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# Blood compatibility of surfaces with immobilized albumin–heparin conjugate and effect of endothelial cell seeding on platelet adhesion

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Received 1 September 1998; revised 28 January 1999; accepted 2 March 1999

**Abstract:** Endothelial cell (EC) seeding significantly improves the blood compatibility of artificial surfaces. Although a coating consisting of albumin and heparin (alb-hep) is a suitable substrate for seeded ECs, binding of ECs to the substrate further improves when small amounts of fibronectin are present in the alb-hep coating. Alb-hep conjugate was immobilized on carbon dioxide gas plasma-treated polystyrene (PS-CO<sub>2</sub>), thereby significantly increasing the recalcification time of blood plasma exposed to this surface. Furthermore, surface-immobilized alb-hep conjugate inhibited exogenous thrombin. Heparin activity was reduced by adding fibronectin on top of a monolayer of alb-hep conjugate, but not by simultaneous coating of fibronectin and alb-hep conjugate. Coating of PS-CO<sub>2</sub> with alb-hep conjugate significantly decreased contact activation (FXII activation). The number of platelets deposited from blood plasma on PS-CO<sub>2</sub> coated with alb-hep conjugate was twice as high as on PS-CO<sub>2</sub> coated with albumin. Addition of fibronectin to alb-hep conjugate-coated PS-CO<sub>2</sub> had no significant effect

on the number of adhered platelets. Seeding of the substrates with ECs significantly reduced the number of adhered platelets under stationary conditions. Platelets deposited onto endothelialized surfaces were primarily found on endothelial cell edges, and sparingly on areas between ECs. In conclusion, alb-hep conjugate-coated surfaces display anticoagulant activity. ECs adhering to and proliferating on this coating significantly decrease the number of platelets which adhere to the surface. Therefore, alb-hep conjugate-coated surfaces form a suitable substrate for seeding of ECs in low density. Although application of fibronectin on top of the coating decreases the anticoagulant activity to some extent, it might be useful in view of the improved adherence of ECs to the coating. © 1999 John Wiley & Sons, Inc. *J Biomed Mater Res*, 47, 279–291, 1999.

**Key words:** small-diameter vascular grafts; endothelial cell seeding; albumin–heparin conjugate; blood compatibility; platelet adhesion

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## INTRODUCTION

Spontaneous outgrowth of endothelium on the inner surface of implanted vascular prostheses occurs in most animal species other than humans.<sup>1,2</sup> Since endothelial cells (ECs) perform a key regulatory role in hemostasis,<sup>3,4</sup> seeding of ECs on the luminal surface of vascular prostheses is a promising method to avoid occlusion of especially small-diameter grafts implanted in humans.<sup>5–8</sup> ECs needed for seeding must be autologous and the efficiency of endothelial cell harvesting from vascular tissue is generally poor. Therefore, the number of ECs available is usually too small to allow immediate formation of a confluent cell layer. During the period in which the layer of ECs is not yet

confluent, activation of the blood coagulation system and platelet deposition must be inhibited. This goal may be reached by using an albumin–heparin conjugate (alb-hep) coating as substrate for the seeded ECs.

As previously shown, a coating of alb-hep conjugate (consisting of a heterogeneous mixture of conjugates with different compositions, such as alb<sub>1</sub>-hep<sub>1</sub>, alb<sub>1</sub>-hep<sub>2</sub>, alb<sub>2</sub>-hep<sub>1</sub><sup>9</sup>) is a suitable substrate for ECs.<sup>10</sup> Human umbilical vein endothelial cells (HUVECs) were grown to confluency on alb-hep conjugate that was covalently immobilized on CO<sub>2</sub> plasma-modified polystyrene. HUVECs cultured on this surface released amounts of von Willebrand factor (vWF) and prostacyclin (PGI<sub>2</sub>) comparable to those of HUVECs grown on fibronectin-coated tissue culture polystyrene (TCPS), which demonstrated the viability of these cells.<sup>11</sup>

For many years, immobilization of heparin has been applied to enhance thromboresistance of artificial sur-

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faces exposed to blood or plasma.<sup>12</sup> Binding of anti-thrombin III (ATIII) to a specific pentasaccharide sequence in heparin results in a marked increase of the inactivation of serine proteases such as Factor Xa and thrombin by ATIII.<sup>13</sup> When heparin is immobilized to a surface in the form of alb-hep conjugate, 27% of its anticoagulant activity is retained.<sup>14</sup>

Contact of artificial surfaces with blood inevitably leads to contact activation, which initiates intrinsic coagulation, fibrinolysis, and activation of the kinin system. The first step in the contact activation process is the activation of the plasma glycoprotein Factor XII (FXII) at the surface of the biomaterial, which results in a conformational change of the molecule. Activated Factor XII (FXIIa) can be cleaved into two polypeptide chains of 52 (aFXIIa) and 28 kD (bFXIIa), of which the latter diffuses away from the surface.<sup>15</sup> Surface-bound aFXIIa is involved in the activation of the intrinsic coagulation, the fibrinolytic system, and the kinin system.<sup>13,16,17</sup> bFXIIa is involved only in the activation of the kinin system.<sup>17</sup>

Deposition of platelets and, to a lesser extent, leukocytes generally occurs on foreign surfaces. Coating of surfaces with von Willebrand factor, fibrinogen, or fibronectin increases adhesion of platelets, whereas coating with albumin or plasma decreases adhesion of platelets.<sup>18–22</sup> Seeding of ECs on artificial surfaces may result in decreased platelet deposition in dogs,<sup>23</sup> baboons,<sup>24</sup> and humans,<sup>5,8,25</sup> although at least one study failed to show this effect.<sup>26</sup> Decreased numbers of platelets adhering to endothelialized surfaces may be explained by the production of PGI<sub>2</sub> and nitric oxide by ECs.<sup>27</sup>

The purpose of the present study was to investigate the blood compatibility of a monolayer of alb-hep conjugate which was immobilized on gas plasma modified polystyrene, and to study the effect of endothelial cell seeding on platelet deposition onto this surface.

## MATERIALS AND METHODS

### Materials

Unfractionated alb-hep conjugates were obtained from Holland Biomaterials Group (Enschede, The Netherlands). Alb-hep 1 [16.5% (w/w) heparin] was based on porcine albumin, and alb-hep 2 and 3 [11.6% and 12.3% (w/w) heparin, respectively] were based on human albumin. Phosphate-buffered saline (PBS) (pH 7.4) was purchased from NPBI (Emmercompascuum, The Netherlands). Polystyrene (PS) and TCPS petri dishes (35 mm in diameter, 9.6 cm<sup>2</sup>) were from Greiner GmbH (Frickenhausen, Germany). TCPS 12-well plates were obtained from Costar (Cambridge, UK). Carbon dioxide (CO<sub>2</sub>) was from Hoekloos (Schiedam, The Netherlands). Pig mucosal heparin sodium (195 U/mg) was

from Bufa Chemie (Castricum, The Netherlands). Gelatin-Sepharose was purchased from Pharmacia (Uppsala, Sweden). Kallikrein/FXIIa chromogenic substrate (S2203) was obtained from Chromogenix (Mölnådal, Sweden). Medium M199, Medium RPMI 1640, glutamax-1, penicillin, streptomycin, fungizone, and trypsin-ethylenediaminetetraacetic acid (EDTA) were purchased from Life Technologies (Paisley, UK). <sup>111</sup>In-oxine was bought from Amersham (Amersham, UK). Tris-(hydroxymethyl)-aminomethane hydrochloride (Tris) and bovine serum albumin (BSA, fraction V) were obtained from Sigma Chemical Company (St. Louis, MO). Dialysis tubing (size 9) was obtained from Medice International Ltd. (London, UK). Human serum was pooled from 12 healthy volunteers. Fresh buffy coats obtained from healthy volunteers were a gift of Bloedbank IJssellanden (Enschede, The Netherlands). A plasma fraction containing human fibronectin (FNc) was a gift from the Central Laboratory of the Blood Transfusion Service (CLB, Amsterdam, The Netherlands). All other chemicals were of the highest purity available from Merck (Darmstadt/Hohenbrunn, Germany).

### Purification of fibronectin

Human fibronectin (FN) was purified from FNc by affinity chromatography on gelatin-Sepharose at room temperature. Phenylmethylsulfonyl fluoride (0.1 mM) was added as protease inhibitor to all buffers. FNc (200 mL, 2.5 mg/mL in 50 mM Tris-HCl, pH 7.5) was applied to a 125-mL column of gelatin-Sepharose. The column was washed with several volumes of Tris-HCl buffer (50 mM, pH 7.5), 2 vol NaCl solution (1M NaCl in 50 mM Tris-HCl, pH 7.5), and again with Tris-HCl buffer (50 mM, pH 7.5). Elution of fibronectin was carried out with 4M urea in Tris-HCl buffer (50 mM, pH 7.5). The absorption of the eluate was monitored at 280 nm (Pharmacia Single Path Monitor UV-1; Pharmacia, Uppsala, Sweden), and the fibronectin-containing fractions were pooled. After dialysis against PBS, aliquots ( $\pm 0.50$  mg FN/mL) were stored at  $-30^{\circ}\text{C}$ .

### Gas plasma treatment and coating of polystyrene petri dishes

Polystyrene petri dishes were cleaned and CO<sub>2</sub> gas plasma treated as previously described.<sup>10</sup> After treatment, surfaces were stored at a temperature of  $-20^{\circ}\text{C}$  and used within 5 days.

Albumin or alb-hep conjugate (alb-hep) were immobilized on PS-CO<sub>2</sub> petri dishes (10.9 cm<sup>2</sup>) by incubating the dishes with the protein or conjugate (1 mL, 15 mg/mL) in borate buffer (5 mM, pH 8.2) for 45 min. Subsequently, the petri dishes were rinsed, using PBS (three times), a low-pH (3.5) phosphate-buffered (NaH<sub>2</sub>PO<sub>4</sub>/Na<sub>2</sub>HPO<sub>4</sub>, 12 mM) saline (1M) solution (three times), and a similar solution with a pH of 10.5 (three times). Finally, the surfaces were rinsed twice with PBS.

A number of the surfaces onto which alb-hep conjugate

had been immobilized were incubated with fibronectin (1 mL, 0.05 mg FN/mL in PBS) for 30 min, after which the surfaces were rinsed three times with PSB (surfaces coded as PS-CO<sub>2</sub>/alb-hep/FN afterward). Analogous to the procedure with alb-hep conjugate, a mixture of alb-hep conjugate (15 mg/mL) and fibronectin (0.05 mg/mL) in borate buffer was added to PS-CO<sub>2</sub> (surfaces coded as PS-CO<sub>2</sub>/alb-hep/FN simultaneous).

The surface concentrations of the different constituents are presented in Table I. Initial surface concentrations were determined using radiolabeled compounds (albumin, alb-hep conjugate, and fibronectin).<sup>11</sup> Small quantities of the immobilized compounds were released during 1 day of incubation in serum-containing cell culture medium, whereas almost all FN was released from the surface of PS-CO<sub>2</sub>/alb-hep/FN afterward.<sup>11</sup>

### Recalcification assay

Activation of the intrinsic coagulation pathway was studied by determining the coagulation time of recalcified human blood plasma in contact with albumin and alb-hep conjugate immobilized on PS-CO<sub>2</sub> petri dishes. Fresh frozen human plasma was thawed at 37°C and stored on ice until further use. Human plasma (900 µL), PBS (100 µL), and rabbit brain cephalin (100 µL) were pipetted into the petridishes and warmed to 37°C for 3 min, after which CaCl<sub>2</sub> (100 µL 0.2M, prewarmed to 37°C) was added. The recalcification time was determined as the time needed to detect the first clot with a stainless-steel hook. As a positive control, recalcification time was also determined for a petri dish to which a glass coverslip was added.

### Thrombin inactivation assay

The activity of surface-immobilized heparin with respect to neutralization of added thrombin was determined using a chromogenic substrate for thrombin in an endpoint assay as described by Chandler et al.<sup>28</sup> The assay was carried out using Tris buffer (50 mM, pH 8.4) containing poly(ethylene glycol) 6000 (1.0 g/L), bovine serum albumin (1.0 g/L), and NaCl (150 mM) (assay buffer). To a petri dish with a coating of albumin, alb-hep conjugate, and/or fibronectin, assay buffer (600 µL) was added and warmed to 37°C for 10 min.

**TABLE I**  
Surface Concentrations of Albumin, Alb-Hep Conjugate, and Fibronectin after Immobilization on PS-CO<sub>2</sub><sup>11</sup>

Coating	<sup>14</sup> C-alb (µg/cm <sup>2</sup> )	<sup>14</sup> C-alb-hep (µg/cm <sup>2</sup> )	<sup>125</sup> I-FN (µg/cm <sup>2</sup> )
PS-CO <sub>2</sub> /Alb	0.43 ± 0.01		
PS-CO <sub>2</sub> /AH		0.57 ± 0.03	
PS-CO <sub>2</sub> /AH/FN afterward		0.51 ± 0.03	0.19 ± 0.01
PS-CO <sub>2</sub> /AH/FN simultaneously		0.24 ± 0.02	0.03 ± 0.00

Next, assay buffer (300 µL) containing chromogenic substrate S2238 (0.2 mg/mL) and ATIII (70 mU/mL), which had been kept at 0°C until use, was added and allowed to warm to 37°C for 5 min. The reaction was started by addition of thrombin (100 µL, 1.2 U/mL) and stopped after 10 min of shaking at 37°C by the addition of an acetic acid solution [100 µL, 40% (v/v) in demineralized water]. The absorbance at 405 nm of the supernatant, caused by *p*-nitroanilin cleaved from the substrate by thrombin, was determined with an enzyme-linked immunosorbent assay (ELISA) reader (340 ATTC, SLT, Austria). The absorbances at 405 nm were converted to mU/cm<sup>2</sup> using a reference curve obtained with heparin samples of known activity.

### Contact activation assay

Activation of the contact system was determined using chromogenic substrate S2302, as described by Van Delden et al.<sup>14</sup> This chromogenic substrate can be cleaved by several proteases, of which FXIIa (both aFXIIa and bFXIIa) and kallikrein are the most important.<sup>29</sup> Since these proteases may be present in supernatant plasma exposed to an activating surface as well as on the surface itself, both the soluble and surface-bound protease activity were measured.

A plasma solution [1 mL, 25% (v/v) in PBS, stored on ice until use] was added to a PS-CO<sub>2</sub> petri dish, after which the dish was placed on an orbital shaker at 37°C for 15 min. Uncoated PS-CO<sub>2</sub> petri dishes were used, as well as PS-CO<sub>2</sub> petri dishes coated with albumin, alb-hep conjugate, or fibronectin. A sample of the supernatant (70 µL) was incubated at 37°C with substrate S2302 solution (110 µL, 1.12 mg/mL in PBS) and the increase in optical density at 405 nm was determined for 30 min using a kinetic ELISA reader (340 ATTC, SLT, Austria). A plasma solution in PBS [25% (v/v)] that was not exposed to the petri dishes was used as control.

After washing the surfaces twice with 3 mL PBS, the enzymatic activity present on the incubated surfaces was measured by incubating the surface with S2302 (1 mL, 0.25 mg/mL in PBS) for 60 min at 37°C. The absorbance of the supernatant at 405 nm was determined using an ELISA reader. A PS petri dish which was not incubated with plasma was used as control.

### Isolation of endothelial cells

Human umbilical vein endothelial cells (HUVECs) were isolated using the method of Jaffe et al.,<sup>30</sup> with slight modifications. The umbilical vein was rinsed with PBS and filled for 20 min at 37°C with trypsin-EDTA solution (0.05% respectively 0.02% in PBS). After harvesting HUVECs, cell culture medium supplemented with 20% (v/v) pooled human serum (CMS) was added to inactivate trypsin. After exchange of the medium by fresh medium, cells were plated in fibronectin-coated TCPS flasks and grown until the third passage at 37°C in 95% air/5% CO<sub>2</sub>, saturated with water vapor. Cell culture medium (CM) consisted of Medium M199 (50% v/v), Medium RPMI 1640 (50% v/v), glutamax-1

(2 mM), penicillin (100 U/mL), streptomycin (100  $\mu\text{g}/\text{mL}$ ), and fungizone (2.5  $\mu\text{g}/\text{mL}$ ). Before passage or seeding, cells were detached from the TCPS surface by incubation with trypsin-EDTA solution, centrifuged, and resuspended in CMS.

### Proliferation of endothelial cells

Before cell seeding, surfaces (9.6  $\text{cm}^2$ ) were placed overnight at room temperature in PBS, containing penicillin (100 U/mL) and streptomycin (100  $\mu\text{g}/\text{mL}$ ). Third-passage HUVECs were seeded at a density of 10,000 cells/ $\text{cm}^2$  and cultured at 37°C in 5%  $\text{CO}_2/95\%$  air saturated with water vapor. Medium (0.20 mL/ $\text{cm}^2$ , CMS) was replaced every other day. At selected times, cells were trypsinized after which cell numbers were determined using a Bürker counting device.

### Preparation of platelet suspensions and $^{111}\text{In}$ labeling of platelets

Fresh buffy coats from healthy volunteers were diluted 2.5 times with Krebs-Ringer's buffer (107 mM NaCl, 4 mM KCl, 20 mM  $\text{NaHCO}_3$ , 2 mM  $\text{Na}_2\text{SO}_4$ , pH 7.3) containing trisodiumcitrate (19 mM) and D(+)-glucose (27 mM), after which platelet-rich plasma (PRP) was obtained by centrifugation (1700  $\times g$ , 4 min, 20°C). Subsequently, PRP was diluted 1:1 with Krebs-Ringer's buffer (pH 5.0) containing 19 mM trisodiumcitrate and 27 mM D(+)-glucose (final pH 6.1). Platelets were isolated according to the method of Cazenave et al.<sup>31</sup> and labeled with  $^{111}\text{In}$ indium oxine.<sup>18</sup> Finally, platelets were resuspended at a concentration of  $1.0 \times 10^5/\mu\text{L}$  in ABO-compatible plasma or in CM with 3.5 mg/mL albumin (CM/alb).

### Deposition of $^{111}\text{In}$ labeled platelets

Stationary deposition of  $^{111}\text{In}$ -labeled human platelets from human plasma or CM/alb was determined using a series of substrates. Test surfaces (petri dishes, 9.6  $\text{cm}^2$ ) were incubated for 4 h with PBS (2 mL) and after removal of the PBS, a suspension of  $1.0 \times 10^5$  platelets/ $\mu\text{L}$  in plasma or CM/alb (1 mL) was added. After 1 h at 37°C the platelet suspension was removed. Next, some of the surfaces were used to determine the number of adhered platelets, whereas others were used to investigate the morphology of the adhered platelets.

To determine the number of adhered platelets, surfaces were washed three times with PBS (3 mL) at 10-min intervals, and subsequently incubated overnight with NaOH (2 mL, 1M). The radioactivity in this solution was determined using a  $\gamma$ -counter (Compugamma 1282; LKB, Stockholm, Sweden). The number of deposited platelets was determined by dividing the radioactivity found in the NaOH solution by the radioactivity incorporated per platelet.

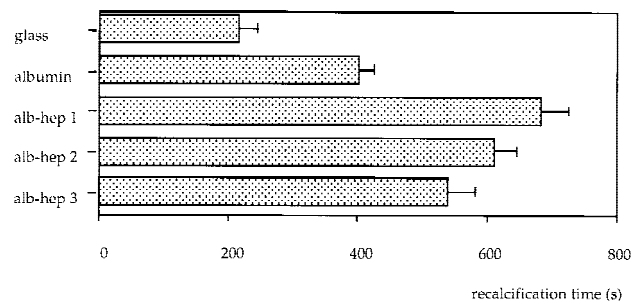
To study the morphology of adhered platelets, surfaces

were washed three times with PBS (3 mL) at 1-min intervals, and subsequently the platelets were fixed with glutaraldehyde [2 mL, 2% (v/v) in PBS]. Samples were kept in this medium at 4°C for at least 52 days, 20 times the half-life of  $^{111}\text{In}$ . After washing three times with water (4 mL, 2 min/step), samples were dehydrated by incubation with a graded series of ethanol solutions in water [4 mL, 25%, 50%, 75%, 90%, and 98% (v/v) of ethanol, two times for 5 min/step] and finally in ethanol (4 mL, four times for 5 min). After drying *in vacuo*, the samples were sputter-coated with gold and examined by scanning electron microscopy using 7 kV accelerating voltage [S-800 field-emission scanning electron microscopy (SEM), Hitachi, Japan]. Stationary deposition of  $^{111}\text{In}$ -labeled human platelets suspended in CM/alb was also determined using a series of substrates onto which HUVECs had been seeded 4 h before the experiment, after which the surfaces were rinsed with PBS at 37°C. In one series used for SEM evaluation, the HUVECs were allowed to proliferate for 48 h prior to incubation with platelets. The control surfaces without HUVECs used in this experiment were incubated with cell culture medium for 4 h before the experiment and rinsed with PBS at 37°C.

## RESULTS

### Recalcification time

All tested surfaces showed longer coagulation times for recalcified blood plasma than glass. Furthermore, for alb-hep conjugate-coated surfaces the coagulation time was significantly longer than for albumin-coated PS- $\text{CO}_2$  ( $p < .05$ ) (Fig. 1). Differences in coagulation times were observed between the three different alb-hep conjugate batches. A significant difference between recalcification time was found for alb-hep 1 and alb-hep 3 ( $p < .05$ ). The alb-hep conjugate batches, as supplied by the manufacturer, differed with respect to heparin content [16.5%, 11.6%, and 12.3% (w/w) heparin, respectively for alb-hep 1, alb-hep 2, and alb-hep



**Figure 1.** Recalcification times of plasma contacted with various surfaces at 37°C. The recalcification time of glass (215 ± 29 s) served as positive control. Heparin content of alb-hep 1, 2, and 3 was 16.5%, 11.6%, and 12.3% (w/w), respectively. The albumin was of porcine origin in alb-hep 1 and of human origin in alb-hep 2 and alb-hep 3. The heterogeneity of the alb-hep conjugates may differ ( $n = 5$ ,  $\pm\text{SD}$ ).

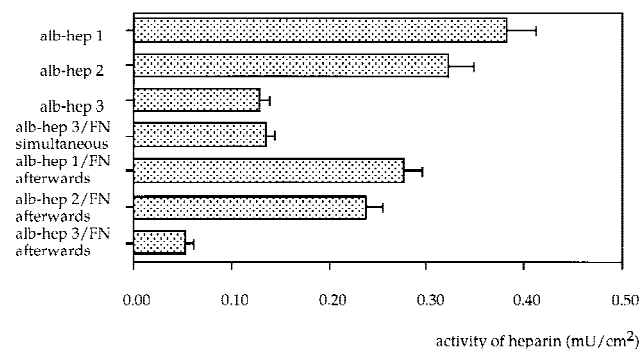
3], and the origin of the albumin used (porcine for alb-hep 1 and human for alb-hep 2 and alb-hep 3). The heterogeneity of the alb-hep conjugates may have differed as well. The highest coagulation time was found for the alb-hep conjugate with the highest heparin content (alb-hep 1).

### Thrombin inactivation assay

Anticoagulant activity, defined as the inactivation of added thrombin, was found for all heparinized surfaces (Fig. 2), but not for control surfaces without heparin. The activity of alb-hep 3 was lower compared to the other two conjugates ( $p < .01$ ). Application of fibronectin to the PS-CO<sub>2</sub> surface together with alb-hep conjugate (alb-hep 3/FN simultaneously) did not influence the anticoagulant activity of the immobilized alb-hep conjugate, whereas application of fibronectin on top of the alb-hep conjugate layer (alb-hep 1, 2, or 3/FN afterward) reduced the anticoagulant activity.

### Contact activation

The cleavage rate of the chromogenic substrate in plasma solutions upon contact with alb-hep conjugate monolayers was two to six times higher compared to the blank (nonactivated plasma,  $1.6 \pm 0.4$  mOD/min). The enzymatic activity of plasma solutions which had been in contact with alb-hep conjugate or albumin was similar and significantly lower compared to fibronectin coating and the reference surface glass (Table II). In



**Figure 2.** Anticoagulant activity determined by a thrombin inhibition assay. Activity found for control surfaces PS, PS-CO<sub>2</sub>, PS-CO<sub>2</sub>/albumin, and PS-CO<sub>2</sub>/FN was  $-0.007 \pm 0.004$ ,  $-0.005 \pm 0.004$ ,  $0.001 \pm 0.002$ , and  $0.005 \pm 0.004$  mU/cm<sup>2</sup>, respectively. Heparin content of alb-hep 1, 2, and 3 was 16.5%, 11.6%, 12.3% (w/w), respectively. The albumin was of porcine origin in alb-hep 1 and of human origin in alb-hep 2 and alb-hep 3. The heterogeneity of the alb-hep conjugates may differ ( $n = 5$ ,  $\pm$ SD).

**TABLE II**  
Contact Activation Measured as Activity in Solution and of Surface after Incubation of Surfaces with 1 mL 25% Human Plasma in PBS for 15 min at 37°C

Surface	Proteolytic Activity in Solution (mOD/min)	Proteolytic Activity on Surface (mOD)
Glass	$59 \pm 1$	$297 \pm 15$
PS-CO <sub>2</sub>	$110 \pm 3$	$306 \pm 5$
PS-CO <sub>2</sub> /alb	$5.4 \pm 0.9$	$270 \pm 19$
PS-CO <sub>2</sub> /alb-hep 1	$7.0 \pm 0.7$	$129 \pm 15$
PS-CO <sub>2</sub> /alb-hep 2	$3.9 \pm 1.2$	$106 \pm 8$
PS-CO <sub>2</sub> /alb-hep 3	$4.1 \pm 0.2$	$190 \pm 11$
PS-CO <sub>2</sub> /FN	$32 \pm 1$	$346 \pm 5$

The rate of cleavage of the chromogenic substrate of non-activated plasma (blank activity in solution) was  $1.6 \pm 0.4$  mOD/min. The absorbance of chromogenic substrate incubated in PS that had not been in contact with plasma (blank activity on surface) was  $47 \pm 6$  mOD. Heparin content of alb-hep 1, 2, and 3 was 16.5%, 11.6%, 12.3% (w/w), respectively. The albumin was of porcine origin in alb-hep 1 and of human origin in alb-hep 2 and alb-hep 3. The heterogeneity of the alb-hep conjugates may differ ( $n = 5$ ,  $\pm$ SD).

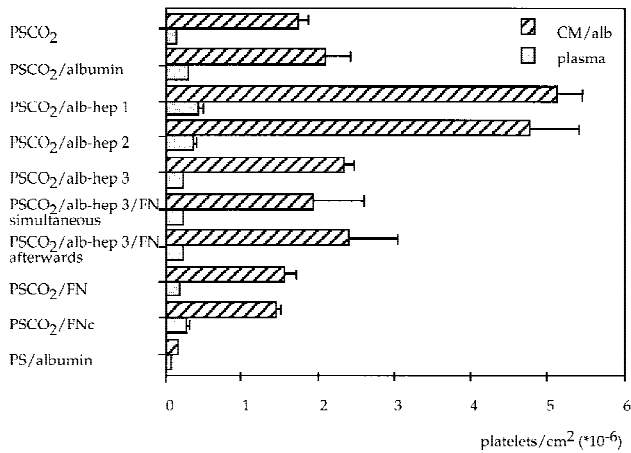
contrast, the contact activation observed for uncoated PS-CO<sub>2</sub> was significantly higher than for glass.

The enzymatic activity of contact-activated proteins which were adsorbed to the surfaces was determined after the diluted plasma had been removed (Table II). The surfaces with immobilized alb-hep 1 and alb-hep 2 showed less cleavage of the chromogenic substrate by contact activation proteases than the other surfaces. Surface-bound enzymatic activity of all surfaces was significantly higher than the blank ( $47 \pm 6$  mOD).

### Stationary platelet deposition in the absence and presence of seeded HUVECs

The number of platelets deposited from CM/alb onto various coatings present on PS-CO<sub>2</sub> and onto uncoated PS-CO<sub>2</sub> did not significantly differ, except for the coatings with alb-hep 1 and alb-hep 2 ( $p < .01$ ) (Fig. 3). On the latter coatings, a twofold increase in the number of adhered platelets compared to uncoated PS-CO<sub>2</sub> was observed. Significantly less platelets adhered to albumin-coated PS compared to coated and uncoated PS-CO<sub>2</sub>. The same trend was observed using plasma instead of CM/alb, but the absolute numbers found for CM/alb were much higher than the numbers found for plasma (Fig. 3).

Deposition of platelets from CM/alb was determined on surfaces seeded with HUVECs using different cell seeding densities (Fig. 4). The number of adhered platelets was significantly reduced on all surfaces where ECs were present. Surfaces seeded with higher cell densities, however, showed a slightly higher number of adhered platelets than surfaces



**Figure 3.** Deposition of  $^{111}\text{In}$ -labeled platelets on PS-CO<sub>2</sub> covered with various coatings, and on albumin-coated PS after incubation for 1 h at 37°C with  $1.0 \times 10^5$  platelets/ $\mu\text{L}$  resuspended in different media: ▨ CM/alb (cell culture medium with 3.5% albumin), □ human plasma. Heparin content of alb-hep 1, 2, and 3 was 16.5%, 11.6%, and 12.3% (w/w), respectively. The albumin was of porcine origin in alb-hep 1 and of human origin in alb-hep 2 and alb-hep 3. The heterogeneity of the alb-hep conjugates may differ ( $n = 3$ ,  $\pm\text{SD}$ ).

seeded with a low cell density. The presence of fibronectin on alb-hep conjugate-coated surfaces seeded with ECs did not influence the number of adhered platelets.

### Morphology of deposited platelets

Samples used for SEM were washed three times for 1 min instead of three times for 10 min as used for determining the number of adhered platelets. In a separate experiment it was shown that both the number of platelets determined at the surface and the morphology of adhered platelets had not changed significantly by this modification of the washing procedure ( $p > .05$ , data not shown).

Scanning electron microscopic examination of the surfaces demonstrated that deposited platelets were distributed homogeneously on nonseeded surfaces. On HUVEC-seeded surfaces, adhered platelets were almost exclusively localized near endothelial cell edges; hardly any platelets were present on uncovered areas between HUVECs.

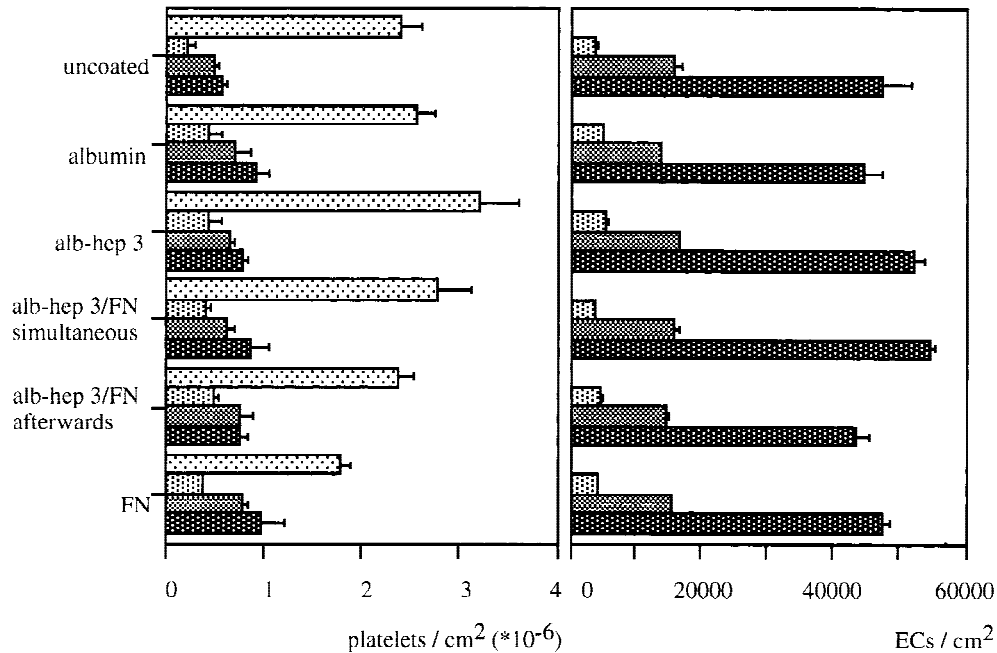
When the morphology of platelets adherent to the surfaces was studied with SEM, it appeared that platelets adhering to alb-hep conjugate- or albumin-coated PS-CO<sub>2</sub> demonstrated various stages of spreading. Some platelets were globular with several pseudopodia, whereas others were almost completely spread [Fig. 5(A–D)]. SEM observations were in agreement with the differences between numbers of adhered

platelets on the conjugates determined by using radio-labeled platelets. Platelets adhering to albumin-coated PS showed less pseudopodia compared to albumin-coated PS-CO<sub>2</sub> and none of the platelets on albumin-coated PS were completely spread [Fig. 5(D,E)]. Extensive spreading of platelets was observed in the case of platelets adhering to fibronectin-coated surfaces as well as to uncoated PS-CO<sub>2</sub> [Fig. 5(F–H)]. Addition of fibronectin to the alb-hep coating (alb-hep 3/FN afterward and alb-hep 3/FN simultaneously) appeared to have slightly increased the percentage of fully spread platelets compared to alb-hep 3-coated PS-CO<sub>2</sub> [Fig. 5(C,I,J)]. Platelets were distributed more or less regularly on all surfaces; platelet aggregates were not observed.

The remarkable reduction in the numbers of adhered platelets by seeding of HUVECs onto the various substrates illustrated in Figure 4 was also observed with SEM. Moreover, platelets were observed almost exclusively at the borders of ECs and not on uncovered areas between the cells. Similar results were found when seeded cells were allowed to adhere for 4 or 48 h [Fig. 6(B,C)]. Figure 6(D) shows discoid platelets attached to the border of the endothelial cells with bridging pseudopod-like structures. No spread platelets were observed on surfaces seeded with HUVECs.

## DISCUSSION

Albumin-heparin conjugate, covalently immobilized to PS-CO<sub>2</sub>, displayed anticoagulant activity as demonstrated by the significant prolongation of the recalcification time of plasma and the inactivation of added thrombin compared to uncoated PS-CO<sub>2</sub> (Figs. 1 and 2). The conjugate with the highest heparin content [16.5% (w/w) heparin, alb-hep 1] showed the largest increase of recalcification time and the highest activity in the thrombin inhibition assay. While the heparin contents of alb-hep 2 and alb-hep 3 [11.6% and 12.3% (w/w) heparin, respectively] were not significantly different, the heparin activity determined by the thrombin inhibition assay was significantly lower on surfaces coated with alb-hep 3 than on surfaces coated with alb-hep 2. This indicates that the heparin content of the alb-hep conjugate is not necessarily a measure of the anticoagulant activity displayed by a surface coated with an alb-hep conjugate. The differences between anticoagulant activity of conjugates with similar heparin content are probably caused by differences in the number of covalent bonds per heparin molecule, or the position of covalent bonds on the heparin chain (modification of the ATIII binding site). The relatively low anticoagulant activity of alb-hep 3 may therefore result from multiple bonds between the heparin and albumin molecules.



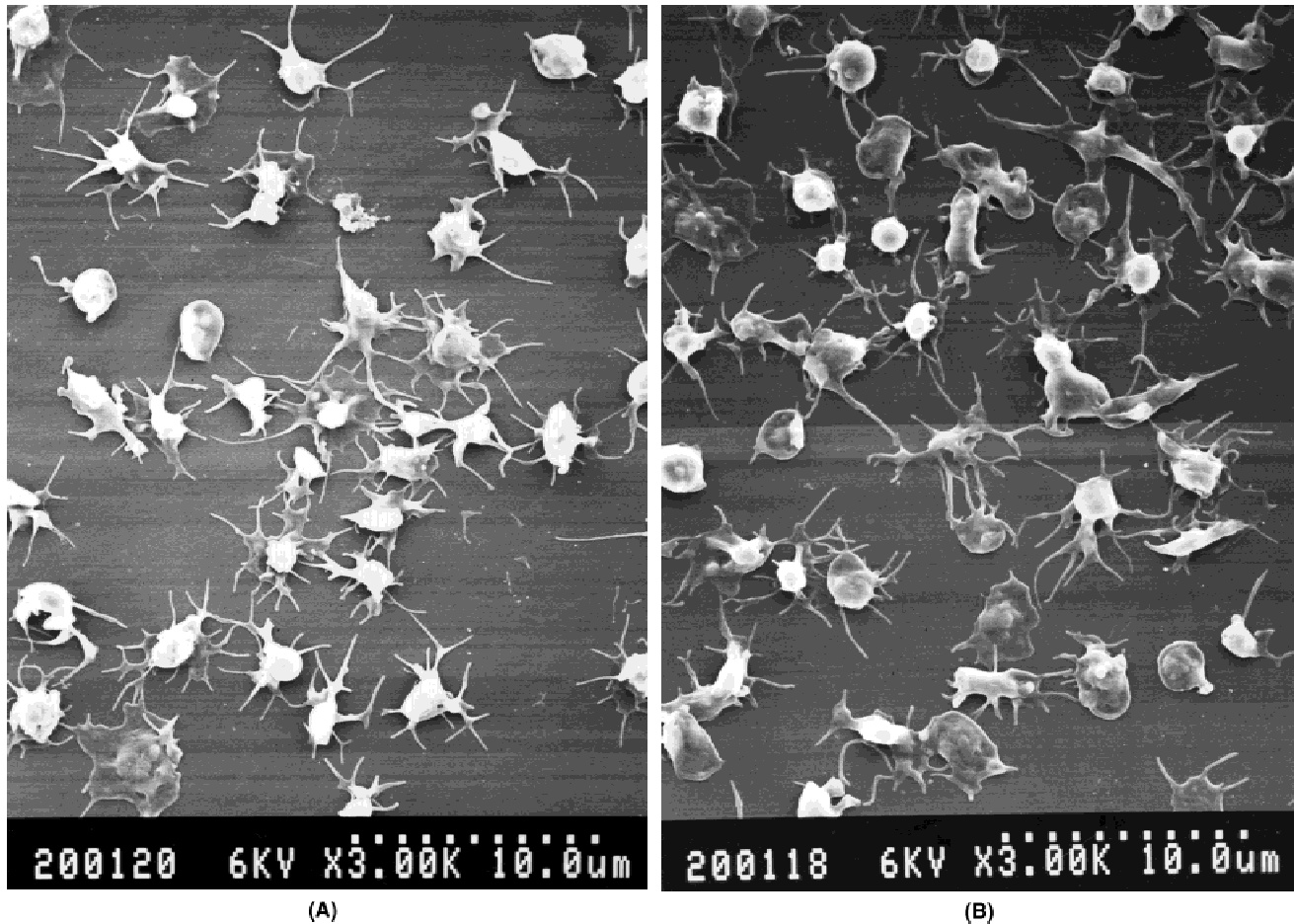
**Figure 4.** Deposition of  $^{111}\text{In}$ -labeled platelets on PS-CO<sub>2</sub> covered with various coatings after incubation for 1 h at 37°C with  $1.0 \times 10^5$  platelets/ $\mu\text{L}$  resuspended in CM/albumin (cell culture medium with 3.5% albumin):  $\square$  surfaces without HUVECs, surfaces with HUVECs seeded at a density of  $\square$  10,000/cm<sup>2</sup>,  $\blacksquare$  40,000/cm<sup>2</sup>, or  $\blacksquare$  100,000/cm<sup>2</sup> 4 h prior to the addition of platelets. Heparin content of alb-hep 3 was 12.3% (w/w); the albumin was of human origin ( $n = 3$ ,  $\pm\text{SD}$ ).

Addition of fibronectin on top of monolayers of alb-hep conjugate (alb-hep/FN afterward) significantly reduced heparin activity of all three conjugates, whereas addition of fibronectin during the coating with alb-hep conjugate (alb-hep/FN simultaneously) did not affect the activity of the immobilized heparin. In the latter case, however, the quantity of fibronectin immobilized on the surface was significantly lower (Table I). Moreover, in this case, a large part of the fibronectin was probably bound directly to the surface instead of via heparin,<sup>11</sup> and therefore hardly influenced the activity of the surface-immobilized heparin. Apparently, the effect of FN on the activity of heparin, which is presumed to be caused by blocking of the ATIII binding sequence of heparin, is concentration dependent. This assumption is affirmed by Byun et al.,<sup>32</sup> who showed that addition of fibronectin to surface-immobilized heparin reduced the binding of ATIII to the surface in a concentration-dependent manner.

Carbon dioxide-treated PS strongly activated the contact system, even more than the positive control surface glass. This high activation may have been caused by the negatively charged groups present at both surfaces (Table II). Coating of PS-CO<sub>2</sub> with either albumin or alb-hep conjugate resulted in a 10- to 30-fold lower rate of cleavage of the chromogenic substrate. Alb-hep conjugate-coated surfaces activate the contact system only minimally. Although surface-immobilized heparin is known to inhibit activation of

FXII and kallikrein via binding of ATIII,<sup>14,32</sup> the low levels found indicate that small quantities of FXIIa and/or kallikrein were present. Furthermore, contact activation caused by alb-hep conjugate coatings, both in solution and at the surface, was significantly lower than the contact activation by fibronectin coatings. This may be a result of the low surface concentration of FN ( $0.23 \pm 0.02 \mu\text{g}/\text{cm}^2$ ),<sup>10</sup> which indicates the presence of uncovered areas. The PS-CO<sub>2</sub> surface at these uncovered areas may account for the high contact activation, and possibly not the FN itself. A surface coating with alb-hep conjugate instead of fibronectin therefore may improve the blood compatibility of surfaces used for seeding of endothelial cells.

Significantly more platelets were deposited under stationary conditions onto the substrates when the platelets were suspended in cell culture medium with 3.5% (w/v) albumin (CM/albumin) instead of citrated plasma (Fig. 3). CM/albumin was used in this experiment because it was found that the spreading of ECs decreased in citrated plasma, probably as a result of the absence of Ca<sup>2+</sup>. In CM/albumin, this phenomenon was not observed. Comparable trends in the numbers of adhered platelets onto the various substrates were found using both suspension media, but when the platelets were deposited from CM/albumin the absolute numbers of adhered platelets were higher. Increased deposition of platelets suspended in CM/albumin instead of plasma may be caused by the presence of higher concentrations of calcium and magnesium in CM/albumin, which stimulate



**Figure 5.** Representative SEM micrographs of platelets deposited on PS-CO<sub>2</sub> with various coatings after incubation for 1 h at 37°C with  $1.0 \times 10^5$  platelets/ $\mu$ L suspended in CM/albumin. (A) PS-CO<sub>2</sub>/alb-hep 1. (B) PS-CO<sub>2</sub>/alb-hep 2. (C) PS-CO<sub>2</sub>/alb-hep 3. (D) PS-CO<sub>2</sub>/albumin. (E) PS/albumin. (F) PS-CO<sub>2</sub>/FN. (G) PS-CO<sub>2</sub>/FNc. (H) PS-CO<sub>2</sub>. (I) PS-CO<sub>2</sub>/alb-hep 3/FN afterward. (J) PS-CO<sub>2</sub>/alb-hep 3/FN simultaneously.

platelet function.<sup>33,34</sup> Immobilization of fibronectin to the surface resulted in extensive spreading of platelets (Fig. 5). This effect of fibronectin was also reported by Beumer et al.,<sup>35</sup> who demonstrated that platelets bind to fibronectin via the so-called RGD-sequence (Arg-Gly-Asp) present in fibronectin molecules.

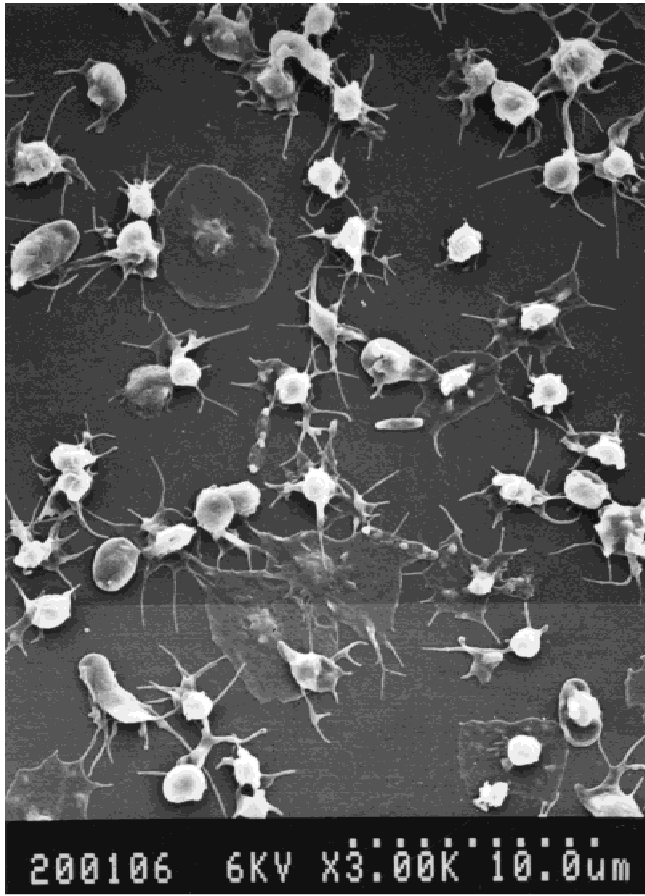
A striking difference was observed between the numbers of platelets adhering under stationary conditions to the various conjugates used in this study. On alb-hep 3 the same number of platelets was deposited as on albumin, and the state of spreading of the platelets was also similar. Significantly more platelets were deposited onto surfaces coated with alb-hep 1 or 2. Furthermore, the average degree of spreading was further progressed on these latter surfaces.

The number of platelets on the alb-hep conjugates showed a positive correlation with the heparin activity as determined by the recalcification and the thrombin inhibition assay. Heparin fractions with a high molecular weight are known to be more reactive toward platelets than fractions with a lower molecular weight.<sup>36–38</sup> Besides the molecular weight, the degree

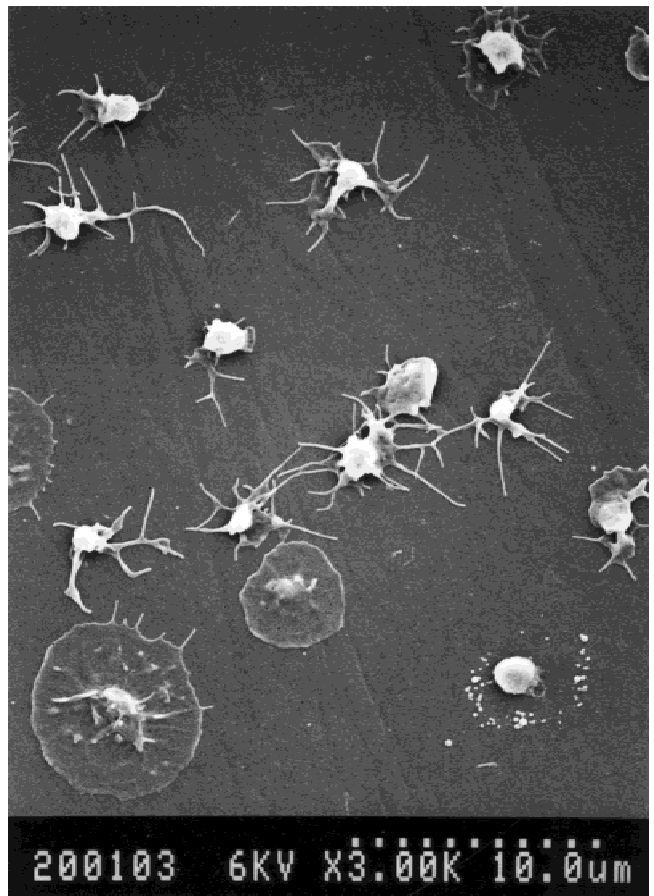
of sulfation plays an important role, and it has been postulated that heparin-platelet binding is principally mediated by an ionic interaction between the negatively charged sulfate groups on heparin and positively charged regions exposed on the platelet surface.<sup>38–40</sup> As suggested earlier, heparin in alb-hep 3 may be coupled to albumin by multiple bonds, and therefore smaller chains of heparin may be exposed from the albumin moiety of the conjugate. This results not only in less heparin activity with respect to inactivation of blood coagulation, but also in reduced interaction with platelets. However, the cause of these effects may be different. Whereas the reduced ability of alb-hep 3 to bind ATIII is probably caused by a decrease in the number of available ATIII-binding pentasaccharides,<sup>13</sup> the reduced binding of platelets to this conjugate may be the result of decreased charge interactions between heterogeneous cationic sites on the platelet membrane and electronegative charge present in the heparin chain.<sup>39,40</sup>

Increased *in vitro* platelet binding to heparinized biomaterial surfaces, as described in this study, as well

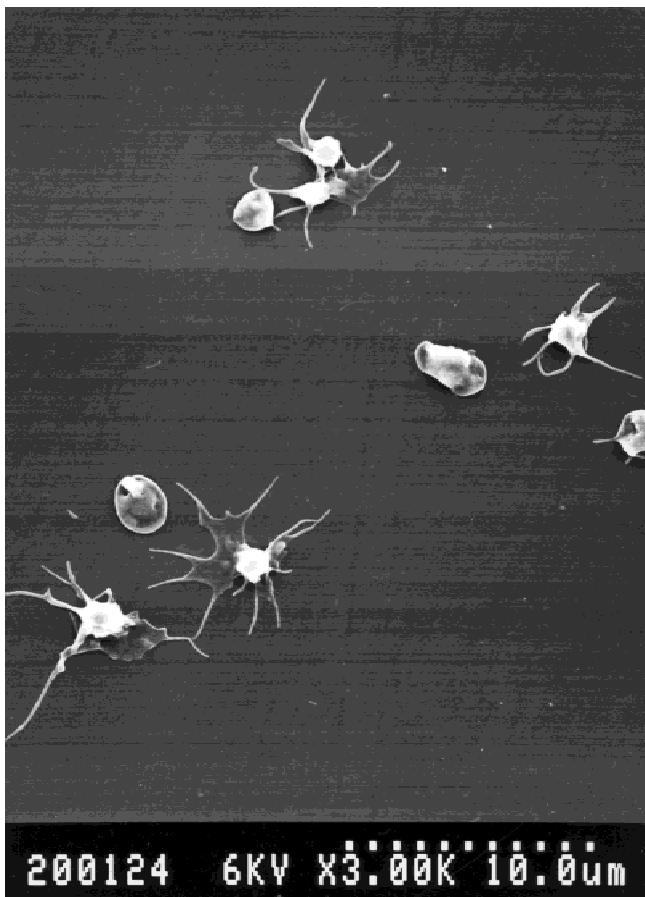




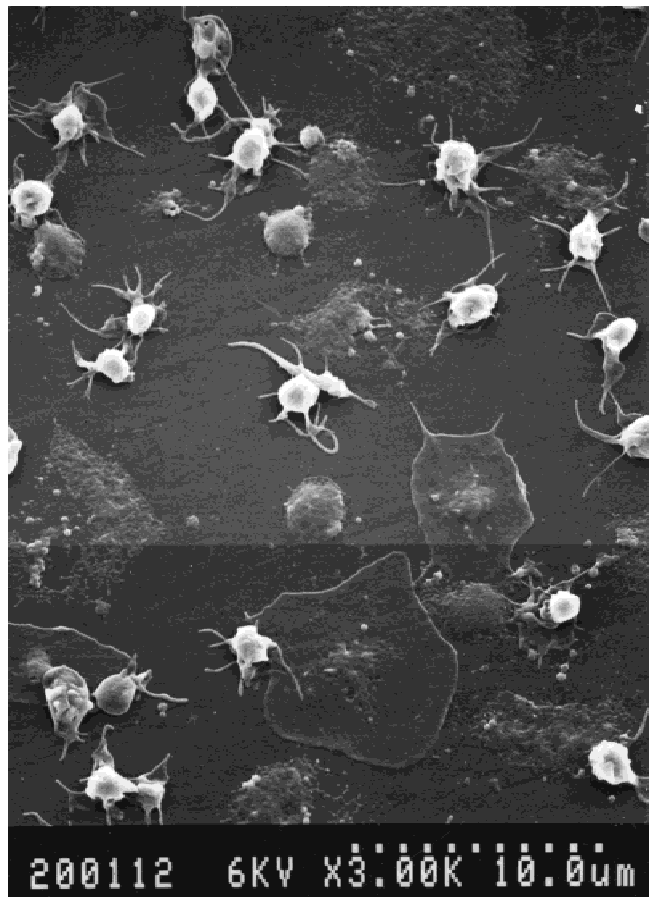
(C)



(D)

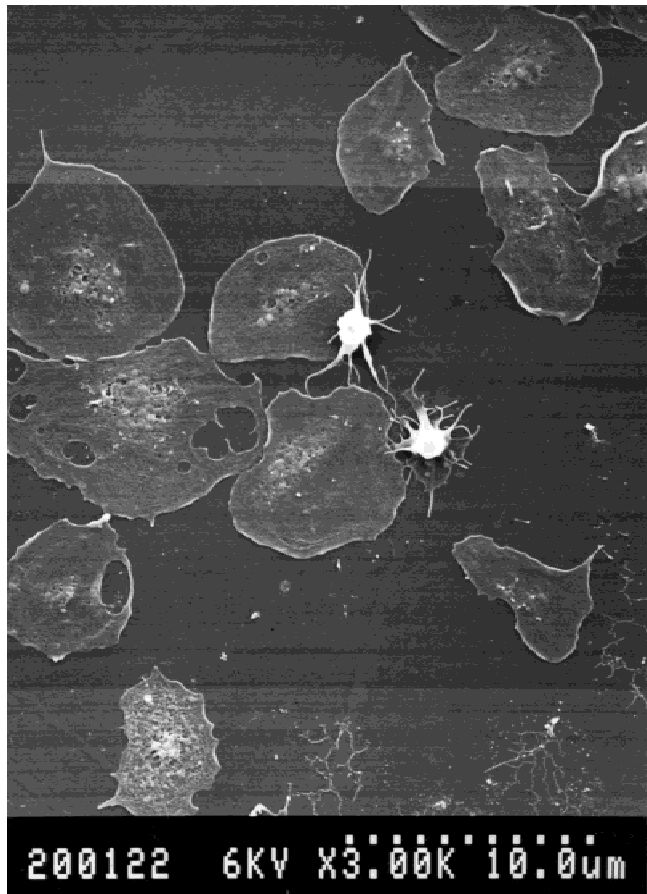


(E)

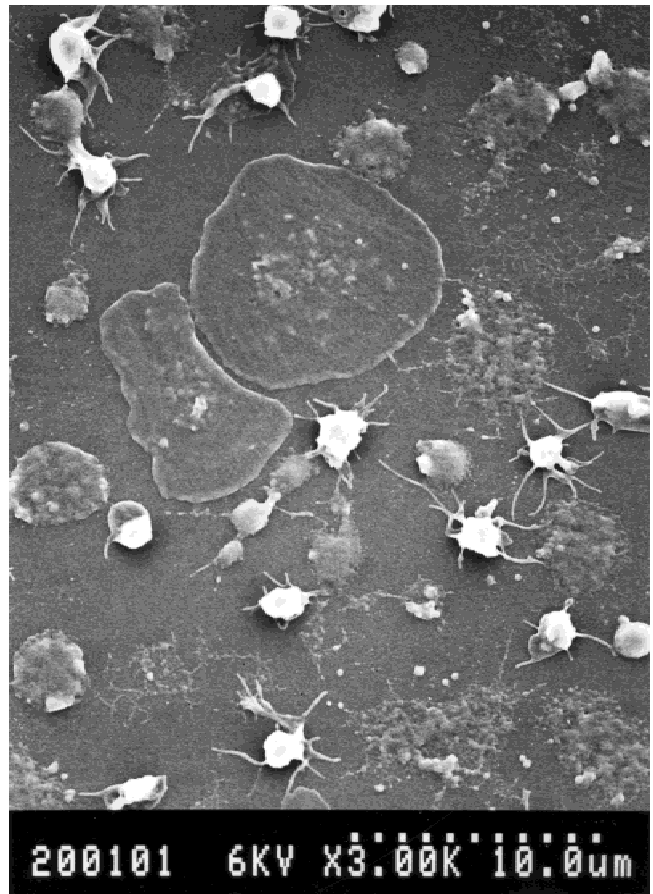


(F)

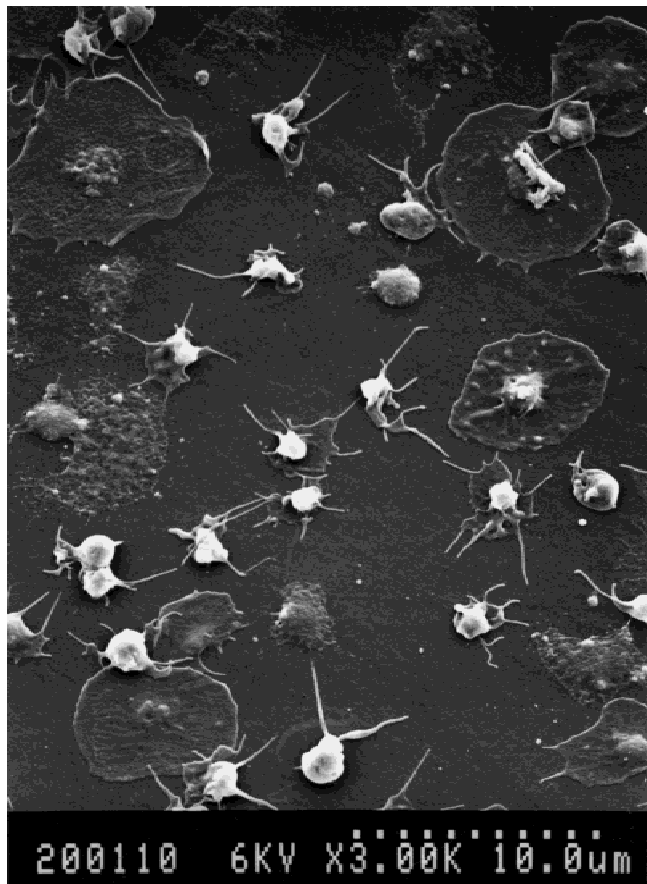
Figure 5. Continued.



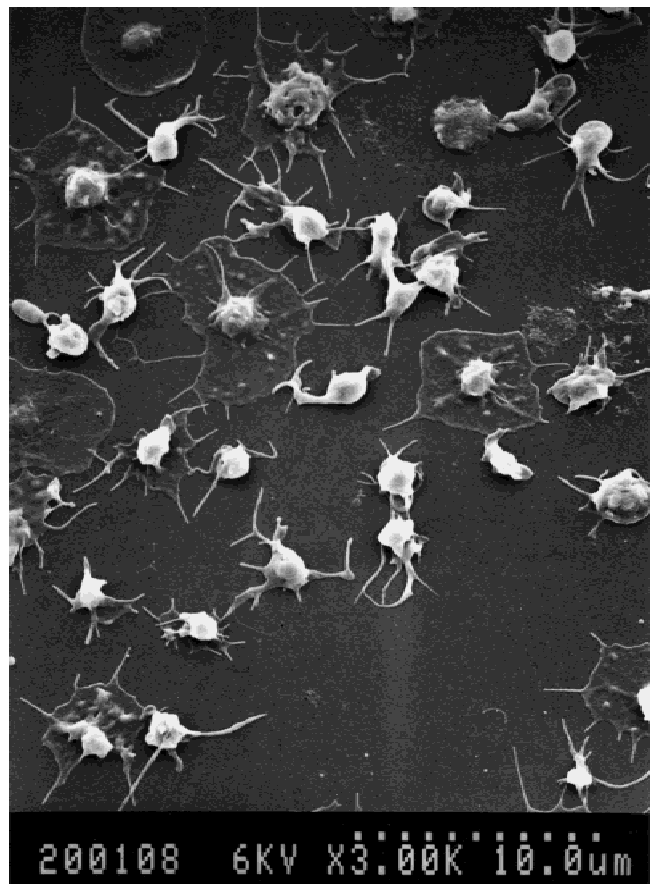
(G)



(H)



(I)



(J)

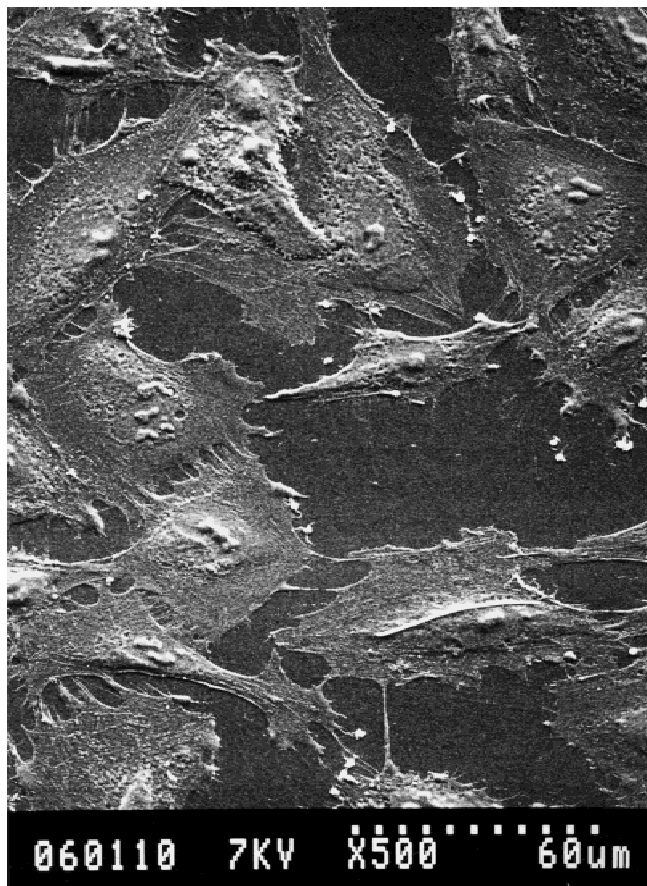
Figure 5. Continued.



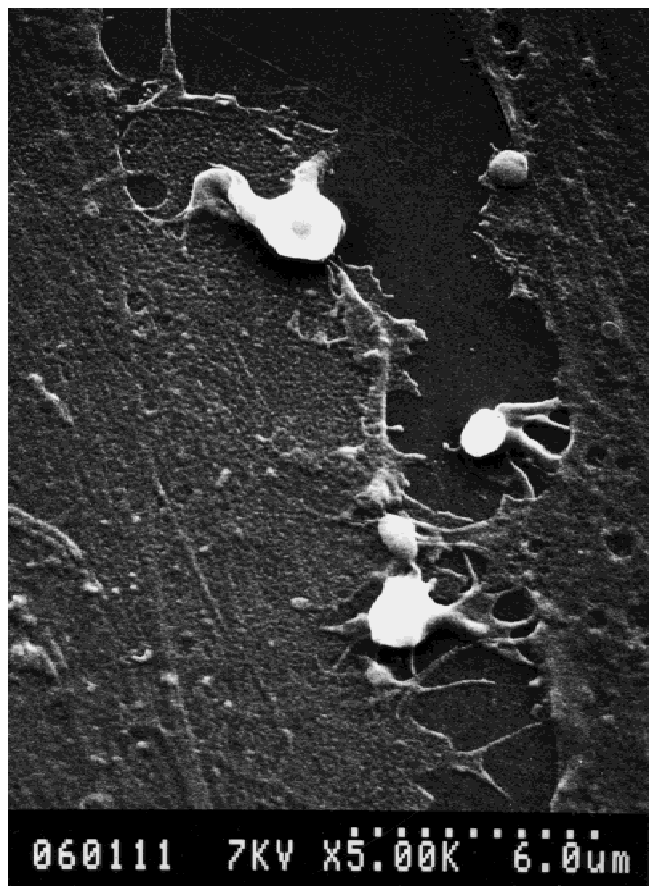
(A)



(B)



(C)



(D)

**Figure 6.** Representative SEM micrographs of platelets deposited onto endothelial cell-seeded coatings present on PS-CO<sub>2</sub> after incubation for 1 h at 37°C with  $1.0 \times 10^5$  platelets/ $\mu$ L suspended in CM/alb. (A) PS-CO<sub>2</sub>/alb-hep 3. (B) PS-CO<sub>2</sub>/alb-hep 3, ECs cultured for 48 h. (C,D) PS-CO<sub>2</sub>/alb-hep 3, ECs cultured for 4 h. Cells were seeded at a density of 10,000/cm<sup>2</sup>.

as decreased binding have been described in the literature.<sup>37–39,42–44</sup> These controversies may be due to the different effects of heparin on platelet activation induced by various agonists.<sup>41</sup> Heparin inhibits the activation of platelets by thrombin in the presence of plasma (or probably ATIII), whereas it is ineffective or sometimes stimulates activation in the absence of plasma.<sup>41</sup> Therefore, it may not be the direct interaction of heparin with platelets that matters, but the interaction of heparin with proteins, such as ATIII and fibronectin, present at the surface and in the medium.

Seeding of substrates with endothelial cells resulted in a significantly reduced number of adhered platelets on all surfaces under stationary conditions (Fig. 4). This is in agreement with data reported previously.<sup>5,8,22–26</sup> On seeded substrates, however, the number of platelets deposited on the substrates under stationary conditions slightly increased with endothelial cell density. Although similar numbers of ECs adhered to both uncoated and coated PS-CO<sub>2</sub> (Fig. 4), differences in EC morphology were observed. As previously shown,<sup>10</sup> spreading of ECs improved on fibronectin-containing substrates. It was observed with SEM that platelets deposited onto endothelialized surfaces were primarily found at the edges of ECs and not on uncovered spots between the cells (Fig. 6). Possibly, ECs exhibit two opposite functions: On the one hand, they prevent adhesion of platelets to the surface, probably caused by PGI<sub>2</sub> and/or nitric oxide secreted by the cells<sup>27</sup>; on the other hand, an interaction of the ECs with platelets occurs. This binding of platelets to the border of ECs may result from the binding of platelets to the platelet-endothelial cell-adhesion molecule (PECAM-1), a homotypic cell-adhesion molecule present on endothelial cells, mainly at cell junctions. The PECAM-1 molecules are also present on platelets, T cells, and monocytes.<sup>45</sup> Apparently, this interaction is not inhibited by PGI<sub>2</sub> and/or nitric oxide released by the ECs. Binding of platelets to HUVECs via PECAM-1 may result from the stationary conditions used in this experiment. The adhesion of platelets to fibronectin-coated polyethylene under flow conditions was previously shown to decrease with increasing endothelial cell density.<sup>46</sup> This difference may be explained by differences in contact time between the platelets and the surface. Under flow conditions, this time is much smaller and perturbation occurs, which may prevent the interaction between platelets and PECAM-1 present on the ECs. Similarly, alb-hep conjugate coating of polyurethane vascular catheters was previously shown to significantly reduce the number of platelets deposited onto the surface under flow conditions.<sup>47</sup> Therefore, less interaction between platelets and alb-hep conjugate coatings seeded with ECs is expected under flow.

## CONCLUSIONS

Albumin–heparin conjugate immobilized on PS-CO<sub>2</sub> retains sufficient anticoagulant activity to increase the recalcification time of blood plasma and significantly decrease contact activation by the surface. However, more platelets adhere to immobilized albumin–heparin conjugate than to immobilized albumin. Addition of a small amount of fibronectin during coating of PS-CO<sub>2</sub> with albumin–heparin conjugate, which was previously shown to improve endothelial cell adherence, decreased anticoagulant activity only slightly, while platelet interaction with the surface did not increase. Seeding of the substrates with endothelial cells significantly reduced the number of adhering platelets which are primarily found on endothelial cell edges. Therefore, coating of vascular graft surfaces with albumin–heparin conjugate supplied with a small amount of fibronectin results in a promising substrate for low-density seeding of endothelial cells.

The authors thank the staff of the department of obstetrics of the Medisch Spectrum Twente (MST, Enschede, The Netherlands) for supplying the human umbilical cords, Bloedbank IJssellanden (Enschede, The Netherlands) for their gift of buffy coats, and Ing. M. A. Smithers for carrying out the SEM experiments.

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