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CD41 Western blotting: a new method to detect platelet adhesion to artificial surfaces used in extracorporeal circulation procedures

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Abstract Cardiopulmonary bypass (CPB) surgery is associated with platelet activation and reduced platelet counts due to artificial surface activation of blood elements and non-physiological flow-patterns. As shown in former studies, coating of medical devices can improve hemocompatibility in extracorporeal circulation systems. In this study, we demonstrate a new method to determine platelet adhesion on 18 coated and non-coated membrane oxygenators in a simulated CPB model with CD41 Western blot. Platelet loss and the release of β-TG (platelet activation marker) were determined during a 120 min recirculation phase. At the end of the run the membrane oxygenators (with tubing system) were rinsed and the amount of adsorbed proteins on the surface was analyzed by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting technique. Uncoated devices showed significantly higher concentrations of CD41 and of fibrinogen adsorption compared to the coated membrane oxygenators. These results correspond with the release of β-TG and platelet loss indicating less platelet adhesion on the coated oxygenators compared with the uncoated group. This new method may be useful in choosing less platelet activating materials for all kind of blood contacting devices to improve thrombogenicity including platelet functionality.

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1 Introduction

Equipment used for extracorporeal circulation (ECC) systems contains a wide spectrum of synthetic materials [1–6]. Although these products possess excellent physical properties, they were primarily developed for industrial use and only later found their way into biomedicine. Thus all these synthetic materials display more or less the same disadvantage: an incompatibility with blood and tissues [7, 8].

Oxygenators are believed to represent the greatest challenge to host inflammation system during cardiopulmonary bypass (CPB) due to the large blood contacting surface (0.6–2 m^2) area and relatively slow blood flow (<3 cm/s). The improvement of biocompatibility of CPB circuits therefore becomes one of the critical factors that affect the performance of CPB circuits. Underlying the response of the organism to the CPB equipment is the initial protein adsorption and subsequent cell interaction at the foreign material–blood interface.

The contact of blood with a synthetic surface leads to plasma protein formation on the surface within seconds. The adsorption of plasma proteins controls the subsequent platelet adhesion by interaction with different platelet-adhesive receptors like glycoprotein (GP) IIb/IIIa and GP Ib. Proteins bound on the surfaces like fibrinogen and von Willebrand factor (vWF) contain the RGD sequence (arginine-glycine-aspartic acid) which possesses a high affinity for GP IIb/IIIa [9]

The adhesion of platelets to artificial surfaces is one of the primary events taking place in the blood–biomaterial interface that influences the hemocompatibility of medical devices. Therefore a number of strategies for detecting platelet function during CPB have been developed, e.g. platelet count, enzyme immunoassays [10–13],

microscopical [14–16] or flow cytometric [14, 15, 17, 18] methods.

Nevertheless until now, the detection of direct platelet adhesion and aggregation on artificial surfaces for evaluation of the hemocompatibility of medical devices was only possible by inaccurate microscopical methods. Equipment like oxygenators or dialysis filters with a huge amount of fibres inside, are especially difficult to analyze. In this study we have developed a reproducible technique for quantification of platelet adhesion on membrane oxygenators (including the tubing system) by using a non-blocking, monoclonal antibody against the glycoprotein receptor GP IIb/IIIa (CD41) of the platelet membrane.

2 Methods

2.1 HLM-model

An established HLM-model was used to simulate an ECC [19]. In brief: During a period of 120 min a 500 ml volume of fresh human heparinized blood (3 IU Liquemin® per ml machine filling volume, Hoffmann-La Roche, Basel, Switzerland) was circulated in this closed system using a roller pump (Sarns Inc., Ann Arbor, MI, USA). A constant blood flow of 3 l/min and a mean arterial pressure of 60 mmHg were maintained while a hypothermia apparatus (Typ Q 102, Haake, Berlin, Germany) held the temperature of the arterial oxygenator exit at 28°C. A mixture of 77% N_2, 20% O_2 and 3% CO_2 provided oxygenation. According to clinical ECC conditions we used a priming volume consisting of 78.3 ml glucose solution 5% (Delta Pharma, Pfullingen, Germany), 15.7 ml sodium bicarbonate 8.4% (Braun Melsungen AG, Melsungen, Germany) and 206 ml Ringer's Lactate (Schiwa, Glandorf, Germany).

Eight blood samples were taken from every trial run, the first one directly from the blood bag (control) and the other seven from the arterial exit of the oxygenator at 1, 5, 10, 20, 30, 60 and 120 min, respectively.

The trial runs consisted of 18 membrane oxygenators of the type Quadrox (Jostra Medizintechnik, Hirrlingen, Germany), divided into three groups: 6 of them had the BioLine heparin-coating, 6 had the SafeLine surface improvement and other 6 were non-coated (control). These control oxygenators are not commercially available.

2.2 SDS-PAGE and immunoblotting

Surface-adsorbed fibrinogen and platelet receptor CD41 were measured by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and the Western blotting technique.

In brief: after 120 min recirculation of fresh human blood in the HLM-model, the oxygenators (including the tubing system) were rinsed with 10 l physiological saline solution (Fresenius Kabi, Homburg, Germany) followed by washing steps with 1 l phosphate-buffered saline (PBS) containing 20 mM EDTA (pH 7.4). In order to determine the time dependent efficiency of protein elution, aliquots were taken from the elution buffer at various times and examined for protein content. In this kinetic experiment (up to 180 min) we found that an elution time of 5–10 min was sufficient to reach a maximum of protein concentration in the eluates (data not published). Therefore we used an elution time of 30 min in all following experiments.

Subsequently, the surface-adsorbed proteins were eluted with 0.5 l lithium dodecyl sulfate (1% [w/v] in PBS). In former experiments, a further elution step with 1 M NaOH confirm complete elution of the adsorbed proteins by LDS. The eluted plasma proteins were pooled, concentrated by ultrafiltration (Centriprep-10, Amicon, Inc., Beverly, MA, USA), assayed for protein concentration, aliquoted, snap-frozen in liquid nitrogen and stored at −20°C. After being concentrated by ultrafiltration, all samples showed identical volumes. This was essential because we wanted to use the exact same volumes of eluates from coated and uncoated oxygenators for the following electrophoresis and blotting experiments.

Protein concentrations were determined by Bradford's method [20] with bovine serum albumin as standard. The samples were stained in microtiter plates (10 µl with 200 µl staining solution) and the adsorbance was determined with a microplate reader (Dynatech, model MR 5000) at 595 nm. We used the samples prepared in this manner without further processing for electrophoresis and immunoblotting.

All electrophoresis reagents were obtained from Biorad (Munich, Germany). The proteins were separated by reduced SDS-PAGE (separating gel 8%, stacking gel 4%) according to Laemmli [21] and transferred by semidry-blotting onto a nitrocellulose membrane (Probind 45 NC, Pharmacia, Uppsala, Sweden). To saturate unoccupied sites, the "blots" were incubated in 5% nonfat dry milk (Sigma) and probed with antibodies to the plasma proteins GP IIb/IIIa and fibrinogen using a double antibody method where the secondary antibody was conjugated with alkaline phosphatase for detection. We used the substrate system 5-bromo-4-chloro-3-indolyl phosphate (BCIP), prepared as described by the supplier (Sigma), to develop a colour reaction for this enzyme.

2.3 Antibodies

CD41 monoclonal antibodies were used against the platelet receptor GP IIb/IIIa (clone SZ22 mouse anti-human,

Beckman Coulter, Krefeld Germany) and polyclonal antibodies against fibrinogen (goat anti-human fibrinogen, Sigma, Deisenhofen, Germany). Secondary alkaline phosphatase conjugated antibodies were obtained from Sigma (donkey anti-sheep IgG) and Immunotech (goat anti-mouse IgG, Marseille, France), respectively.

2.4 Cell count (platelets)

The counts of the blood cells were measured in EDTA-blood immediately after sampling using a fully automized cell counter (Axxon Lab AG, Baden-Dättwi, Switzerland).

2.5 β-Thromboglobulin (β-TG)

A sandwich enzyme-immunoassay was used for the determination of human β-TG in plasma (Boehringer Mannheim, Mannheim, Germany). In brief: platelet-deficient plasma (Anticoagulant CTAD 10% (Vol.): Citrate, theophylline, adenosine, dipyridamol was given to a microtiter plate coated with a specific antibody to β-TG ($F(ab')_2$ anti-β-TG). After the incubation time the plate was washed five times and in a second step we added POD-labeled β-TG antibodies (anti-β-TG peroxidase). The unbound POD conjugate was removed in the following washes (5 times). The activity of the wall-bound POD was determined photometrically at 492 nm after adding the chromogen *o*-phenylenediamine and urea peroxide and stopping the colour reaction with hydrochloric acid 1 mol/l. The concentrations (IU/ml) were read from a double-logarithmic calibration curve.

2.6 Statistical procedure

All values determined of the diluted samples were corrected for haematocrit to differentiate between the effects of dilution (hemodilution) and the changes in plasma protein levels based on consumption. The results were expressed as mean (M) ± standard error of mean (SEM). Statistical analysis was performed by the statistics software package SPSS (SPSS Software Inc., Chicago, IL, USA). Differences between the groups were calculated by univariate analysis of variance. We considered values of $P < 0.05$ as being significant.

3 Results

3.1 SDS elution of adsorbed proteins

The amounts of total crude protein after SDS elution were determined by Bradford's method [20] and the results indicate that heparin-coated PVC tubes adsorb less plasma proteins compared to the uncoated ones (data not shown).

3.2 Western blot

Experiments with the CD41 antibody indicated a major band with a molecular mass of approximately 116 kDa (Fig. 1) which is nearby to the molecular mass of this protein (120 kDa) according to literature [22]. Devices without surface coatings showed significantly higher concentrations of CD41 compared to polypeptide- (lane 1) and heparin-coated (lane 2) surfaces. Nevertheless, some low molecular weight fragments could be detected in the uncoated group which are nearly invisible in the eluates from the coated devices.

In contrast to the coated group we were able to detect a stronger concentration of fibrinogen (Fig. 2) in the uncoated oxygenators (lane 3). A dominant band at approximately 66 kDa and some diffuse bands especially in the uncoated oxygenators could be determined. Human fibrinogen has a molecular weight of approximately 330 kDa and is subdivided into three units with a molecular mass of 63, 56 and 47 kDa [23].

Through the means of Western blot and the modified ELISA technique it was possible to compare the amount and the activated forms of immobilized proteins on coated and uncoated surfaces. In addition, the soluble activated plasma proteins in whole blood, measured by ELISA, correlated with the adhesion proteins in the same samples.

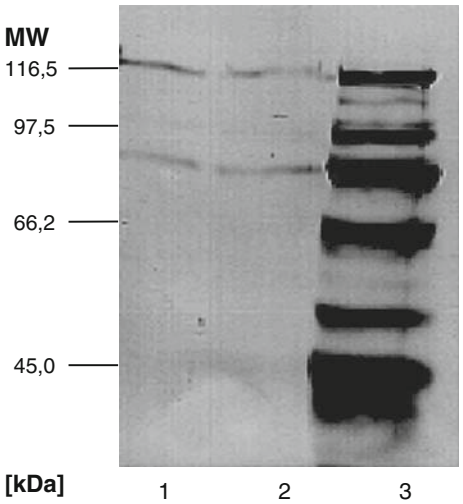

Fig. 1 Surface-bound platelet receptor GP IIb/IIIa (CD41) protein on membrane oxygenators after contact with blood (120 min) in a simulated CPB model. Proteins were eluted from (1) biopassive, (2) bioactive, and (3) non-coated surfaces and investigated by Western blot technique. Significantly more CD41 was determined on the non-coated oxygenators (lane 3) compared to surface-coated membrane oxygenators (lanes 1 and 2)

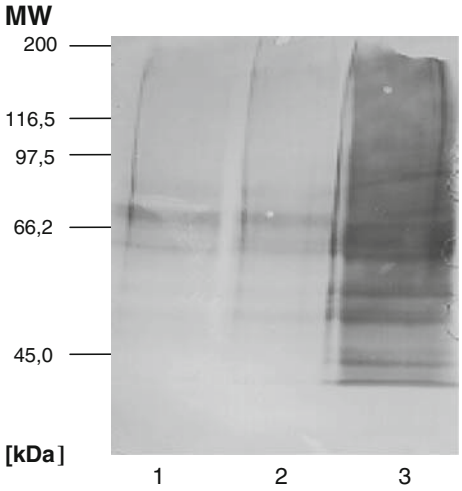

Fig. 2 Surface-adsorbed fibrinogen to membrane oxygenators following contact with blood (120 min) in a simulated CPB model. Proteins were eluted from (1) biopassive, (2) bioactive, and (3) non-coated surfaces and subjected to electrophoresis following Western blot analysis. Significantly less fibrinogen was determined on the surface-coated membrane oxygenators (lanes 1 and 2) compared to non-coated oxygenators (lane 3)

3.3 β-TG levels and platelet count

During the experiment, β-TG levels increase significantly (Fig. 4) in the uncoated group. This is caused by stronger platelet adhesion and thereby stronger activation of platelets. Contrary, both coated oxygenators groups indicate better characteristics concerning platelet activation. Platelet count in the uncoated system (Fig. 3) dropped nearly to null during CPB during the first 30 min of recirculation and then raised again. In contrast, the platelet numbers show similar characteristics in both coated oxygenators during the run in the CPB model. These results are in accordance with the Western blot experiments.

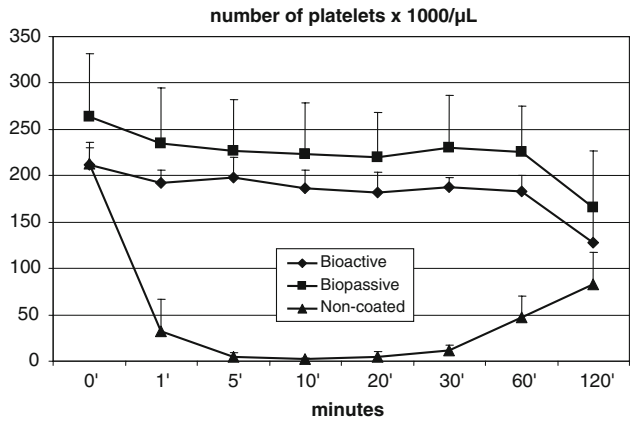

Fig. 3 Platelet counts (M ± SEM) during 120 min circulating fresh human blood in a simulated CPB model. Blood samples were taken from 0 (control), 1, 5, 10, 20, 30, 60 and 120 min respectively. After 1 min platelet count dropped extremely in the control group without coating (*black triangle*) compared to bioactive (*black diamond*) and biopassive (*black square*) coated membrane oxygenators

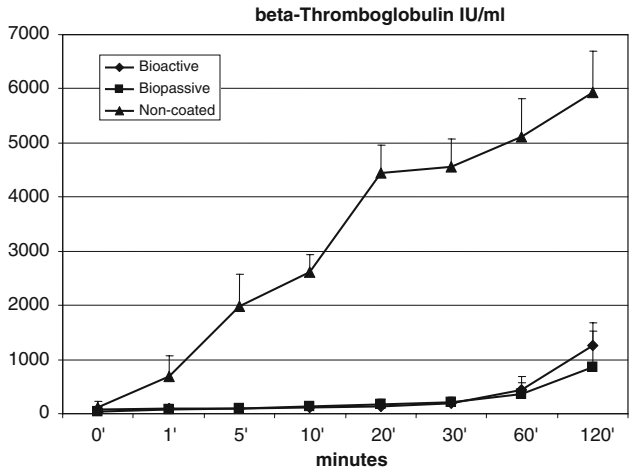

Fig. 4 Platelet activation measured by levels of β-TG (M ± SEM) in three groups of membrane oxygenators during 120 min recirculation of fresh human blood in a simulated CPB model. The β-TG concentration in the uncoated membrane oxygenators (*black triangle*) showed a continuously increase compared to the bioactive (*black diamond*) and biopassive (*black square*) coated membrane oxygenators

4 Discussion

Plasma protein adsorption plays an important role at the blood–material interface and has a strong impact on subsequent cellular interactions and clot formation. The research groups of Vroman and Brash [24–28] demonstrated that plasma proteins on artificial surfaces (e.g. F XII, fibrinogen, vitronectin, HMWK) have an important function in further thrombogenicity.

Surface bound fibrinogen has been shown to play a key role in the adhesion and activation of platelets to artificial surfaces [29]. The detection of adhered platelets on biomaterial surfaces is therefore an important test to assess thrombogenicity. For measurement of platelet adhesion the test surface is usually rinsed to remove non-adherent cells and is then analyzed by enzyme immunoassays [10–13], microscopically [14–16], or by flow cytometry [14, 15, 17, 18]. Common methods, like measuring of β-TG levels and platelet count are not sufficient to determine direct platelet adhesion to the inner surfaces of CPB circuits. Especially binding of the platelet receptor GP IIb/IIIa to the adsorbed fibrinogen is essential, because this receptor mainly mediates the adhesion of platelets to surface bound fibrinogen and thereby contributing to surface induced thrombosis [30]. Therefore we investigated different coated membrane oxygenators in a simulated CPB model by Western blot with antibodies directed against a platelet

receptor GP IIb/IIIa. Monoclonal CD41 clone SZ22 is a non-blocking antibody that reacts with the α-chain of the CD41 antigen on platelets. By using this method we were able to quantitatively determine the attachment of human platelets to the biomaterial devices which reflects thrombogenicity of the surface. We compared non-coated devices with (biopassive) polypeptide- or (bioactive) heparin-coated devices in respect to platelet adhesion, total platelet count and levels of β-TG release. The Western blots results show that protein bands in eluates from the surface coated devices were much weaker than in eluates from the noncoated control group demonstrating the effectiveness of the surface-coatings in reducing fibrinogen adsorption and GP IIb/IIIa binding. In contrast high concentrations of surface bound GP IIb/IIIa and fibrinogen were detected on non-coated membrane oxygenators (Figs. 1 and 2). Occurrence of multiple diffuse bands in the uncoated devices (lane 3) is founded to degradation products of the proteins due to the presence of proteases produced by cell damage or cell release mechanisms during the investigation. These results corresponded with the lower total platelet count (Fig. 3), the elevated β-TG levels (Fig. 4) within the uncoated devices. After 5 min recirculation in the CPB model, the platelet number in the uncoated group dropped nearly to zero. We explain this phenomenon by an immediate adhesion of the platelets to the artificial surfaces (tubing system, oxygenator membranes) that have a surface area of approximately 3 m^2. After a period of time the number of platelets increased again, suggesting that the adhered platelets were released back in the system. This observation could not be detected in the coated groups. Similar results were obtained by Li et al. [31]. They investigated accumulations of platelets in pump-oxygenator systems employed in CPB by gamma scintigraphy. This effect is generally not distinctly seen in clinical CPB. We assume, that due to the small blood volume (500 ml) in our CPB system the platelet attachment will be stronger visible within the platelet counts compared to the clinical situation because of the total lower platelet number. Furthermore, platelets in the uncoated group were highly activated demonstrated by elevated levels of β-TG levels. This is in accordance with the results obtained from Western blot and platelet count. In contrast, levels of β-TG in coated devices displayed a reduced activation of platelets.

5 Conclusion

Platelet adhesion and aggregation at the inner surfaces of the ECC devices remains an unsolved phenomenon. Nevertheless, thrombogenicity of CPB devices is highly dependent on the degree of platelet adhesion and loss of functional reactivity. We were able to demonstrate that the coated devices attenuate platelet adhesion significantly by using Western blot technique with CD41. These data correspond to the lower platelet count and the higher β-TG levels and fibrinogen concentration within the uncoated group.

Our newly developed immunological technique for quantification of platelet adhesion enables us to evaluate the affinity of platelets to the artificial surfaces used for CPB. This may be useful in choosing less platelet activating materials for CPB procedures which may ameliorate the various post-pump syndromes, including blood loss caused by loss of platelet functionality.

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