INTERACTION OF ALBUMIN-HEPARIN CONJUGATE PREADSORBED SURFACES WITH BLOOD

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In a number of medical therapies blood is contacted with foreign materials for varying periods of time. When no anticoagulant is administered, this contact can lead to the formation of thrombi on the surface of the material[1,2]. Numerous investigators have tried to prevent thrombus formation by binding heparin, either ionically or covalently, onto various material surfaces[3,4]. Recently we reported on the synthesis of covalently bound conjugates of albumin and heparin, which were developed for the improvement of the blood compatibility of polymeric surfaces[5]. After adsorption onto different materials both the presence of heparin at the interface and a slow release of the conjugate from the surface might aid in the prevention of thrombus formation. Albumin-heparin conjugate easily adsorbs onto hydrophobic surfaces as albumin does. Moreover, it is expected that heparin as part of the conjugate is positioned away from the surface by the albumin spacer. Thus, contrary to ionically or direct covalently bound heparin, heparin bound through an albumin spacer should be more available for complex formation with antithrombin III and activated clotting factors.

It was shown that albumin-heparin conjugate has heparin like activity as determined by APTT, Factor Xa and thrombin time assays[5]. After precoating of materials with albumin-heparin conjugates, prolongation of both the plasma recalcification time and whole blood clotting time was observed, indicating that surface induced coagulation was inhibited. Although conjugate desorption was observed, the adsorbed albumin-heparin conjugate showed a prolonged clotting time and its activity, like heparin in solution, was dependent on complex formation with antithrombin III[6].

Besides surface induced coagulation, blood platelet adhesion is an important factor in the blood compatibility of a biomaterial. The interaction between platelets and surfaces preadsorbed with proteins has been studied by different investigators. In general, preadsorbed fibrinogen and γ-globulin promote platelet adhesion, whereas albumin adsorption decreased platelet adhesion[7-9]. In this paper we will present the results of platelet adhesion studies on different surfaces (PVC, Silastic, Biomer, cellulose acetate and glass) with and without preadsorbed albumin-heparin conjugate.

MATERIALS AND METHODS

Albumin-Heparin Conjugates. Albumin-heparin conjugates were synthesized as previously described[5]. In brief: albumin (fraction V, Sigma, St. Louis, USA) and heparin (Diosynth, Oss, The Netherlands) were dissolved in water and the pH was adjusted to pH 5.1-5.2. After addition of the coupling agent EDC (1-ethyl-3-[3-dimethyl-aminopropyl]-carbodiimide), the reaction mixture was stirred for 18 hrs at room temperature. Albumin-heparin conjugates were separated from unreacted albumin and heparin by DEAE-cellulose and Cibacron Blue Sepharose chromatography. Low and high antithrombin III (AT III) affinity conjugates were obtained by fractionation with immobilized AT III[5].

Radiolabeling of Albumin-Heparin Conjugate with ^{51}Cr. Albumin-heparin conjugate (2 gm) was dissolved in 80 ml of phosphate buffered saline (PBS) (0.9% [w/v] NaCl, 10 mM NaH_2PO_4 adjusted to pH 7.4 with 0.1 N NaOH). To this solution, 2 mCi $^{51}CrCl_3$ (New England Nuclear, Boston, MA) dissolved in 100 μl of 0.5 N HCl was added and the pH was adjusted to pH 7.4 with 0.1 N NaOH. Quantitative binding of the $^{51}Cr^{3+}$-ion on the albumin-heparin conjugate occurs within 15 mins. A stock solution of radiolabeled albumin-heparin conjugate (20 mg/ml, specific activity 1 μCi/mg) was obtained by diluting the former solution with PBS up to 100 ml. This stock solution was stored at 4°C. The stability of the ^{51}Cr labeled albumin-heparin conjugate was determined at various times using a Sephadex G25 (Pharmacia Fine Chemicals, Uppsala, Sweden) gelfiltration column. After 10 days storage, free ^{51}Cr was not detected in the stock solution of conjugate by the chromatographic procedure used.

Adsorption and Desorption Studies. The adsorption of albumin-heparin conjugate onto Silastic® (Dow Corning, Midland, MI), polyvinyl chloride (PVC, Bentley Laboratories, Irvine, CA), Biomer® (Ethicon Co., Somerville, NJ) and Cellulose acetate (CA acetyl content 39.8%, Aldrich Chemical Co., Milwaukee, WI) was measured using ^{51}Cr labeled albumin-heparin conjugate prepared as described above. A solution of ^{51}Cr labeled albumin-heparin conjugate with the appropriate concentration (1 ml) was added to 1 ml of deaerated PBS containing a piece of polymer film (1 cm^2). During the addition the polymer film was completely submersed in the PBS solution. After the required adsorption time, 1 ml of the solution was removed and 5 ml of PBS was added. Subsequently, 5 ml of the solution was removed and 5 ml of fresh PBS were added. This procedure was repeated 4 times. During the washing procedure, special attention was paid to prevent the polymeric films contacting the air-solution interface. Finally, polymer films were removed from the tubes and washed for one minute in a

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beaker containing 2 L of PBS. This procedure was followed to avoid formation of a Langmuir-Blodgett layer on the material surfaces. The radioactivity on the polymer film was measured with a gamma counter (Beckman). Assuming that no preferential adsorption of labeled albumin-heparin conjugate had occurred, the surface concentration of albumin-heparin conjugate was calculated using the following equation:

$$\text{Surface concentration of albumin-heparin conjugate } (\mu g/cm^2) = \frac{\text{Counts* per } cm^2 \text{ of polymer}}{\text{Counts* per } \mu g \text{ of albumin-heparin conjugate}}$$

*Corrected for background

For the determination of the rate of adsorption, a bulk concentration of albumin-heparin conjugate of 2 mg/ml was used and the adsorption time was varied (15 mins-5 hrs). Adsorption isotherms of albumin-heparin conjugate were determined by varying the bulk concentration of albumin-heparin conjugate (0.2, 0.5, 1.0, 2.0, 5.0 and 10 mg/ml) and applying adsorption times of one hour. For the determination of the desorption of albumin-heparin conjugate, polymer films preadsorbed with ^{51}Cr labeled albumin-heparin conjugate (2 mg/ml, one hour) were contacted with a solution of citrated (3.8% w/v) bovine plasma 1:9 (v/v). The remaining surface concentration of albumin-heparin conjugate was determined at different time intervals (15 mins-24 hrs). All adsorption and desorption studies of albumin-heparin conjugate were performed at room temperature.

The films of Biomer and PVC were prepared by casting solutions of 10% (w/w) and 15% (w/w) in dimethyl acetamide, respectively, on glass plates. The solvent was removed in an oven for 6 hrs at 60°C followed by vacuum drying overnight at the same temperature. A CA film was obtained by casting a 10% (w/w) solution in acetone on a glass plate. The solvent was removed by slow evaporation for 6 hrs at room temperature in an acetone atmosphere. Pieces of Silastic were washed for 24 hrs with double distilled water before use. Glass (glass plates used for TLC, Merck, F.R.G.) was precleaned in chromic acid for 24 hrs and subsequently extensively washed with double distilled water before use.

In Vitro Platelet Adhesion Studies. The adhesion of blood platelets on materials preadsorbed with albumin-heparin conjugate was studied at 38°C using 2 types of laminar flow cells (dimensions: Figure 2) as described by Olijslager[10,11]. A healthy dog was anesthetized with nembutal and a 14 GA 2 inch Angiocath® teflon catheter (The Deseret Co., Sandy, UT) was inserted into the jugular vein. Blood samples of 25 ml were drawn using a double syringe technique and 2.5 ml of a 3.8% (w/v) sodium citrate solution, adjusted to pH 7.4, was added to prevent coagulation. Blood was pumped with a rate of 2 ml/min/cell through 2 identical cells. One cell contained a material treated for one hour with a solution of 5 mg/ml of albumin-heparin conjugate in PBS and the other cell was equipped with the same material which was pretreated with PBS for one hour. Before blood contact, both cells were flushed for 5 mins with PBS with a rate of 2 ml/cell/min. PBS was then replaced by blood avoiding an air/blood interface. After 5 mins of blood contact, the cells were perfused for 10 mins with PBS (2 ml/min/cell) followed by a 2% solution of glutaraldehyde in PBS to fix adhered cells. After one hour fixation the cells were flushed with double distilled water and disassembled. The surfaces were air dried and the adhered blood platelets were stained with a 0.1% cresylviolet solution in water. Of each material, the adhered blood platelets of 30 areas (10 at each position as shown in Figure 2) of 1000 μm^2 were counted with a phase contrast microscope (Nikon Biophot Research Microscope). A magnification of X1000 with oil immersion was used. The average blood platelet number on a surface treated with albumin-heparin conjugate was divided by the average blood platelet number of the corresponding untreated material yielding a blood platelet adhesion ratio. The L-cell (wall shear rate 0.31 $secs^{-1}$, Figure 2[10]) was used for the determination of blood platelet adhesion ratios on glass, Biomer, PVC and CA pretreated with albumin-heparin conjugate, whereas the O-cell (wall shear rate 30 $secs^{-1}$, Figure 2) was used for the determination of blood platelet adhesion ratios on films of Biomer, PVC, CA and Silastic pretreated with either albumin-heparin conjugate, high affinity conjugate, low affinity conjugate or albumin.

Blood Platelet Morphology. The morphology of adhered blood platelets on Silastic with or without albumin-heparin conjugate, was studied with a Jeol (type 35 CF) scanning electron microscope. Surfaces were dehydrated through ethanol/water mixtures[10]. For the morphology studies only the L-cell was used.

Statistical Analysis. The significance level of determined average platelet adhesion numbers was calculated using Fisher's t distribution (paired variates)[12].

RESULTS

Figures 1a-c shows the adsorption and desorption characteristics of ^{51}Cr labeled albumin-heparin conjugate on different surfaces. PVC, Silastic and Biomer showed a strong resemblance with respect to adsorption and desorption data, whereas CA behaved differently. The plateau value for adsorption of albumin-heparin conjugate onto CA is reached within 15 mins, whereas for the other polymers 30 to 60 mins are required (Figure 1a). At a bulk concentration of albumin-heparin conjugate of 10 mg/ml, for CA a surface concentration of 0.2 $\mu g/cm^2$

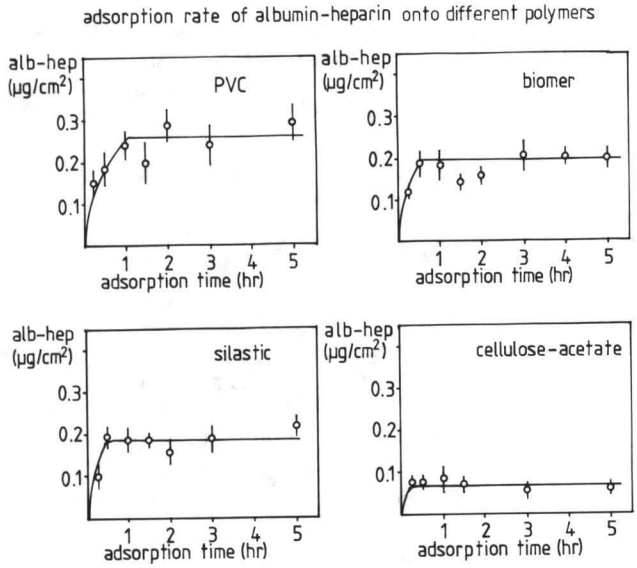

Figure 1a. Adsorption of albumin-heparin conjugate onto different polymer surfaces as a function of time. All points are M ± SD of 6 to 9 measurements using ^{51}Cr-labeled conjugates.

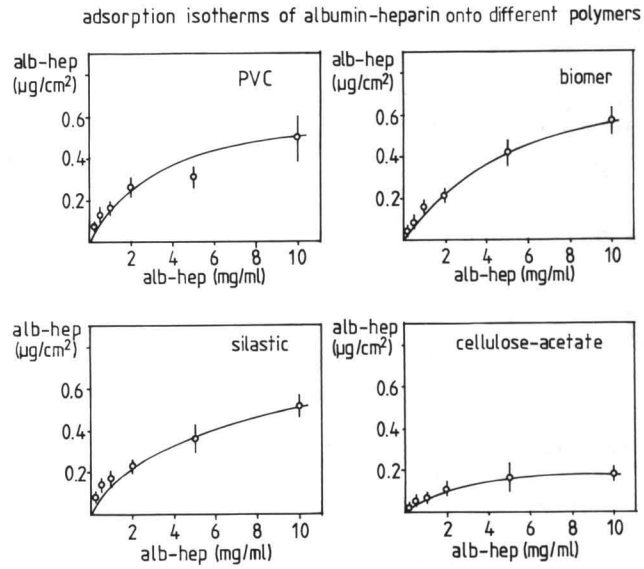

Figure 1b. Adsorption isotherms of albumin-heparin conjugate onto different polymers using ^{51}Cr labeled albumin-heparin conjugate. All points are M ± SD of 6 to 9 measurements.

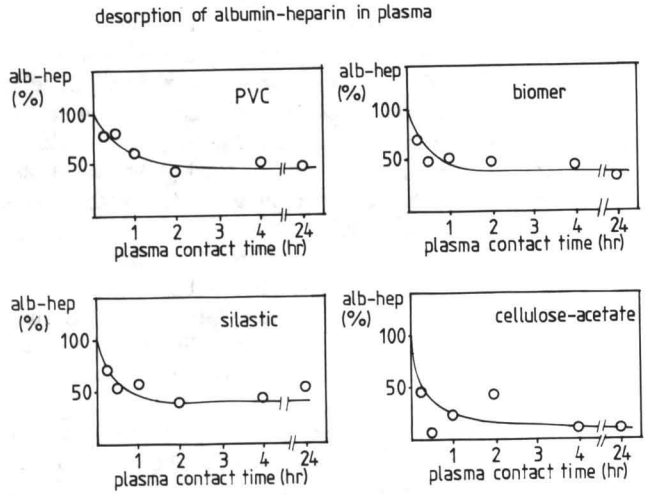

Figure 1c. The desorption of albumin-heparin conjugate from different surfaces in the presence of bovine plasma as a function of time.

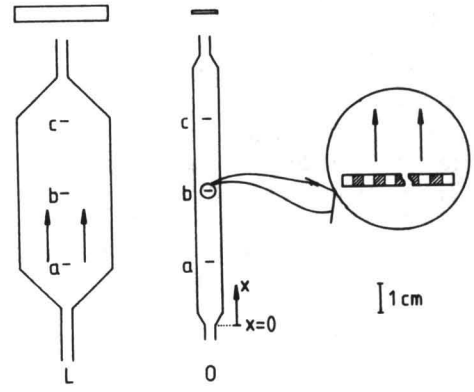

Figure 2. The dimensions of the L-type and the O-type cell. Also indicated are the sites (a,b,c) where the platelet adhesion numbers are determined. From reference 10.

is observed; for the other polymers this value was approximately 3 times higher (Figure 1b). In contact with blood, preadsorbed albumin-heparin conjugate gradually released from the surface. During the first 2 hrs of contact between plasma and PVC, Biomer or Silastic preadsorbed with albumin-heparin conjugate, 40 to 60% of the amount of conjugate present before plasma contact was exchanged by plasma proteins and no further exchange was observed. On the other hand, almost complete exchange of preadsorbed conjugate on CA occurred within 4 hrs (Figure 1c).

Table I presents the results of blood platelet adhesion onto untreated and albumin-heparin conjugate pretreated materials, obtained with sets of laminar flow cells of the L and O-type. Except for Silastic, a statistically significant ($p < 0.05$) reduction in numbers of adhered blood platelets was measured when treated and untreated materials were compared using the O-cell, a reduction of the number of adhered blood platelets on surfaces preadsorbed with albumin-heparin conjugate was also observed. However, the decrease was only statistically significant for Biomer and glass.

TABLE I. (MEAN ± SD) BLOOD PLATELET ADHESION NUMBERS ON DIFFERENT MATERIALS (UNTREATED AND ALBUMIN-HEPARIN CONJUGATE TREATED)

Material	Blood Platelet No. (per 1000 m^2) Untreated	Treated	Mean Adhesion Ratio	Number of Experiments	p value	Cell Type Used
Biomer	10.9 ± 5.9	7.0 ± 2.9	0.64	12	0.02	O
Biomer	2.2 ± 0.9	0.8 ± 0.9	0.36	5	0.05	L
PVC	9.4 ± 3.4	4.9 ± 2.4	0.52	6	0.05	O
PVC	2.1 ± 1.7	1.4 ± 0.6	0.67	4	0.3	L
CA	24.1 ± 4.4	16.9 ± 1.9	0.70	5	0.01	O
CA	3.4 ± 0.5	3.0 ± 0.9	0.88	5	0.4	L
Silastic	7.6 ± 4.0	5.0 ± 1.8	0.66	5	0.1	O
Glass	2.6 ± 0.7	1.6 ± 0.7	0.62	4	0.05	L

The blood platelet adhesion on materials pretreated with albumin and albumin-heparin conjugates with different affinities for AT III was investigated using the O-cell (Figure 3). As mentioned above, treatment of materials with unfractionated albumin-heparin conjugate led to a statistically significant (except for Silastic) reduction of adhered blood platelets. By precoating surfaces with high affinity albumin-heparin conjugate, the numbers of adhered blood platelets were equally reduced as observed with unfractionated conjugate, except for Silastic. Using the latter surface, high affinity conjugate showed lower numbers of adhered platelets compared with unfractionated conjugate. Blood platelet adhesion numbers observed on low affinity albumin-heparin conjugate pretreated materials were not statistically different significantly from the corresponding untreated materials. Precoating of materials with albumin led to reduction of adhered blood platelets. However, no statistical calculations could be made because of the small number of experiments.

With the SEM, no significant differences were seen in morphology of blood platelets adhered on surfaces with albumin-heparin conjugate and untreated materials. There was also no significant difference in numbers of blood platelet aggregates on treated and untreated materials.

DISCUSSION

Adsorption of albumin and other plasma proteins onto various materials has been studied using radiolabeled (i.e. ^{125}I and ^{131}I) proteins[13-19]. However, different results were obtained for the type of adsorption isotherms (i.e. Langmuir or high affinity) and plateau values. Although preferential adsorption of radiolabeled proteins has been observed[19], presently, no alternative method is available for the quantitative measurement of protein adsorption onto polymer films. For the adsorption and desorption studies of albumin-heparin conjugate on PVC, Biomer, Silastic and CA, we applied the ^{51}Cr label, because on glass, no preferential adsorption of ^{51}Cr labeled albumin-heparin conjugate was detected[6]. Linear relationships ($r > 0.97$) were obtained when the logarithm of the solution concentration of albumin-heparin conjugate is plotted versus the logarithm of the surface concentration of albumin-heparin conjugate. From Figures 1a-c it was concluded that PVC, Biomer and Silastic surfaces behaved similarly, whereas CA deviated with respect to its rate of adsorption of albumin-heparin conjugate, the maximum amount of adsorbed albumin-heparin conjugate and the rate of desorption of adsorbed conjugate in plasma. This may be ascribed to the fact that PVC, Biomer and Silastic are rather hydrophobic polymers, whereas CA is more hydrophilic.

The effect of heparin in solution on the aggregation of blood platelets as well as the effect of immobilized heparin on the adhesion of blood platelets is still not fully understood. Salzman et al[20] demonstrated that heparin enhances ADP-induced aggregation of blood platelets in platelet rich plasma. Salzman et al showed that for

heparin fractions with high affinity for AT III this platelet aggregation activity was lower than for fractions with low affinity for AT III. On the other hand, Yamamoto et al[21] reported opposite findings.

The adhesion of blood platelets onto heparinized biomaterials has been the subject of many conflicting reports. Compared with control materials, both increased[22,23] and decreased[24,25] platelets adhesion onto heparinized materials have been observed. This can be ascribed to the fact that different investigators used different method for heparinization of materials as well as different test systems for the measurement of blood platelet adhesion. Table I shows that treatment of materials with albumin-heparin conjugate led to a reduced number of adhered blood platelets during the initial contact of citrated canine blood and foreign material. This indicated that surface bound albumin-heparin conjugate had a protective effect on the adhesion of blood platelets. The observed decrease in the number of adhered blood platelets was lower for CA than for the other materials tested, which might be partially ascribed to the lower surface concentration of albumin-heparin conjugate on CA compared with the other materials tested. Another explanation may be the different conformation of albumin-heparin conjugate on CA compared to the other surfaces. From Figure 3 the following general tendency can be observed. For all the polymers tested, precoating of these polymers with unfractionated albumin-heparin conjugate led, as mentioned before, to a significant decrease in platelet adhesion numbers. Approximately, the same reduction was observed for high affinity albumin-heparin conjugate, whereas the blood platelet numbers on materials preadsorbed with low affinity albumin-heparin conjugate were not significantly different from the corresponding untreated materials. Albumin preadsorbed onto the materials slightly reduced the adhesion of platelets; however, not enough experiments were performed for a statistical analysis. The results obtained indicate that complexes of AT III and either high affinity or unfractionated conjugate formed at the material surface after contact with blood, reduced the platelet adhesion. This is in agreement with the results published by Lindon et al[26] for heparin immobilized on agarose beads. Our results also support the observations made by Ebert and Kim[27] that immobilized heparin is covered with a layer of adsorbed proteins, probably AT III, which prevents direct contact between immobilized heparin and platelets.

CONCLUSION

The results presented here show that albumin-heparin conjugate preadsorbed surfaces reduce initial blood platelet adhesion. Albumin-heparin conjugates can be used to improve the intrinsic blood compatibility of materials which are used in contact with blood. The adsorbed conjugate can prolong clotting time and also reduce platelet adhesion.

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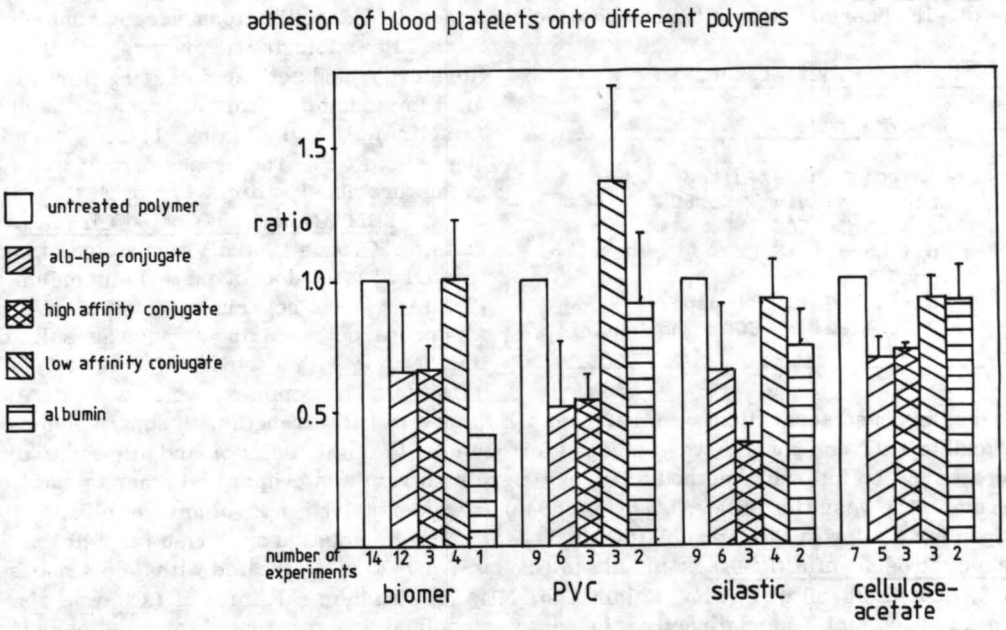

Figure 3. Mean platelet adhesion ratios (± SD) for different materials pretreated with different albumin-heparin conjugates and albumin. The platelet adhesion numbers for the untreated materials are normalized to 1.00.