THE ROLE OF THE EXTRACORPOREAL CIRCUIT IN THE TRAPPING AND DEGRANULATION OF PLATELETS

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ABSTRACT

**Background**: Although platelet (PLT) activation and degranulation are well-known phenomena during hemodialysis (HD), controversies still exist about their nature and origin. **Methods**: PLT characteristics [PLT numbers, mean PLT volume (MPV), PLT distribution width (PDW), PLT large cell ratio (p-LCR), immature PLT fraction] and activation status [CD62p expression, platelet factor 4 (PF4) and β-thromboglobulin (BTG) plasma levels] were estimated in 19 patients before and during HD. Blood was sampled from both the afferent and efferent lines. Additionally, the influence of low-molecular-weight heparin (LMWH) on PF4 and BTG concentrations was analyzed. **Results**: CD62p expression increased in the extracorporeal circuit (ECC) in the first 30 min. Simultaneously, PLT numbers dropped markedly within the ECC. MPV, PDW and p-LCR decreased over time. Like CD62p expression, BTG reached peak values at t30, was exclusively released within the ECC and was not influenced by the application of LMWH. In contrast, PF4 was significantly released outside the ECC in response to LMWH. **Conclusions**: PLTs are predominantly activated within the ECC and not on a remote distance. PLTs stick to the ECC, particularly after first passage. BTG is an appropriate marker for HD-induced PLT degranulation, whereas PF4 originates from both activated PLTs and LMWH-induced detachment from the endothelium. PLTs are not exhausted due to the repetitive stimulation of clinical HD. Hence, dialysis modalities with longer duration or greater frequency may be associated with a less beneficial PLT activation profile, which may counteract their clinical benefits.
INTRODUCTION

During hemodialysis (HD) various side effects occur, including the activation of protein systems in the blood and the stimulation of circulating cellular elements. The sum of these undesirable side effects has been termed bioincompatibility [1]. With respect to platelets (PLTs), during HD both an increase in the expression of cell surface molecules and the release of intragranular substances has been well documented. Recently, we showed that the upregulation of the PLT surface marker P-selectin (CD62p) is an early process, occurring over the entire length of the extracorporeal circuit (ECC) [2]. Of the many PLT granule products that may increase during the course of HD, platelet factor 4 (PF4) and β-thromboglobulin (BTG) are well documented [3]. Activated PLTs adhere to various blood cell elements, including other PLTs, neutrophils, lymphocytes [4], monocytes and erythrocytes [5]. As a result of these cell-cell interactions, a mixture of microaggregates is formed, with specific pathophysiological effects. Flow cytometric analysis showed production of reactive oxygen species after the intradialytic formation of platelet-neutrophil aggregates [6]. In vitro, it was shown that PF4 binds directly to ox-LDL and increases ox-LDL binding to vascular cells and macrophages, thus contributing to the development of atherosclerosis [7]. In HD patients, PLT activation correlated both with bleeding and thrombotic diathesis [8], and vascular access failure [9]. In the clinical situation, PLT reactivity corresponded positively with the subsequent incidence of cardiovascular morbidity and mortality, both in nonrenal patients [10] and in patients with end-stage renal disease [11]. Although PLT activation and degranulation are well established phenomena during HD [12], controversies remain about the nature of PLT activation, intermittent or continuously [5], and the origin of PF4 and BTG, which are generally considered as markers of HD-induced PLT degranulation [2]. Therefore, 19 patients were included in the present analysis. PLT characteristics and activation status were estimated immediately before and during a single HD session. Not only the expression of the cell surface marker CD62p was measured, but also the plasma levels of PF4 and BTG. In addition, a variety of PLT indices, such as PLT numbers, mean PLT volume (MPV), PLT distribution width (PDW), PLT large cell ratio (p-LCR), and immature PLT fraction (IPF) was estimated. To establish the role of the ECC, blood was sampled both from the afferent (before the blood pump and analyzer) and efferent (after the dialyzer) lines at various time points. In an additional study, the influence of low-molecular weight heparin (LMWH) on the plasma concentrations of both PF4 and BTG was analyzed to determine the involvement of this anticoagulant in PLT degranulation.

SUBJECTS AND METHODS

Patients
Nineteen stable patients (11 males and 8 females, median age 63 years, range 28–82) undergoing regular HD treatment for at least 2 months (median 25 months, range 7–83 months) were included. Criteria for exclusion were age under 18 years, severe incompliance and a life expectancy less than 3 months due to nonrenal disease. In the present study,
patients with clinical signs of infection, autoimmune disease or malignancy as well as the use of medication that might interfere with PLT function were excluded. The etiology of renal insufficiency was hypertensive nephrosclerosis in 8 patients, diabetic nephropathy in 5 and adult dominant polycystic kidney disease in 3 patients. The other 3 patients had one of the following diagnoses: IgA nephropathy, membranous nephropathy and tubulo-interstitial nephritis. Written informed consent was obtained in all cases. The protocol was approved by the local medical ethics committee.

**Study Design and Blood Sampling**

All parameters were measured over time, i.e. before HD by sampling from the fistula (t0) and after 1 (t1), 5 (t5), 30 (t30), 60 (t60) and 150 (t150) min from the efferent line. Furthermore, samples were taken from the afferent lines after 5 min (t5aff) and 30 min (t30aff). The calculated differences over the ECC (Δ, efferent value – afferent value) at t1, t5 and t30 were designated Δt1 (first passage), Δt5 and Δt30, respectively. Results were corrected for changes in plasma volume, based on hematocrit (Ht) measurements (corrected value_{tx} = value_{tx} \times [Ht_0 / (1 – Ht_0)] \times [(1 – Ht_{tx})/Ht_{tx}]). PLT numbers were corrected for changes in plasma volume based on a different formula: corrected value_{tx} = value_{tx} \times [Ht_0 / Ht_{tx}]. All measurements were performed in duplicate.

**Additional Study**

The influence of LMWH on BTG release was studied in 5 patients during and just before another single HD session. Conforming to our previous study [2], LMWH (dalteparin, median 5000 IU, range 2500–7500) was administered in the AV fistula 10 min before the actual start of HD. Blood was drawn directly from the AV fistula 10 min before dialysis, just before dalteparin injection (t–10). Ten minutes after dalteparin injection, and just before starting HD, a second blood sampling was performed (t0). After the start of HD, samples were taken from the afferent line after 5 (t5aff) and from the efferent line after 1 (t1) and 5 (t5eff) min. All results (CD62p, BTG and PF4) were corrected for changes in plasma volume, based on Ht measurements.

**HD Procedure and Materials**

Only first use low-flux polysulfone dialyzers were utilized (F8 HPS; Fresenius Medical Care, Bad Homburg, Germany; ultrafiltration factor 18 ml/h x mm Hg, surface area 1.8 m², steam-sterilized) on Fresenius 4008 and 5008 series dialysis machines. Bicarbonate dialysate was used with a dialysate flow of 500 ml/min, whereas dialysate temperature was kept at 36°C. For dialysate preparation, tap water, purified by reverse osmosis, was used for the dilution of a concentrated bicarbonate solution to the following concentrations (mmol/l): 138 Na⁺, 2.0 K⁺, 1.50 Ca²⁺, 0.50 Mg²⁺, 109 Cl⁻, 2.5 CH₃COO⁻ and 32.5 HCO⁻₃ (SK-F 216/1; Fresenius Medical Care). All dialyzers were prerinsed with 1000 ml 0.9% NaCl. Individual doses of dalteparin, which is the standard type of anticoagulation in our center, were based on body weight (50 IU/kg) and duration of dialysis and given as a bolus injection at the beginning of the dialysis session (median 5000 IU, range 2500–7500).

**Analytical Methods**

**PLT Surface Markers.** The PLT surface markers CD62p (P-selectin; clone CLB Thromb
6; Beckman Coulter, Mijdrecht, The Netherlands) and CD41 (clone P2, Beckman Coulter) were detected by direct labeling and flow cytometry. Blood was drawn into K2EDTA (ethylenediaminetetraacetic acid) tubes and within 2 h after collection incubated with a glycoprotein-specific fluorochrome-labeled monoclonal antibody. A flow cytometer (Epics XL; Beckman Coulter) was used to determine the percentage of PLTs with CD62p surface expression. CD41 served as a PLT-specific label.

**BTG, PF4**. Blood samples were drawn into CTAD tubes (Vacutainer® CTAD; Becton Dickinson, Plymouth, UK), cooled on ice and centrifuged for 20 min at 2500 g. Plasma samples were stored at −70°C until measurement. BTG and PF4 were determined using commercially available sandwich ELISA kits (Asserachrom b-TG® and Asserachrom PF4®; Diagnostica Stago, Asnières, France).

**PLT, PDW, MPV, p-LCR, and IPF**. Blood samples were collected in K2EDTA tubes (Becton Dickinson). PLT counts, PDW, MPV, p-LCR and IPF are measured using a Sysmex XE-2100 hematology analyzer with a dedicated IPF-Master software package (Sysmex Corp., Kobe, Japan). A fluorescent dye reagent containing polymethine and oxazine is used with the flow cytometric IPF test methodology. After injecting the dye through the cell membrane, RNA in reticulated red blood cells and reticulated PLTs is stained. The stained cells pass through a semiconductor diode laser beam and the resulting forward scatter intensity (measure for cell volume) and fluorescence (measure for RNA content) are measured. IPF

![Figure 1](image.png)

**Figure 1.** **PLT CD62p expression (% PLT, mean ± SD) during HD.** Concentrations in both the afferent and the efferent lines are depicted in the line plot. Changes in concentrations in the efferent line from t0 (fistula) over time: * p < 0.001. The difference between the efferent line at t1 and the afferent line at t5 was significant (p < 0.001). Gray bars represent the absolute changes in PLT CD62p expression (% PLT, mean ± SD) over the ECC during HD: ° p < 0.001.
count is calculated from the combination of intensity of the fluorescence signal and the forward scattered light signal from the XE-2100 reticulocyte measurement channel by application of a Sysmex algorithm.

Statistical Analysis
All analyses were performed with the SPSS 15.0 software system. Data were tested for normality. A general linear model for repeated measures was applied to specify the effects of treatment time (based on measurements from the fistula at t0 and from the efferent line thereafter) and sampling point (efferent vs. afferent lines) on the course of PLT activation during a single session of HD. Paired-samples t tests were used as post-hoc analysis. Correlations between BTG, PLT numbers and CD62p expression were calculated and expressed as Pearson’s coefficients. Data are expressed as mean ± standard deviation. Differences were considered significant at p < 0.05.

Figure 2. PLT numbers (x 10^9/l, mean ± SD) during HD. PLT numbers in both the afferent and efferent lines are displayed in the line plot. Changes in concentrations in the efferent line from t0 (fistula) over time: * p < 0.001. Changes in PLT numbers (x 10^9/l, mean ± SD) over the ECC are represented by the gray bars: ° p = 0.002. Effects of time: • p = 0.002.
RESULTS

CD62p

Changes over Time: $t_0$ (Fistula) $\rightarrow t_{150}$. As shown in the line plot (Figure 1), CD62p expression showed an increase over time ($p < 0.001$) reaching a maximum at $t_{30}$: from $24 \pm 13\%$ at $t_0$ to $45 \pm 22\%$ at $t_{30}$. Thereafter, CD62p expression declined gradually.

Changes over the ECC ($\Delta t_1$, $\Delta t_5$ and $\Delta t_{30}$). As illustrated by the gray bars (Figure 1), CD62p expression increased similarly over the ECC after first passage, $t_5$ and $t_{30}$ (effect of sampling point: $p < 0.001$).

PLT Numbers

Changes over Time: $t_0$ (Fistula) $\rightarrow t_{150}$. As shown in the line plot (Figure 2), PLT numbers decreased over time: from $212 \pm 48 \times 10^9/l$ at $t_0$ to $194 \pm 43 \times 10^9/l$ at $t_{30}$ (efferent line) and $206 \pm 48 \times 10^9/l$ at $t_{150}$ ($p < 0.001$). At $t_{60}$, a modest rebound was observed.

Changes over the ECC ($\Delta t_1$, $\Delta t_5$ and $\Delta t_{30}$). As demonstrated by the bars (Figure 2), PLT numbers dropped considerably over the ECC only at first passage: $\Delta t_1$ $-31 \pm 31 \times 10^9/l$ (effect of sampling point: $p = 0.002$, effect of time: $p = 0.002$).

Figure 3. BTG (IU/ml, mean ± SD) concentrations during HD. Concentrations in both the afferent and efferent lines are depicted in the line plot. Changes in concentrations in the efferent line from $t_0$ (fistula) over time: * $p < 0.001$. Gray bars represent the absolute changes in BTG (IU/ml, mean ± SD) concentrations over the ECC during HD: ° $p < 0.001$. 
β-Thromboglobulin
Changes over Time: $t_0$ (fistula) → $t_{150}$. As shown in the line plot (Figure 3), during HD a highly significant increase was observed reaching maximum values at $t_{30}$: from $180 \pm 45$ IU/ml at $t_0$ to $364 \pm 138$ IU/ml at $t_{30}$ ($p < 0.001$). At $t_{150}$, BTG levels were still considerably elevated if compared to baseline. BTG levels at $t_5$ in the afferent line were fairly similar to those in the efferent line at $t_1$ (dotted part, $p = \text{NS}$), suggesting no substantial release from outside the ECC.

Changes over the ECC ($\Delta t_1$, $\Delta t_5$ and $\Delta t_{30}$). As illustrated in the bar chart (Figure 3), marked increments across the ECC were observed after first passage, $t_5$ and $t_{30}$ (effect of sampling point: $p < 0.001$), and were similar over time.

Platelet Factor 4
Changes over Time: $t_0$ (Fistula) → $t_{150}$. As shown in the line plot (Figure 4), PF4 levels increased over time ($p < 0.001$) and maximum values were observed at $t_5$: from $13 \pm 8$ IU/ml at $t_0$ to $114 \pm 31$ IU/ml at $t_5$. Although PF4 levels declined gradually thereafter, at $t_{150}$ PF4 levels were still elevated if compared to baseline ($39 \pm 21$ IU/ml). Interestingly, the considerable difference in PF4 observed between the efferent line at $t_1$ and the arterial
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line at t5 (dotted part, p = 0.001) implies substantial release from outside the ECC shortly after the start of HD.

Changes over the ECC (Δt1, Δt5 and Δt30). As demonstrated by the gray bars (Figure 4), marked increments across the ECC were observed at t1, t5 and t30 (effect of sampling point: p < 0.001). Interestingly Δt1 was significantly higher than Δt5 and Δt30 (effect of time: p = 0.001).

MPV, PDW, and p-LCR

Changes over Time: t0 (Fistula) → t150. As shown in Table 1, from t60 onwards MPV, PDW and p-LCR declined, reaching their nadirs at t150 (p < 0.001 for each parameter).

Changes over the ECC (Δt1, Δt5 and Δt30). Neither parameter showed changes over the ECC.

Immature PLT Fraction

Changes over Time: t0 (Fistula) → t150. Neither the absolute value (table 1) nor the percentage of immature PLTs changed over time.

Changes over the ECC (Δt1, Δt5 and Δt30). Immature PLTs decreased over the ECC both at first passage and at t5 (effect of sampling point: p = 0.005). Considering the change of immature PLTs as a percentage of the total number of PLTs, only at t5 a slight decrease was observed over the ECC (p = 0.037).

Correlations between BTG, CD62p and PLT Counts

There was a correlation between the increase in CD62p expression and the release of BTG over the ECC at all time points measured (Δt1 CD62p vs. Δt1 BTG: r² 0.28, p = 0.020, Δt5 CD62p vs. Δt5 BTG: r² 0.41, p = 0.003 and Δt30 CD62p vs. Δt30 BTG: r² 0.19, p = 0.063), suggesting that BTG is a proper marker for PLT activation in clinical HD. The drop in PLT counts at t1 correlated with the increase in CD62p expression over the ECC at t5 (r² −0.26, p = 0.027) and t30 (r² −0.24, p = 0.035).

Additional Study

After dalteparin injection (t −10) BTG levels remained stable from 165 ± 51 IU/ml at t −10 to 164 ± 48 IU/ml at t0 (before pump start) and increased thereafter to 185 ± 69 IU/ml at

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Effect of time: * p < 0.001; effect of sampling point: ** p = 0.005.
t1 and 254 ± 122 IU/ml at t5eff (p = 0.009, Figure 5). In line with the results of our previous study [2], CD62p expression remained stable after dalteparin injection and increased only after the start of dialysis (data not shown), whereas PF4 levels increased immediately after dalteparin injection from 10 ± 6 IU/ml at t −10 to 90 ± 34 IU/ml at t0 (p = 0.009) just before pump start.

**DISCUSSION**

PLT activation and degranulation are well-known phenomena in HD patients. However, the exact mechanism of these processes and the specific contribution of the ECC herein are incompletely understood. To elucidate these issues 19 chronic HD patients were included in the present analysis. Blood samples were drawn at various time and sampling points during treatment. Calculated differences between the efferent and afferent sampling points at t0/1 (first passage), t5 and t30 were designated ∆t1, ∆t5 and ∆t30, respectively. In line with previous observations [2], CD62p expression increased over time, followed by a progressive decline after 30 min. The increase over the ECC after first passage (∆t1) was comparable to both ∆t5 and ∆t30, indicating that PLTs are predominantly activated within the ECC and not on a remote distance. This view is further substantiated by the observation that at all time points measured, CD62p expression in the efferent line was higher than in the afferent line.
With respect to PLT numbers, a marked drop occurred after first passage, followed by an incomplete rebound at t5. As the drop over the ECC at first passage amounted to approximately 15%, the combined data of CD62p and PLT numbers suggest that a considerable number of activated PLTs stick to the ECC, most probably the dialyzer membrane [3] directly after the start of HD. Interestingly, in this respect, PLT behavior is different from that of polymorphonuclear cells, as these cells attach mainly to the lung vasculature after activation in the ECC and only to a limited extent to the dialyzer [13]. Whether the increase at t5 is due to mobilization of fresh PLTs from pools and/or detachment from the ECC is not readily apparent from this study.

With respect to PLT degranulation, BTG levels reached peak values at t30 and decreased gradually over time thereafter. The increase over the ECC was highly significant, without marked differences between Δt1, Δt5 and Δt30. There was in fact a significant correlation between ΔCD62p and ΔBTG at t1 and t5. As BTG levels were lower in the afferent line at all time points, these data suggest that BTG is almost exclusively released within the ECC. With respect to PF4, a quite different profile was observed. After an initial increase over the ECC, at t5 a sharp increase was observed in the afferent line, suggesting considerable release outside the ECC. Indeed, we have recently shown that the increase in PF4 during HD is mainly caused by LMWH-induced release from outside the ECC, most likely due to detachment from the endothelium [2]. Interestingly, the additional experiment in the present study showed that BTG release was not attributable to LMWH-induced release from outside the ECC. Our current and previous data taken together, it seems reasonable to conclude that PF4 is mainly released from the endothelium after the administration of LMWH and to a more limited extent from PLTs that are activated during first passage through the ECC. With respect to PF4 and BTG, only the latter substance is an appropriate parameter for the assessment of PLT degranulation during clinical HD. Moreover, its abundant release indicates that PLTs are not exhausted due to the repetitive stimulation of HD treatment, as was suggested before for PLTs [14, 15] and polymorphonuclear cells [16].

With respect to the PLT indices MPV, PDW and p- LCR, all values decreased over time, whereas significant changes over the ECC were absent. Previously, it was suggested that PLT volume decreases due to degranulation [14]. In that analysis, however, neither volume parameters nor degranulation products were measured over the ECC. In the present study, BTG was released continuously over the ECC, whereas volume parameters remained unaltered. Therefore, it is less likely that degranulation itself is responsible for the changes in volume parameters over time. Hence, we propose an alternative mechanism which might explain the observed changes. It has been well established that during the course of HD artificial membranes are progressively covered with protein depositions [17]. In the present study, PLTs adhered to the ECC, but mainly after first passage, on native material, before the deposition of proteins. As shown in this study, activated PLTs still released their granule content thereafter, but did hardly stick any further to the dialyzer membrane, most probably as a result of the protecting protein layer. Thus, with the exception of first passage, activated and degranulated PLTs leave the ECC during the entire course of HD and enter the circulation of the patients [5]. Subsequently, these activated PLTs are trapped and removed by the reticuloendothelial system of the body. As younger and larger PLTs are more prone to activation, during HD volume parameters in the circulation may decrease over time due to a proportional increase in older and smaller PLTs.
With respect to IPF, marked changes did not occur. However, at t5 the decline of this parameter was proportionately greater than the decrease in PLT, suggesting that in the first minutes of HD especially the younger and larger PLTs with a relatively high RNA content stick to the ECC.

The findings of our study may have important practical consequences. Cardiovascular disease is the most important cause of death in patients with ESRD. Besides traditional risk factors and the accumulation of middle and large-molecular-weight uremic toxins [18], HD-related risk factors, such as the bioincompatibility of the ECC, may contribute to the development of cardiovascular disease. Interestingly, in this respect, PLT activation is thought to play a causal role in atherogenesis, especially in the early stages [19]. As mentioned above, PLTs are not exhausted due to the repetitive stimulation of repeated HD. Moreover, during HD activated PLTs and their degranulation products leave the ECC and enter the circulation. Therefore, it is conceivable that modalities of longer duration or greater frequency, such as nocturnal HD and daily HD, are associated with a less beneficial PLT activation profile. Hemodiafiltration, which is characterized by a higher transmembrane pressure and considerable hemoconcentration, may also negatively influence PLT activation. Hence, it is conceivable that the beneficial effects of these modalities on hemodynamics [20] and/or bone mineral disease [21] are counterbalanced by an adverse profile of various biocompatibility parameters. Unfortunately, little or no information is available on these issues in either of these modalities.

To summarize, as demonstrated by the expression of CD62p, PLTs are activated over the ECC during the first 30 min of HD. Similar to leukocytes, a sharp drop in numbers is observed shortly after the start of treatment. However, whereas the drop in leukocytes is caused mainly by the attachment of activated polymorphonuclear cells to the lung vasculature, activated PLTs stick to the ECC, most probably to the membrane of the dialyzer. PLT degranulation, as measured by an increase in BTG, parallels CD62p expression, whereas the increase in PF4 levels is caused by release from both inside and outside the ECC. From these data it can be concluded that PLTs are not exhausted, possibly due to the short half-life time, especially in uremic conditions. As volume parameters declined gradually over time, it is conceivable that especially younger, larger and more viable PLTs are activated during treatment and entrapped in the reticuloendothelial system of the body. Whether the potentially negative effects of fashionable modalities, such as nocturnal HD, daily HD and hemodiafiltration, on PLT activation and degranulation counterbalance their supposed beneficial effects on clinical endpoints is currently unknown.
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