The Effects of Heparin Bound Surface Modification (Carmeda® Bioactive Surface) on Human Platelet Alterations During Simulated Extracorporeal Circulation

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Abstract

To determine if treatment with covalently bound heparin (Carmeda Bioactive Surface (CBAS)) to the synthetic surface of the extracorporeal circuit (ECC) would alter the stereotypic pattern of adverse platelet alterations, 450 ml of heparinized blood (IU/ml) was recirculated at a flow rate of twice the circulating volume (L/min) for 2 hrs at 37°C through either untreated (CONT, n=7) or treated (CBAS, n=7) circuits constructed of identical components including a pediatric (0.8m²) reversed hollow fiber membrane oxygenator. In CONT circuits, platelet count maintained 88±1% (x±SEM) of its initial level in the circuit prime sample, dropped to 36±6% after 5 min, and returned to 56±2% following 2 hrs of ECC. In CBAS circuits, platelet count in the circuit prime sample demonstrated 90±4%, decreased to 68±10% after 5 min (p<0.05) and declined further to 45±5% after 2 hrs (NS).

Although platelets from both groups retained reactivity to ADP after priming the circuit, only at 5 min of recirculation did CBAS circuits significantly preserve this responsiveness. In CONT circuits, baseline plasma levels of platelet factor 4 rose from 24±3 to 581±82 ng/ml in the primed circuit and continued to rise to 2933±276 ng/ml by 2 hrs of ECC. In contrast, CBAS circuits markedly reduced this release after 2 hrs (577±165 ng/ml). Furthermore, by 2 hrs of ECC, plasma levels of thromboxane B₂ in the CBAS circuits were significantly reduced when compared to CONT circuits (3035±1529 vs 2991±16293 pg/ml, respectively). We conclude that CBAS modification of the simulated extracorporeal circuit preserved the initial circulating platelet count with retained ADP reactivity and markedly decreased release of platelet factor 4 and thromboxane B₂.

Introduction

Contact between blood and the synthetic surfaces of an extracorporeal circuit results in plasma protein absorption with subsequent platelet activation (1,2). The combination of platelet activation and the need for systemic anti-coagulation contributes to the prolonged postoperative bleeding times which can increase chest tube drainage following cardiopulmonary bypass (3-5). Modification of the reactive synthetic
surface may help to prevent these untoward complications. Consistently, much research has focused on attempts to alter surface composition to improve biocompatibility and thus reduce platelet adhesion (6,7). One such attempt has been to covalently link heparin via an end point attachment, resulting in a surface modification termed Carmeda Bioactive Surface (8). Since surface composition affects plasma protein binding, which in tum mediates platelet activation, we evaluated the effects of this modification on platelet alteration during simulated extracorporeal circulation.

**Materials and Methods**

**Perfusion Circuits**

Perfusion circuits with a surface area of 1.5 m² were assembled from standard medical grade polyvinylchloride tubing, polycarbonate connectors, venous reservoir bag, and a 0.8 m² pediatric reversed hollow-fiber membrane oxygenator. Blood and gas compartments were flushed with 100% carbon dioxide for 15 min prior to priming. Tubing and oxygenators were primed by permitting whole blood to enter the circuit by gravity flow. Blood was recirculated by a precisely shimmed, barely occlusive, calibrated double-roller pump for 2 hrs at a rate of two times the circulating volume in liters per minute.

Blood temperature was maintained at 37°C. The oxygenator was ventilated with a 95% oxygen/5% carbon dioxide mixture at a rate of one liter per minute. For seven experimental circuits, the surfaces of the oxygenator and all components of the perfusion circuit were treated with Carmeda Bioactive Surface. Control circuits (n=7) were untreated. Otherwise all circuits were handled identically.

**Sample Acquisition**

These studies were approved by Temple University Committee on Human Investigations and the National Institutes of Health. Written and verbal informed consent were obtained from each volunteer.

Blood for recirculation trials was drawn from volunteers who abstained from anti-platelet medication for at least two weeks prior to donation. All blood samples were drawn into syringes containing 3.8% trisodium citrate (9:1 V/V). Four hundred and fifty ml of blood were drawn directly into a venous reservoir containing one U/ml of beef lung heparin and 1.65 gm of glucose. One U/ml of heparin is the lowest amount of heparin necessary to allow recirculation through the simulated loop without evidence of macroscopic clot formation. Two 25 ml baseline samples were obtained directly from the volunteer and incubated at 37°C. Prior to the start of recirculation, a 25 ml circuit prime sample was drawn from the venous reservoir. Additional 25 milliliter samples were withdrawn from the circuit at 5 min and 2 hrs of recirculation and
Platelet Studies

Platelet Counts

Whole blood platelet counts were obtained with a Coulter ZBI cell counter (this needs a citation) and checked when necessary by phase microscopy (9).

Platelet Aggregation

Platelet-rich plasma (PRP) was prepared from aliquots of citrated whole blood by centrifuging samples at 150 g for 10 min at 25°C. Following gentle aspiration of the PRP (350,000±50,000 platelets/µl), the remaining blood was centrifuged at 12,000 g for five min at 25°C in a microcentrifuge to obtain platelet-poor plasma (PPP) with a platelet count less than 1000/µl (10). Platelet aggregation studies were performed as described previously (11). We determined that the platelet release reaction is complete when ADP-induced aggregation exceeds 62% light transmission through PRP at five minutes (12). The threshold concentration of aggregating agent is defined as the lowest concentration necessary to produce irreversible or second wave aggregation, indicating that platelet granule release has occurred. This value is determined for the baseline incubated samples and used to test the responsiveness of the circuit prime and recirculated platelets at the corresponding time points. Percent reactivity to ADP is then expressed as the response of the circuit prime or recirculated platelets divided by the response of the incubated baseline platelets. Normal platelet reactivity was demonstrated in baseline samples for all studies using 1-5 uM ADP (13).

Platelet Factor 4

The appearance of the platelet-specific protein platelet factor 4 (PF₄) in plasma was used to indicate the occurrence of the platelet release reaction. Aliquots (2.7 ml) of citrated whole blood were transferred to plastic tubes containing 10% disodium ethylenediaminetetraacetic acid (EDTA), 5.4 mg/ml theophyllin, and 3x10⁻³ M PGE₁ and immediately centrifuged at 2000 g for 20 minutes at 4°C to obtain platelet-poor plasma. The PPP was again centrifuged at 12,000 g at room temperature for two minutes in a microcentrifuge (14). Plasma levels of PF₄ were quantitated by radioimmunoassay with a specific antibody as previously described (15). The sensitivity of this assay is 1 ng/ml.

Thromboxane B₂

Thromboxane B₂ (TXB₂), the stable end-product of thromboxane A₂, is a potent platelet activator and vasoconstrictor. Aliquots (3.7 ml) of citrated whole blood were transferred to a plastic tube containing EDTA for a final concentration of 10 mM and spun for PRP. Prior to high speed centrifugation (2000 g at room temperature for 10 min) to prepare PPP, enough indomethacin dissolved in 100% ethyl alcohol was added to give a final concentration of 10 uM (14).

Plasma levels of TXB₂ were measured by radioimmunoassay...
say with a specific antibody as previously described (16). The sensitivity of this assay is 25 pg/ml.

**Statistical Analysis**

Mean, standard deviation, and standard error of the mean were calculated for each determinant at each sampling period. One-way analysis of variance was performed in each oxygenator group to determine significance over time. A Duncan’s multiple range t test was used to determine significance between untreated and treated oxygenator groups at a specific time point. A p value of <0.05 was considered significant. A Mann-Whitney-U test was used in cases of unequal variances.

**Results**

**Platelet Count**

Mean platelet counts (Figure 1) are expressed as a percentage of the initial platelet count obtained from baseline samples drawn directly from the volunteers. In CONT circuits (n=6), platelet count after circuit prime was 88±1% (mean±SEM), and decreased to 36±6% after 5 min of recirculation, returning to 56±2% after 2 hrs (p<0.05). Compared to CONT circuits, platelet counts at corresponding time periods in CBAS circuits (n=7) were 90±4% (p=NS), 68±10% (p<0.05), 45±5% (p=NS) after prime, 5 min and 2 hrs of recirculation, respectively. Thus, the CBAS treatment reduced the early platelet depletion during recirculation.

**Platelet Aggregation**

In CONT circuits (n=7), platelet reactivity to ADP (Figure 2), expressed as a percentage of baseline activity, decreased to 60±12% after circuit prime, declining further to 28±9% and 22±5% after 5 min and 2 hrs of recirculation, respectively. In CBAS circuits (n=7), priming decreased platelet responsiveness to 68±10% (p=NS) while platelet reactivity was 56±9% (p<0.05) after 5 min and 40±8% (p=NS) after 2 hrs of recirculation. Early loss of platelet reactivity to ADP was therefore delayed by the CBAS surface treatment.

**Platelet Factor 4 Levels**

In CONT circuits (n=6), baseline plasma PF4 levels (Figure 3) were 24±3 ng/ml, but increased to 581±82, 1650±220, and 2933±276 after priming, 5 min and 2 hrs of recirculation, respectively (p<0.05). PF4 levels in CBAS-treated circuits (n=7) rose from a baseline value of 37±7 ng/ml to 449±64, 492±86, and 577±165 after priming, 5 min and 2 hrs of recirculation, respectively (p<0.05). At 5 min and 2 hrs of recirculation, PF4 levels in CBAS-treated circuits were significantly less (p<0.05) than in CONT circuits.

**Thromboxane B2 Levels**

In CONT circuits (n=6), the baseline plasma thromboxane B2 level (Figure 4) was 333±94 pg/ml. Thromboxane B2 levels were 428±110 after priming, and 3997±3123 and 29,916±16,293 following 5 min and 2 hrs of recirculation, respectively. The thromboxane B2 levels in CBAS-treated circuits (n=7) were 589±191 at baseline and 779±236, 542±184, and 3035±1529 after priming, 5 min and 2 hrs of recirculation, respectively. Thromboxane B2 levels were lower after two hrs of recirculation in CBAS circuits when compared to CONT circuits (p<0.05).

**Discussion**

Adverse platelet alterations resulting from exposure of blood to the synthetic surfaces of the extracorporeal circuit result in prolonged postoperative bleeding times and can increase chest tube drainage following cardiopulmonary bypass (1,17,18). These alterations result from platelet interaction with the adsorbed plasma proteins on the synthetic surface. Within seconds of blood contact with the synthetic surface an absorbed and highly reactive protein layer of approximate thickness of 200 angstroms results (1). This layer is composed primarily of reactive fibrinogen which bridges the platelets to the synthetic surface (19). Platelets will then adhere, undergo shape change and finally release their granule constituents while generating thromboxane A2 (1,20). The latter two phenomena serve to further propagate the platelet response.

Attempts to ameliorate these predictable patterns of platelet activation have focused on either pharmacologic change of the hemostatic response or modification of the synthetic surface. Unfortunately, both approaches have had limitations. The use of prostaglandins and their analogues, for example, has been associated with intraoperative hypotension (13,21). Use of agents which interfere with coagulation, such as ancrod, have resulted in increased post-operative bleeding (22). Attempts to produce a less reactive artificial surface have included alterations of the protein adsorbate and changes in the molecular composition of the polymer. However, neither approach has produced the sought after “holy grail” of true biocompatibility (23). For example, while adsorption of albumin to the extracorporeal circuit decreases surface affinity for platelets in-vitro, in clinical trials using a membrane oxygenator, the salutary effects have been less encouraging (6,24).

Recently, interest has focused on a new technique to covalently bind heparin to synthetic surfaces in order to reduce systemic heparin levels. Heparin serves as a catalyst to bind thrombin and antithrombin III (AT III), preventing clot formation. Ionic attachment of heparin to the surface, such as the complex formed with tridodecylmethylammonium chloride (TDMAC-heparin), has demonstrated poor stability during blood contact. Previous attempts at non-directed covalent bonding of heparin to the surface caused steric interference with the AT III receptor site and were ineffective in inactivating
thrombin (25). The Carmeda process, which covalently links heparin via a spacer molecule to the surface, preserves orientation of the active site. In-vitro studies have demonstrated both the stability of the heparin preparation and its efficacy in catalyzing the irreversible binding and inhibition of thrombin by AT III (8,26).

Our studies indicate that the Carmeda Bioactive surface modification did transiently preserve both circulating platelet count and reactivity to ADP. The surface modification also caused a sustained reduction in platelet alpha granule (PF 4) release, and markedly reduced thromboxane B2 generation. Clearly, platelet affinity for the surface was changed during the early phases of blood contact. Whether this was due to direct effects on platelet membrane surface affinity or was mediated by alterations in the interaction of the surface with plasma proteins remains unknown.

Since platelet factor 4 is an anti-heparin protein which neutralizes heparin (20), it is possible that the observed decrease in platelet factor 4 may reflect its binding to the CBAS-treated surfaces. Because of this property of platelet factor 4, specificity studies have been performed on normal human plasma prior to and after the addition of 1, 10, and 50 U/ml of heparin with no effect on the assay (Written Communication, Abbott Laboratories).

Nevertheless, additional platelet release markers with less affinity for heparin, such as beta thromboglobulin, should be measured.

In summary, the Carmeda Bioactive Surface modification partially reduced platelet activation in the simulated extracorporeal circuit. Interestingly, this treatment caused a dissociation between surface-induced platelet adhesion and surface-induced platelet activation. Further studies focused on the effects of this surface modification on platelet membrane receptor expression should reveal additional information and, ultimately, a mechanism to account for the fundamental interaction between platelets and synthetic surfaces.

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