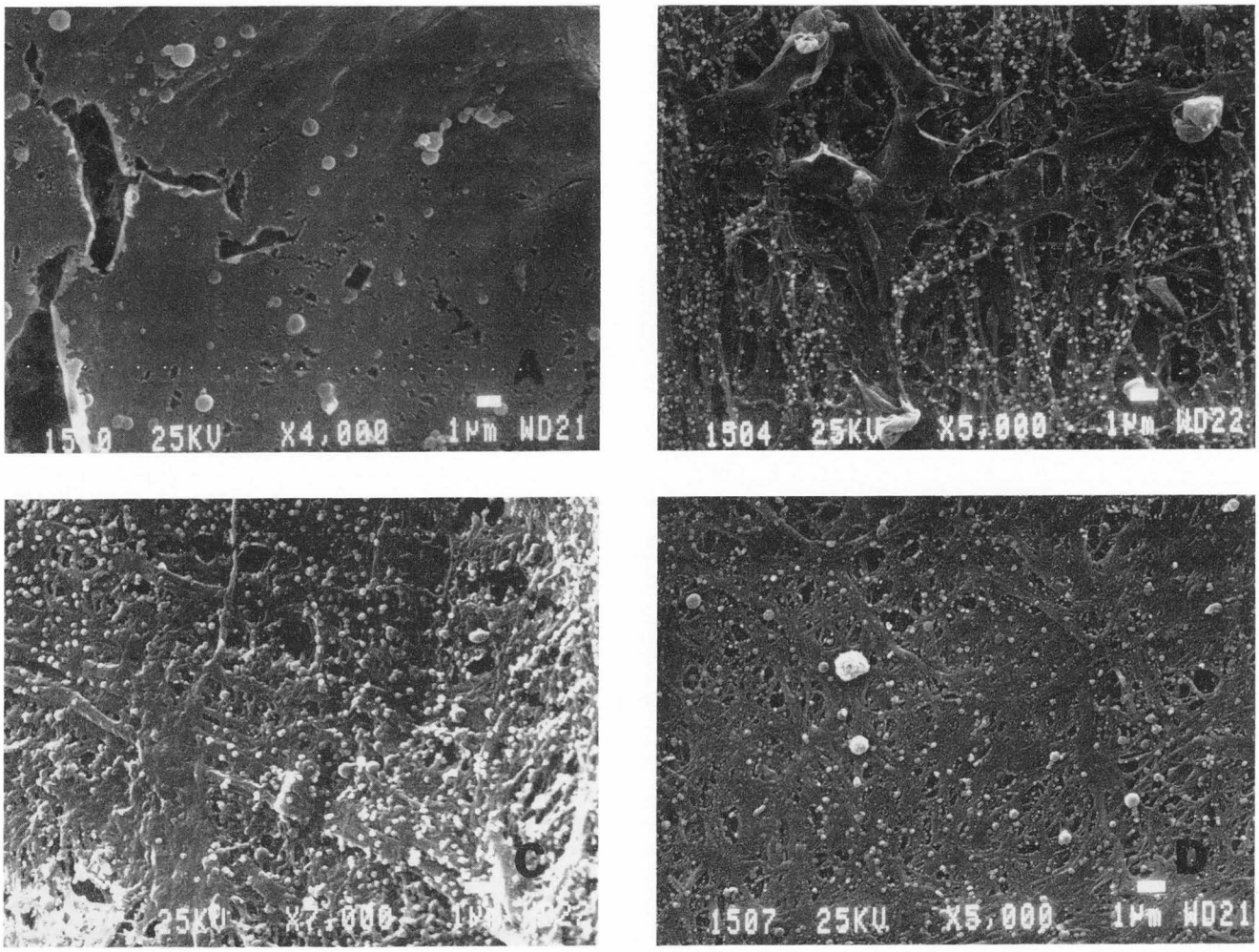


Figure 2. Detection of protein adsorption on a retrieved human ePTFE graft removed due to graft infection. Protein adsorption was determined as described in the methods using antibodies for: **A.** Control, 1% ovalbumin in PBS. The presence of bacteria is seen but no silver beads are visualized. The pseudointima completely covers the surface. **B.** Fibronectin. Several cells can be seen along with an abundant amount of silver beads particularly in the acellular areas. **C.** Fibrinogen. A diffuse pattern is observed, similar to fibronectin but fewer beads. **D.** Factor VIII/vWF. The pattern for this protein is diffuse with a few bacteria present. Bar = 1 μm for all photomicrographs.

Results

By our modification of methods used for routine immunocytochemical analysis or scanning electron microscopy, adsorbed proteins were identified on vascular grafts retrieved from humans. Rabbit antihuman antibodies, specifically IgG fractions obtained commercially or purified in our laboratory from crude antisera, were used as primary antibodies. Protein A-gold was used to label the primary antibodies and was subsequently silver coated using enhancing reagents as described earlier [25, 33]. Silver beads were then visualized using scanning electron microscopy at magnifications as low as x4,000. EDX analysis (Fig. 1) showed an intense peak for silver on the surface of the retrieved grafts. Because metal is required to nucleate the silver enhancing reaction, the silver peak indicated on EDX represents silver deposition on colloidal gold particles.

Our silver enhanced gold labelling technique differed from other methods [28] by using a blocking agent (protein A/milk solution) to prevent non-specific adsorption of the primary antibodies and/or protein A--gold. The controls in our system were essential such that non-specific binding of primary antibody could be ruled out. The background level of our assay is shown in Figure 2A, with virtually no beads present. Antibodies to alpha-fetoprotein, a protein not expected to be found in normal human circulating blood and therefore not deposited on surfaces represented another control and few beads were present (data not shown). In contrast, when stained for fibronectin (Fig. 2B), a

pattern of diffuse protein adsorption occurred on this graft. A similar pattern was observed for identification of fibrinogen (Fig. 2C) on the same graft. However, fewer beads were evident when labelled for fibrinogen as compared with fibronectin. We determined that trace proteins (<0.1 mg/ml) could be identified on the retrieved grafts. Factor VIII/vWF, a protein found in trace amounts in blood (10 μg/ml), was easily identified on this graft (Fig. 2D) but again fewer beads were found compared to the detection of fibrinogen or fibronectin.

In another ePTFE graft (Fig. 3), the detection of the trace protein, Hageman factor (factor XII), on the graft surface (Fig. 3A) showed a diffuse pattern similar to factor VIII/vWF seen in the previous graft. The amount of beads attributed to fibrinogen (Fig. 3B) and fibronectin (Fig. 3C) were fewer than seen in Figure 2, but again, were distributed uniformly.

The detection of protein adsorption on a retrieved HUV graft (Fig. 4) was less than on the explanted ePTFE grafts. Fibronectin (Fig. 4A) and Hageman factor (Fig. 4B) were observed but the number of beads was sparse.

Discussion

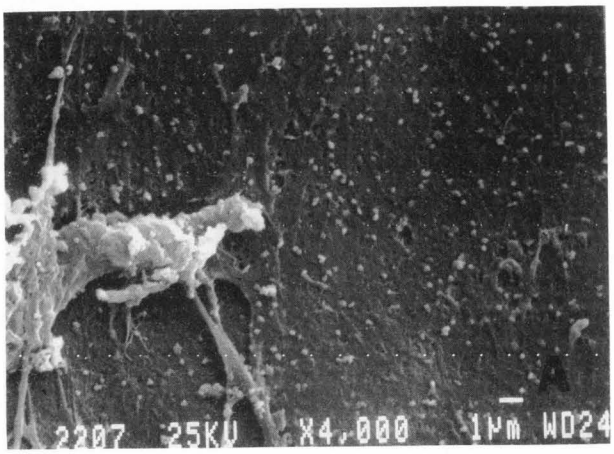
The development of small diameter vascular prostheses for treatment of peripheral vascular disease has been hampered by the occurrence of thrombosis and other problems [13]. There is a rather poor understanding of the interaction of blood with graft materials during the early and late stages of implantation. The types of adsorbed proteins, their morphological distribution and their interaction with cells may play a significant role in determining the ultimate biocompatibility and/or thrombogenicity of the graft.

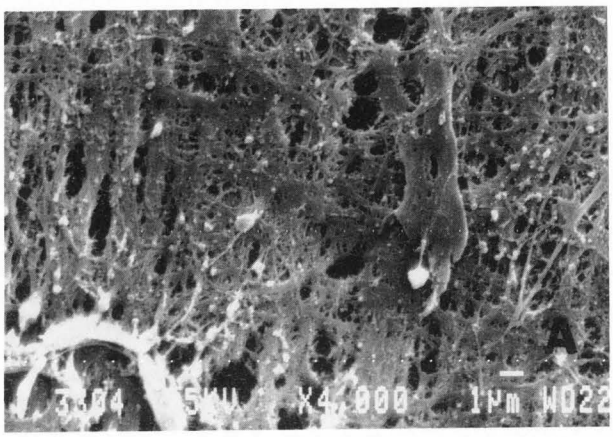
The use of immunological labels to study protein adsorption on biomaterials have been used by others [23, 26-29]. Many of these studies have used single purified proteins or combinations under static non-flow conditions or have used non-human blood, plasma or proteins. For example, in a series of papers, Park and co-workers [26, 27, 29] described the use of immunologic labels to study adsorption of human and canine blood proteins on biomaterials. Albumin, fibronectin, and fibrinogen were visualized on polyvinylchloride, silicon rubber, polyethylene, oxidized polyethylene, Biomer, and Teflon. Complete coverage of these surfaces by fibrinogen occurred and to a lesser extent with albumin. Formation of fibronectin networks on the surfaces of polyethylene were suggestive of incomplete or altered adsorption due to intermolecular association of proteins.

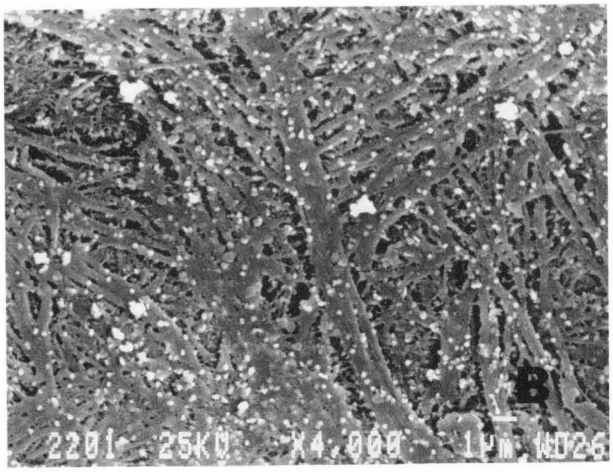
We have previously investigated short term ($\leq$ 3 hours) blood protein adsorption using an in vitro recirculation system [25, 39]. Anticoagulated or non-anticoagulated human whole blood was circulated through clinically relevant small diameter (4 mm) and NHLBI-DTB reference materials. We detected the adsorption of proteins important to coagulation, fibrinolysis, cell adhesion and cell activation including Hageman factor, factor VIII/vWF, fibronectin, fibrinogen, IgG, albumin, and hemoglobin. Protein A-gold labelling with SEM revealed that proteins from human whole blood adsorbed to surfaces in either a uniform or patchy pattern. For example, on ePTFE after 5 minutes of in vitro recirculation, adsorbed Hageman factor was distributed evenly on the node and fibrils. Factor VIII/vWF showed a similar pattern, whereas the adsorption pattern of fibrinogen was different in that there was a preference for the fibrils of the material and it was multilayered, while the nodes showed less adsorption.

In the present studies explanted human vascular grafts adsorbed proteins in a more uniform and disperse pattern with fibronectin displaying the greatest number of gold beads. We did not find patchy distribution of any protein and thus these studies differed from our earlier studies with an in vitro model. However, it is important to realize that the retrieved grafts had been implanted for long periods compared to our short term in vitro studies. Significant accumulation of matrix proteins such as fibronectin in the developing pseudointima, either deposited from blood or produced by cells, may account for the uniform pattern found in these studies. Other proteins, such as fibrinogen, were readily detected and other immunocytochemical studies have shown this protein and related fibrinogen molecules within diseased blood vessels [18]. The importance of other proteins such as Hageman factor and factor VIII/vWF detected on the retrieved grafts is unclear but both Hageman factor and factor VIII/vWF are important proteins involved in coagulation, fibrinolysis and complement activation [30]. Factor VIII/vWF, fibrinogen and fibronectin are important in the adhesion of platelets and leukocytes to surfaces [7, 10, 17, 21, 23, 25, 35, 39]. Once activated, these cells can release a variety of mediators that may further influence the composition of the graft [37, 38]. Thus, the proteins deposited on the retrieved grafts have implications in the activation of blood coagulation and platelet and leukocyte adhesion and activation.

The methods used to identify proteins in this study and developed in our previous studies are potentially useful to the study of protein adsorption on surfaces. The advantage of our method is the ability to identify by immunologic techniques virtually any protein that adsorbs to a surface provided an antibody is available for that specific protein. It is also an advantage to identify a specific protein (antigen) by antibody binding to that protein in a complex solution containing many proteins such as those found in blood. In addition, we controlled for the non-specific binding of antibodies to the surfaces by using antibodies to proteins not normally found in human blood, alpha-fetoprotein and Herpes virus. Secondly, non-specific protein binding sites on the polymer surface were blocked by the use of non-fat dry milk. This blocking agent has been used in immunocytochemical and immunoblotting applications [5] and we also used the milk solution with







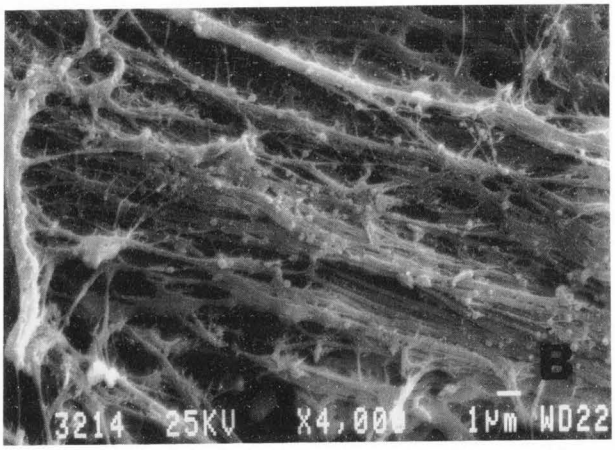


Figure 4. Detection of protein adsorption on retrieved human umbilical vein graft. Protein adsorption was determined as described in the methods using antibodies for:
A. Hageman factor (factor XII). The surface of this graft is different from the previous ePTFE grafts in that no pseudointima is present. Note that the number of beads is fewer than in the ePTFE grafts.
B. Fibronectin. There are more silver beads present than in A. Bar = 1 μm for all photomicrographs.

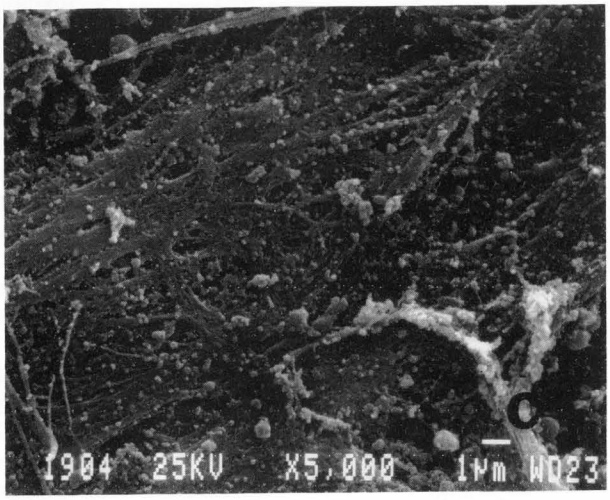


Figure 3 (at left). Detection of protein adsorption on different retrieved human ePTFE grafts removed for graft infection. Protein adsorption was determined as described in the methods using antibodies for:
A. Hageman factor (factor XII). The pattern of Hageman factor on this graft is diffuse.
B. Fibrinogen. There is an appreciable number of silver beads comparable to the previous graft detected with fibrinogen.
C. Fibronectin. There are fewer beads on this graft compared to the graft in Fig. 1B.
Bar = 1 μm for all photomicrographs.

non-gold labelled protein A to prevent any additional non-specific binding of protein A. Therefore, low background detection of gold beads was observed. Thirdly, the use of silver enhancement provided better visualization of labelled proteins since beads were enlarged from 10 nm to between 60 and 100 nm, and easily visualized at magnifications between x2,000-x10,000. In addition, the relationship of the beads with the surface features of the pseudointima, such as adherent cells or matrix components, can be readily observed.

In summary, we describe the identification of proteins on retrieved vascular grafts in an attempt to identify proteins on the surface that may be involved in the failure of grafts due to thrombosis. The adsorption of proteins onto a material is an important event that can modulate thrombotic or antithrombotic responses. Further work is necessary in understanding the complex interaction of blood with surfaces in order to design and develop new materials for use as cardiovascular devices, prostheses or artificial organs.

Acknowledgments

These studies were supported by the National Institutes of Health Grant No. HL-27277.

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Discussion with Reviewers

Reviewer I: The protocol of freezing at 0°C, then shipping on dry ice would produce very slow freezing. Storing at 0°C is probably alright (since it is doubtful that freezing would occur), but the subsequent handling would cause a great deal of ice crystal damage as the crystallization front migrated across the sample. Following such a protocol I would not expect the morphology of the sample to be representative of it in life.
R.M. Albrecht: Do the slow freezing procedures employed have effects on structure or antigenicity due to large crystal size and/or solute concentrations and possibly selective dehydration during freezing?
Authors: We have other evidence to address the concern regarding potential freezing artifacts during specimen handling. An ePTFE vascular graft (4 mm diameter) was exposed to whole human blood, with no anticoagulant, for one minute. The graft was then cut into 1 cm segments and one half of these segments were frozen at 0°C for two days (slow freezing). The remaining segments (fresh samples) were subjected to the protein detection methods described in this paper and fibrinogen (FB), fibronectin (FN), factor VIII/vWF (F-VIII), and Hageman factor (HF) were detected. Alpha-fetoprotein and ovalbumin were used as controls as described in the manuscript. The frozen segments were thawed at room temperature and subjected to the same procedure. SEM was then used to visualize the silver enhanced gold beads on both the frozen and fresh samples. The beads were counted and expressed as the number of beads per cm^2. These values were then plotted against each other and a correlation coefficient (R value) of 0.995 was determined (see Table 1 and Fig. 5).

These data suggest that the potential for artifact as a result of freezing the explanted vascular grafts was minimal. The slow freezing method may affect structures on the surface but this was not the main goal of the study, rather it was to study protein adsorption patterns. The extent to which freezing affects antigenicity of proteins is unknown but it may have less effects than other preservation methods such as glutaraldehyde or paraformaldehyde.

Reviewer I: What was done to ensure that plasma proteins were not adsorbed after retrieval? Was the

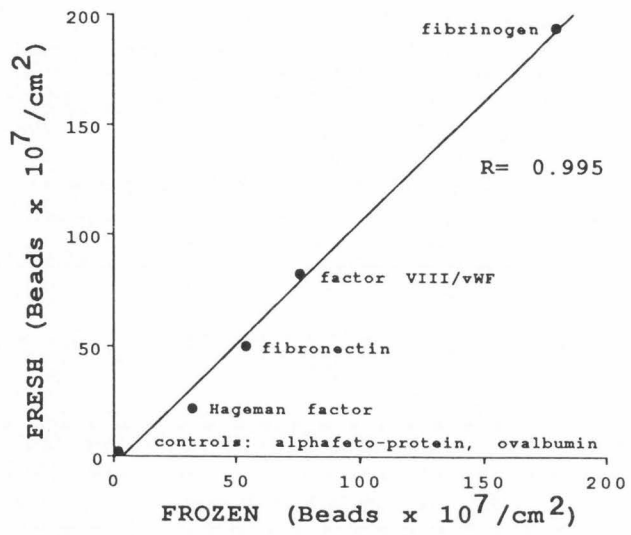

Figure 5. Comparison of fresh versus frozen techniques on protein adsorption of ePTFE grafts.

Table 1. Quantification of Silver Enhanced Gold Beads

Each value is expressed as the number of beads x 10^7/cm^2. The error expressed is a counting error.

	FB	FN	F-VIII /vWF	HF	Oval	AP
Fresh	194±20	50±10	82±10	22±2	2±2	2±2
Frozen	180±20	54±10	76±10	32±2	2±2	2±2

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preparation rinsed after explantation?
Authors: Since these were long term implants, we would not expect the composition of the adsorbed protein layer to change significantly even if there was residual plasma in the lumen upon removal. We could not control rinsing at the operation site so we cannot address the issue of specimen handling prior to shipment.

Reviewer I: In the "checkerboard" assay, was adsorption measured onto the same materials, i.e., ePTFE and HUV? Is only 1 hour adsorption really relevant for this assay since proteins continue to denature for several hours, if not days, following adsorption? Were these samples also treated with glutaraldehyde? The glutaraldehyde will alter protein antigenicity and thus affect labelling.
Authors: The purpose of the checkerboard analysis was not to mimic the in-vivo adsorption of proteins but to test our antibodies for cross-reactivity. Ideally, this kind of test would simulate precisely the in-vivo situation, but we could not do that and be able to detect cross-reacting antibodies from the general milieu. The only purpose of this analysis was to obtain reasonably convincing evidence, but not necessarily proof, that our antibodies did not cross-react with other proteins of interest. While glutaraldehyde may alter the antigenicity of the protein in question, we would expect that this systematic error would hold true in the checkerboard analysis since these samples were treated the same way.

Reviewer I: With silver enhanced gold technique each and every gold label is not enhanced or enhanced evenly. This can make it difficult to determine, especially, the relative levels of labelling on different samples. Why not use gold labels large enough to be detectable in the SEM without silver enhancement (e.g., 20-50 nm gold)?
R.M. Albrecht: In the present case of surface labelling, is there evidence to suggest that the efficiency of labelling is better with the smaller probe diameters, i.e., 5 to 10 nm? If not, might it be more expedient to simply label with a larger, 20 to 40 nm, probe size and avoid problems of variable levels of enhancement inherent in the silver process? Would direct conjugation of the label to the primary antibody be more useful than indirect labelling for quantitative studies? Do the authors have any experience to suggest directly conjugated probes have reduced or increased labelling efficiency?
Authors: Early studies showed that increasing the bead size decreased the labelling specificity, probably due to stearic hindrance of protein-A gold/antibody binding. In addition, 20-50 nm gold beads are harder to visualize using SEM than 100 nm silver enhanced beads. The silver enhancement process allows for greater specificity and improved visualization of the label. This is valid and perhaps future work should address this question directly. Performing these studies with only a single antibody step with a larger bead would have the possible advantage that it would avoid a second antibody and its associated binding uncertainties. One disadvantage is that more antibodies would have to be gold-labelled. We do not have any direct experience in this regard but we would expect that directly conjugated probes would have decreased binding efficiency from stearic hindrance and possibly from antibody conformational changes. This is generally thought to be true with tissue immunocytochemistry.

Reviewer I: Could not differences in the denaturation of protein after adsorption, in the short term in-vitro versus the long term implantation, affect these results?
Authors: We would certainly expect long-term degradation of adsorbed protein to partially affect these results. That is the reason for undertaking this study, so that comparisons can be made with similar experiments with no long term degradation (in-vitro studies).

Reviewer I: In Fig. 2a, what are the small round spheres (about 0.5 mm on the micrograph), if they are not "beads"? Since the size of the silver reaction product is not easily controlled, some means other than size must be used to determine that they are not silver-enhanced gold. An EDX map could serve this purpose.
Authors: The small round spheres are bacteria. Figure 1, an EDX spectrum for Hageman factor detection by immunogold labelling with silver staining, shows that the major reaction product is silver, although palladium is also identified. We have performed other EDX analyses for each protein determined on these surfaces and the data are similar in that the major peak is always silver.

G. Pasquinelli: How many vascular grafts did you study? Did you find any difference between ePTFE and umbilical vein grafts?
Authors: We tested a total of 5 grafts that were suitable for this study. Minimal differences, with this small number studied, were found between ePTFE and HUV that we feel are convincing enough to publish.

G. Pasquinelli: You state that fibronectin was the most abundant protein detected on your samples. What method did you use to compare the results? Did you perform a count on the enhanced gold particles?
Authors: We did not count beads to compare amounts of protein present. Visual inspection was used to make obvious comparisons.

G. Pasquinelli: How old were the human implants? As regards protein adsorption, is there any difference between grafts which failed due to thrombosis or infection?
Authors: The implants had been in place for months to years. Our study is too small to determine differences between thrombosis and infection. More study is definitely needed. We would expect bacteria to degrade and modify proteins, and produce and deposit some of their own unique, and possibly cross-reacting, proteins on the surface.

G. Pasquinelli: Have you performed analogous experiments without preliminary fixation with glutaraldehyde? Did you find differences in antibody binding?
Authors: No experiments were done without preliminary fixation. This will be considered in future work.

R.M. Albrecht: The preservation and storage procedure could be a source of variability. Slow freezing of large specimens results in extremely large ice crystals and slowly moving freezing fronts (the temperature listed was 0°C, but this is actually above the freezing point of the tissue). The water activity gradients can be relatively high with solutes concentrating along the front and between crystals. Since considerable migration of water molecules occurs to the expanding but lower vapor pressure ice crystal, dehydration also becomes important. These factors may have a deleterious effect on protein antigenicity. Different epitopes have different sensitivities. Thus if one is to label subsequently and compare relative amounts of label, it is essential that some sort of control (i.e., unfrozen-fixed, unfixed, or ultra rapidly frozen/rapidly thawed) must be done to ensure that the loss of antigenicity does not affect the estimates of one surface bound component relative to another. The problem is generally more severe when dealing with cell surface bound antigens than with proteins on surfaces as is the case here. Nevertheless, I think the controls are advisable. Simple structural displacement due to the crystal size also could be a problem.

Small gold probes are useful for a variety of reasons including increased accessibility, penetration, and to provide a better quantitative estimation where density of epitope is such that a larger bead could overlap variable numbers of sites. Smaller particles also afford a more precise level of positional resolution.

In the present case however, it might be better to use larger 20-40 nm probes to begin with and avoid all the trouble of silver enhancing with its highly variable level of enhancement of individual gold particles. I am not sure I fully appreciate the need for larger probe size, it may be due to instrument limitations or just that they are easier to see at low magnification micrographs. If the latter is the case, BSE imaging with various image processing techniques can provide good visualizable localization without having to grow giant labels.
Authors: Thank you for your comments.

R.M. Albrecht: Is antigenic specificity of the species of interest retained in the face of glutaraldehyde fixation?
Authors: By all indications in our checkerboard analysis, antigenic specificity is preserved. In order to minimize non-specific cross-linking of antibodies to the surface, glutaraldehyde treatment was always followed by glycine "neutralization".

R.M. Albrecht: Is it clear that the protein A used to block the Fc fragments of non-specifically adsorbed immunoglobulin cannot also bind Fc of subsequently added polyclonal antibodies? Protein A has been shown to bind Fab ends of some antibodies. Does protein A bind equally well to all types of rabbit antibodies? Has, for example, anti-Fc-gold or anti-human IgG-gold been used to see if Fc of plasma adsorbed Igs are actually present in sufficient concentration to be a problem?
Authors: We are assuming that once protein A is bound to an Fc site on the surface, it does not subsequently migrate to another. We do not know if this is a valid assumption. According to the manufacturer, protein A binds "strongly" to rabbit Ig. We do know that plasma adsorbed Igs are on the surface since this was one of our test antibodies. Since they are present in detectable amounts, we felt a blocking step for the

other proteins was warranted. Otherwise, there might be increased background staining since protein A could potentially bind to these antibodies as well.

K. Park: The authors used protein A-gold complex at the concentration which was not described in the manuscript. They simply diluted the 10 nm gold beads obtained from Janssen by 100 folds. Since the concentration of the gold beads is very important in determining the staining time, the concentration should be described. For example, it is not clear whether staining for 1 hour at 37°C is enough to have full staining of the sample. How did the authors know that staining for longer period of time is not necessary?
Authors: Dr. Park is quite right in this point and we do not know the precise concentration of protein A-gold in our solution. We tried various staining times and dilutions by trial and error, and, while we do not know the precise optimum, this combination worked well. More concentrated solutions seemed to consistently give high background staining of controls while more dilute solutions showed poor staining even for proteins we would expect in abundance (such as albumin). Future work should look at the staining time since this variable was not systematically examined by us. One to two hours was a convenient amount of time but more dilute solutions may work with lengthened staining time.

K. Park: One of the dangers in interpreting the colloidal gold staining is that the extent of gold staining may not be related to the concentration of target proteins, except when the target protein is covered as a monolayer. Since immunogold staining is also related to the exposure of target proteins, the fewer beads on the surface do not necessarily mean that fewer protein molecules exist. Some proteins may be hidden by other proteins so that they may not be detected by immunogold staining. The authors need to reflect this aspect of immunogold staining in their results.
Authors: While there has been much work concerning the initial events in protein adsorption that could determine final clinical outcome, this study looked at the other extreme: the end point. This study does not ask what is the composition of the pseudointima but what is on the surface at the time of failure.

K. Park: Protein adsorption and thromboembolism are dynamic events. The immunogold staining data, however, represent protein adsorption profile at a particular time point. Please comment on how this snap-shot examination is related to the overall dynamic events?
Authors: This is related to Dr. Park's previous question. We are looking at the end point in this study. While initial events are probably extremely important in determining outcome, it is not known if the final surface composition is important. This kind of study may help to answer that question.