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Original Article

Intra- and post-dialytic platelet activation and PDGF-AB release: cellulose diacetate *vs* polysulfone membranes

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Abstract

Background. During haemodialysis the blood-membrane contact causes a release of platelet granule content, which contains platelet-derived growth factor AB (PDGF-AB). In view of the potential role of this in altering biocompatibility during haemodialysis, we evaluated the intra- and post-dialytic changes in PDGF-AB serum levels during haemodialysis sessions performed with cellulose diacetate (CDA) and polysulfone (PS) membranes respectively.

Methods. PDGF-AB, platelet factor 4 (PF4), beta thromboglobulin (βTG), and mean platelet volume (MPV) levels were determined in 30 patients, each of whom underwent six dialysis sessions: three with a CDA and three with a PS membrane. Blood samples were taken at times 0, 15, 30, 120, 180, and 240 min during dialysis and at 1, 4, and 20 h after the end of the session. Statistical analysis was performed using a one-way ANOVA and Student's *t* test.

Results. PDGF-AB at 15 min was increased to $+41\pm9\%$ with CDA *vs* $+20\pm5\%$ with PS ($P<0.001$) from the T0 values, and at 120 min it was $+19\pm8\%$ with CDA *vs* $-25\pm9\%$ with PS ($P<0.001$) from T0 levels. At 240 min it was $+95\pm14\%$ with CDA *vs* $+49\pm15\%$ with PS ($P<0.001$) from the T0 values, returning to basal only 20 h after the end of the session. βTG at 15 min was $+60\pm8\%$ for CDA *vs* $+24\pm7.5\%$ for PS ($P<0.001$) from the T0 values. PF4 showed a similar trend to βTG. MPV at 30 min from the start of dialysis was 7.4 ± 0.3 fl with CDA and 8 ± 0.3 fl with PS ($P<0.001$), and at 240 min MPV was 7.9 ± 0.3 fl with CDA and 8.4 ± 0.3 fl with PS ($P<0.001$).

Conclusions. Platelet activation and platelet release reactions are lower with PS than with CDA membranes. PDGF-AB, released during and after dialysis, represents a clear biocompatibility marker. Its slow return to basal values and its action on vascular cells make it a potential risk factor for atherosclerosis in uraemic patients.

Keywords: biocompatibility; cellulose diacetate; haemodialysis; PDGF-AB release; platelet activation; polysulfone

Introduction

The incidence of cardiovascular disease, mainly related to atherosclerosis, significantly increases in dialysis patients and is the major cause of death among these subjects [1]. It is still controversial whether these patients are prone to accelerated atherogenesis as a consequence of uraemia *per se* or dialysis therapy and in particular the bioincompatibility of dialysis membranes, or whether the high cardiovascular mortality is a consequence of various risk factors already present in the patient at a predialysis phase [2,3].

We believe that both platelet activation, one of the earliest biological reactions to take place following the first minutes of extracorporeal circulation, and the possible release of growth factors acting as mediators, require further investigation.

The circulating platelet mass is normally a heterogeneous mixture of intact larger platelets, shape-changed platelets, and partially or completely degranulated platelets, all of which have a low mean platelet volume (MPV). During extracorporeal therapy, platelets are continuously removed from and added to the blood stream, creating a further heterogeneous population of new (larger) and old (reduced) platelets. Thus, during haemodialysis, the assessment of the MPV may be as important as the platelet count in

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the study of thrombocyte response to the blood–membrane contact. The younger larger platelets show higher reactivity than the older smaller (lower MPV) platelets [4].

As is well known, contact of blood with artificial material during haemodialysis causes both platelet-dense granules (adenosine diphosphate (ADP) and serotonin) and the α-granules to release their contents, which contain platelet-derived growth factor (PDGF), platelet factor-4 (PF4, 358 000 Daltons) and β-thromboglobulin (βTG, 35 800 Daltons). Regarding the 'release reaction', there is general agreement that PF4 and βTG can be used as indices of platelet activation and of membrane biocompatibility [5]. The cyclical platelet and coagulative activation could lead to endothelial injury and may play a triggering role in the accelerated atherosclerosis of dialysis patients. Intradialytic administration of heparin into the extracorporeal circuit has been shown to stimulate platelet aggregation [6]. PDGF is one of the most important growth factors acting on all cells of mesenchymal derivation. Its molecular weight ranges from 28 000 to 35 000 Daltons, with a half-life of less than 2 min. It is synthesized by fibroblasts, endothelial cells, macrophages and platelets. PDGF is composed of two different glycoprotein chains (A and B), connected by covalent linkages. The two chains are codified by two different genes, which are expressed independently and enable cells to synthesize three isomorphic molecules: PDGF-AA, PDGF-AB, and PDGF-BB. The AB isoform is mainly stored inside α-platelet granules [7]. The serum level for PDGF-AB in the normal population is 29.7 ± 14 ng/ml. PDGF (mainly its AB isoform) causes proliferation of fibroblasts, smooth-muscle cells, and any kind of mesenchymal cell, all of which, in turn, can synthesize PDGF itself. PDGF stimulates extracellular matrix synthesis, and activates both migration and contraction of smooth-muscle cells [8]. As is well known, all these changes are typical of the evolution of atherosclerotic plaque [2,3].

In our previous paper we studied the PDGF-AB release and platelet activation during and after haemodialysis with a cuprophane membrane, and confirmed a sizeable PDGF-AB release [9]. In the present study we evaluated the intra- and post-dialytic changes in PDGF-AB serum levels, together with βTG and PF4 plasma levels and the mean platelet volume during haemodialysis sessions performed with cellulose diacetate (CDA) and polysulfone low-flux (PS) dialysers, to establish whether differences exist in platelet activation between a cellulosic and a synthetic membrane.

Subjects and methods

Subjects

Thirty patients on regular dialysis treatment for at least 6 months were included in the study. No patient had diabetes, myeloproliferative disorders, or coagulative alterations, and none was being treated with anticoagulant/antiaggregant drugs at any stage during the study. Patient characteristics are reported in Table 1.

Table 1. Characteristics of selected patients

Patients (*n*)	30
Age (years)	58.5 ± 11
Sex (M/F)	18/12
Renal dialysis therapy (months)	53.7 ± 43.9
Kt/V	1.4 ± 0.2
Shunt recirculation rate (%)	7 ± 3
Haematocrit (%)	31 ± 4
Platelets ($1000/mm^3$)	174.1 ± 23.5
Prothrombin time (%)	98 ± 7
aPTT (s)	38 ± 4

Dialysis and heparinization schedule

Each patient underwent three consecutive haemodialysis sessions with a CDA filter (Althin Althane 15, hollow-fibre dialyser, 1.5 m^2 surface area, Kuf 8.1 ml/h/mmHg, gamma ray sterilization), followed by three sessions with a PS filter (Fresenius F7 HPS, hollow fibre dialyser, 1.6 m^2 surface area, Kuf 9.8 ml/h/mmHg, steam sterilization).

During all dialysis procedures, conventional dialysis with a bicarbonate buffer (calcium concentration: 3.5 mEq/l) 4 h duration, was used. A volumetric ultrafiltration control monitor was used, body weight decreased approximately 750 ± 50 ml/h, blood flow was 310 ± 30 ml/min, and dialysate flow was 500 ml/min. No foam or air was detected in the extracorporeal circuit in any of the sessions.

As a wash-out between CDA and PS tests, one dialysis procedure was interpolated employing PS membrane.

In all procedures the same heparinization modalities were employed. In the washing phase, 2 l of saline solution containing 20 000 IU of standard heparin (Sodic Heparin, Vister, Parke–Davis) were used, then 500 IU during the connection procedure, followed by 3500 ± 500 IU in continuous infusion during the first 3 h of dialysis.

Sample collection

We evaluated: PDGF-AB measured in serum, PF4 and βTG in plasma, and platelet count and MPV in whole blood.

Blood samples were taken at dialysis times 0, 15, 30, 120, 180, and 240 min from peripheral blood in the arm contralateral to the AV fistula. During haemodialysis sessions the blood pump was kept near 100 ml/min and the ultrafiltration rate near 0 for 1 min before the blood sampling collection phase. Post-dialytic evaluation samples were taken at 1, 4 and 20 h after the end of the session. The platelet count (and MPV determination) was measured from the peripheral blood both during and after dialysis at the same time intervals. To obtain serum samples for PDGF-AB determination, we used serum separator tubes, and allowed samples to clot for at least 30 min at room temperature. Samples were centrifuged for 10 min at approximately 1000 *g*. This procedure was repeated until a clear serum was obtained without corpuscle elements. Serum was collected in a second tube, and stored at $-20^{\circ}C$ until needed.

For PF4 and βTG plasma values, blood was collected in DiaTubes (Becton Dickinson), containing a 0.5-ml solution consisting of citrate (0.109 mol/l), adenosine (3.7 mmol/l), theophylline (15 mmol/l), and dipyridamole (0.198 mmol/l).

After venepuncture the first 1 ml was discarded, and 4.5 ml was collected and added to the anticoagulant solution. Tubes were placed in melting ice for 15 min and then centrifuged at 2000 *g* for 30 min. One-third of the plasma was collected in the middle region of the supernatant, aliquoted, and immediately frozen at −80°C. Quantitative tests were performed within 2 weeks. PDGF was measured by ELISA (Quantikine, R&D System), using commercial kits. βTG and PF4 were also determined by ELISA, using commercial kits (Stago, Boehringer Mannheim). The platelet count and mean platelet volume (MPV) changes were assessed in the peripheral blood at the same time intervals as target molecules. Platelet volume variations were investigated using a cytometric technique (Bayer–Advia) to evaluate the MPV (fl), a statistical mean value of the platelet size distribution.

Data analysis

Because of the high individual variability of PDGF, PF4, and βTG basal values, we assessed the percentage variation of serum levels against time 0 min (T0) for both intra- and post-dialytic evaluation. In contrast, MPV and activated partial thromboplastin time (aPTT) variations are reported as absolute values. We compare the data obtained between the two membranes tested.

The data are presented as means ± SD. Statistical evaluation was performed by the statistical program Statview 4.01 for Macintosh. One-way ANOVA was used to test statistical significance; the paired Student *t* test was used to confirm the data obtained by ANOVA and for comparative analysis between the two membranes tested. Linear regression was performed to correlate aPTT with PF4, βTG and PDGF-AB and to highlight the potential effect of heparinization on the platelet release reaction.

Results

PDGF-AB

The PDGF-AB serum levels in our test dialysis population were 31.7 ± 11.9 ng/ml at T0 for dialysis sessions performed with CDA and 29.1 ± 11.0 for those with PS membrane (P = n.s.).

A comparative analysis from T0 levels between the two membranes showed a difference in PDGF-AB values that was already present at 15 min (+41 ± 9% with CDA *vs* +20 ± 5% with PS, $P < 0.001$) (Figure 1). At 30 min we observed a peak with CDA (+80 ± 10 *vs* +24 ± 10% with PS, $P < 0.001$). At 120 min there was a nadir more marked with PS (+19 ± 8% with CDA from the T0 levels *vs* −25 ± 9% from T0 levels with PS, $P < 0.001$). The end-session and the post-dialytic PDGF-AB values confirmed the significant difference between CDA and PS (at 240 min, +95 ± 14% with CDA *vs* +49 ± 15% with PS, $P < 0.001$). Four hours after the end of the session we observed +28 ± 7% with CDA *vs* +14 ± 4%, $P < 0.001$). With both membranes there was a slow progressive return to near pre-dialytic values by 20 h after the end of the session.

βTG

βTG percentage variations from T0 levels were found to be higher with CDA than with PS (at 15 min, by +60 ± 8%, $P < 0.001$, for CDA *vs* +24 ± 7.5% for PS, $P < 0.001$) (Figure 2). At 240 min, βTG percentage variations increased by +63 ± 9% with CDA *vs* 19 ± 5% with PS ($P < 0.001$). One hour after the end of the session, βTG increased by +37 ± 4% for CDA *vs* +15 ± 9% for PS ($P < 0.001$) (Figure 2).

PF4

With CDA membranes, PF4 percentage variations from T0 values were significantly higher than with PS (at 15 min they were +38 ± 10% with CDA *vs* +29 ± 7.5% with PS, $P < 0.001$). At 120 min PF4 levels increased by +24.5 ± 7% with CDA *vs* +21 ± 5% with PS ($P < 0.05$). At 240 min PF4 levels were +40 ± 8% with CDA *vs* +21 ± 4% with PS, and 1 h after the end

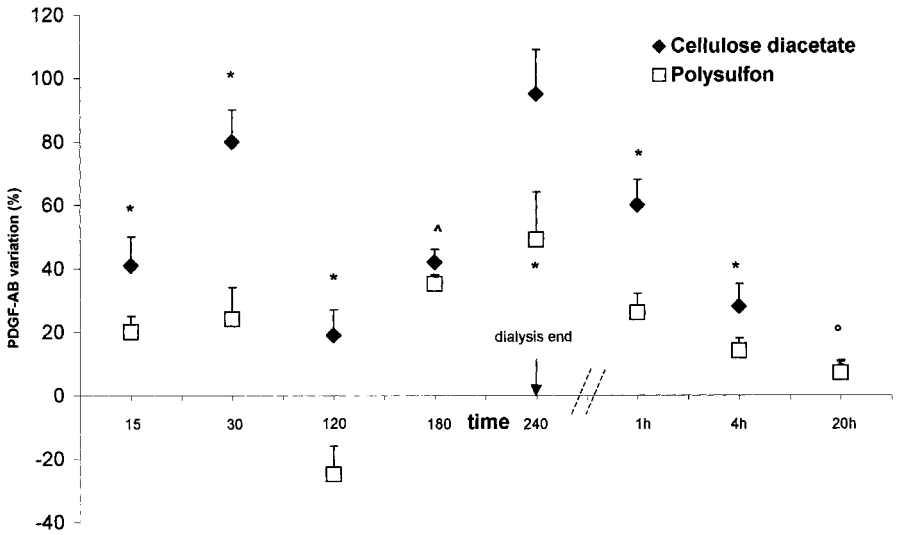

Fig. 1. PDGF-AB levels during and after dialysis sessions; ◆ CDA membrane *vs* □ PS membrane. The data are presented as percentage variations with respect to basal levels (means ± SD); the statistical evaluation was performed by one-way ANOVA. *$P < 0.001$; °P = n.s.

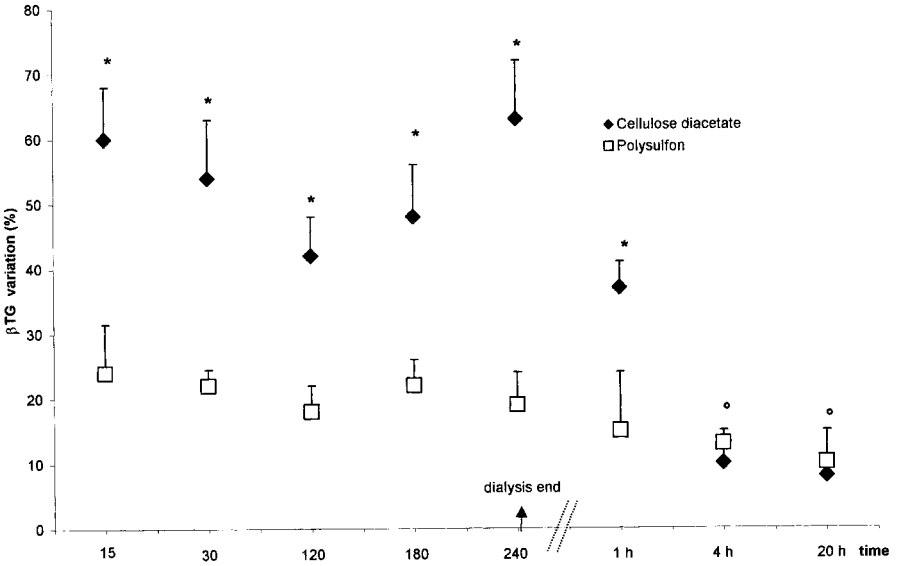

Fig. 2. βTG levels during and after dialysis sessions; ◆ CDA membrane *vs* □ PS membrane. The data are presented as percentage variations with respect to basal levels (means ± SD); the statistical evaluation was performed by one-way ANOVA. $^{*}P<0.001$; $^{\circ}P$ = n.s.

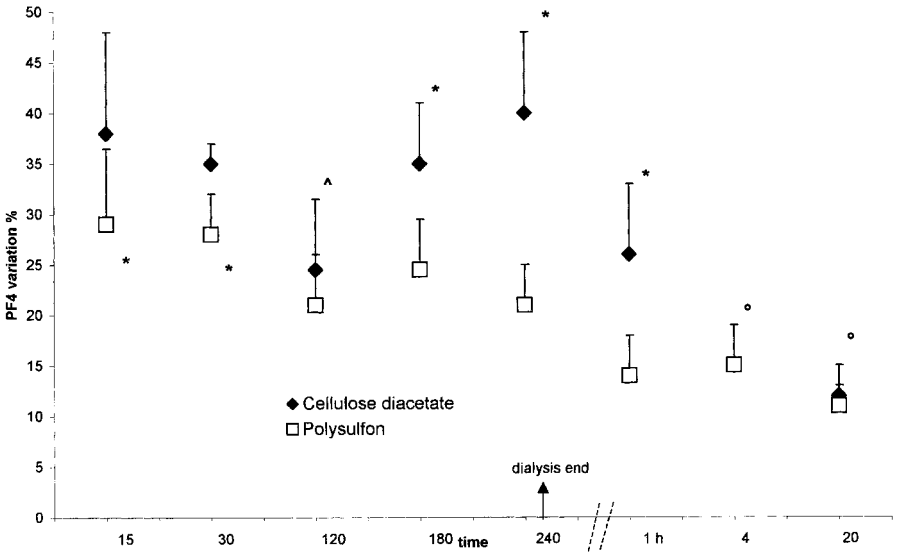

Fig. 3. PF4 levels during and after dialysis sessions; ◆ CDA membrane *vs* □ PS membrane. The data are presented as percentage variations with respect to basal levels (means ± SD); the statistical evaluation was performed by one-way ANOVA. $^{*}P<0.001$; $P<0.05$; $^{\circ}P$ = n.s.

of dialysis PF4 levels were +26 ± 7% with CDA *vs* +14 ± 4% with PS (Figure 3).

Platelet count and mean platelet volume

In the intradialytic phase, the platelet count after 30 min showed a transient fall, to 15 ± 5% with CDA and to 7 ± 4% with PS ($P<0.001$). Thereafter, the platelet count remained stable during the rest of the session and during the post-dialytic period.

Again, a statistically significant difference was found in MPV between the two membranes at each of the time intervals considered. Thus at 30 min from the start of dialysis MPV was 7.4 ± 0.3 fl with CDA and 8 ± 0.3 fl with PS ($P<0.001$). At 240 min MPV was 7.9 ± 0.3 fl with CDA and 8.4 ± 0.3 fl with PS ($P<0.001$), and then returned to basal values within 20 h of the end of the session (Figure 4).

aPTT

Basal values of aPTT (T0) did not differ between the two membranes tested (38 ± 10 s for PS and 37 ± 9 s for CDA (Figure 5). At 30 min, we found a peak in aPTT values with standard heparin (SH), due to both the rinsing solution heparin and the continuous infusion heparin. Thus, at 30 min, aPTT blood level was 106 ± 20 s with PS and was 110 ± 23 s with CDA.

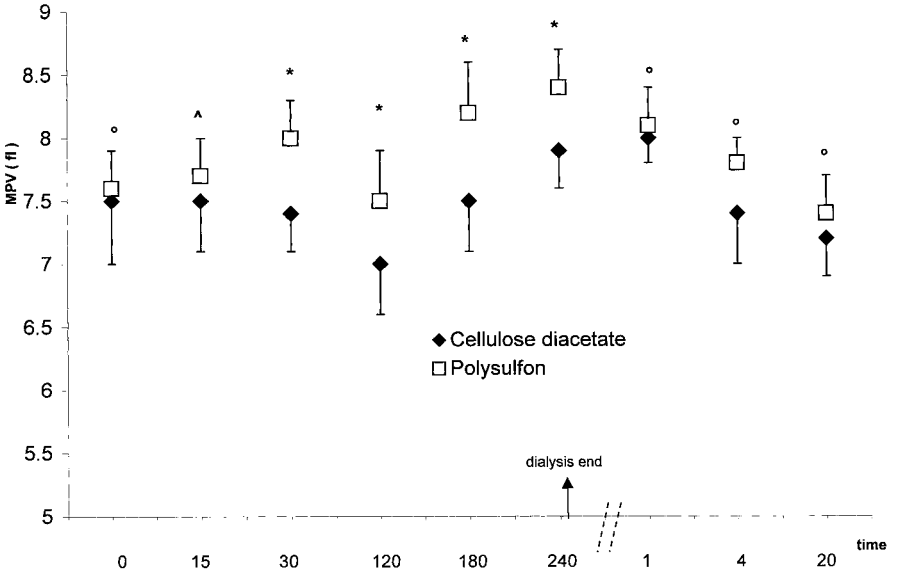

Fig. 4. MPV levels during and after dialysis sessions, ◆ CDA membrane *vs* □ PS membrane. The data are presented as absolute values with respect to basal levels (means ± SD); the statistical evaluation was performed by one-way ANOVA. *$P < 0.001$; °P = n.s.

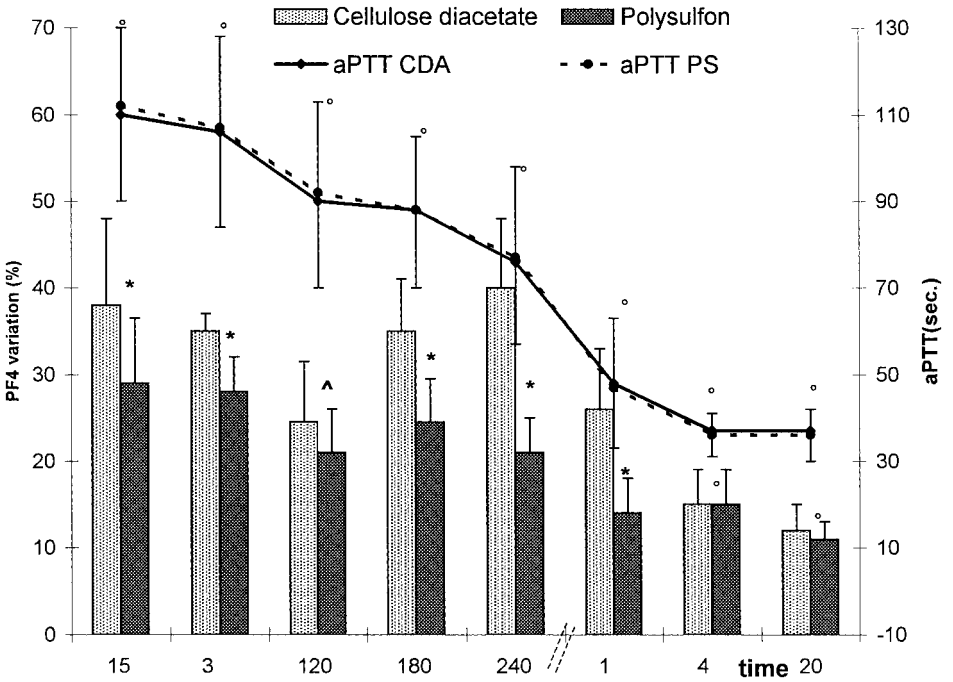

Fig. 5. aPTT and PF4 levels during and after dialysis sessions with CDA membrane *vs* PS membrane. PF4 data are presented as percentage variation with respect to basal levels (means ± SD); aPTT data are presented as absolute values with respect to basal levels (means ± SD); the statistical evaluation was performed by one-way ANOVA. *$P < 0.001$; $P < 0.05$; °P = n.s.

At the following intervals, we observed stable aPTT values in the therapeutic range without any significant difference between the two membranes tested. One hour after the end of the session, aPTT was 48 ± 15 s for PS and was 46 ± 11 s for CDA. Four and 20 h from the end of the session, no significant aPTT values were found for either membrane considered.

Linear regression did not show a clear correlation between aPTT and the above-mentioned markers of platelet release reaction (Tables 2, 3) at any of the time intervals tested with either dialyser.

Discussion

Platelet activation and aggregation, and coagulative activation are the earliest and most important phenomena that occur after contact between blood and artificial membranes [10].

It is widely agreed that platelet activation and the consequent release of active biological molecules is mainly due to platelet–membrane contact, and represents an appreciable index of membrane biocompatibility. Nevertheless, platelet activation is

Table 2. Linear regression (R) between aPTT and markers of platelet activation during and after CDA dialysis

Time	PF4	βTG	PDGF-AB
During dialysis			
15 min	0.16	0.25	0.2
30 min	0.19	0.18	0.4
120 min	n.s.	0.4	0.3
180 min	n.s.	n.s.	0.17
240 min	n.s.	n.s.	n.s.
After dialysis			
1 h	n.s.	n.s.	n.s.
4 h	n.s.	n.s.	n.s.
20 h	n.s.	n.s.	n.s.

Table 3. Linear regression (R) between aPTT and markers of platelet activation during and after PS dialysis

Time	PF4	βTG	PDGF-AB
During dialysis			
15 min	n.s.	n.s.	0.1
30 min	n.s.	n.s.	n.s.
120 min	n.s.	n.s.	n.s.
180 min	n.s.	n.s.	n.s.
240 min	n.s.	n.s.	n.s.
After dialysis			
1 h	n.s.	n.s.	n.s.
4 h	n.s.	n.s.	n.s.
20 h	n.s.	n.s.	n.s.

also known to be affected by shear stress as well as by other biological reactions triggered by the blood–membrane contact, including protein adsorption, complement, coagulative and leukocyte activation [11,12].

Our study confirms the finding that during dialysis sessions there is a considerable platelet activation. PDGF-AB, βTG, and PF4 are released into the blood from the first minutes of the dialysis session, and they may therefore be considered, along with other factors, as suitable markers of platelet activation. These markers showed different behaviours according to the two tested membranes in both the intradialytic and the post-dialytic periods.

Peripheral blood levels of PDGF-AB increase to different degrees with the two membranes by as soon as 15 and 30 min, with a difference in intradialytic platelet degranulation. The greater PDGF-AB concentrations observed with CDA than with PS could be related to the high thrombogenicity of the CDA membrane. The effective PDGF-AB release could be underestimated, especially considering the short half-life (<2 min) of this molecule and its fast removal from the blood circulation.

At 120′ min we observed a fall in PDGF-AB concentrations and a statistically significant difference between the two membranes. This event may be explained by the reduced platelet response that occurs when functionally exhausted (degranulated) platelets come to prevail in the circulation [13]. Two further mechanisms might be related: firstly, the high initial plasma levels of PDGF-AB could imply a negative feed-back mechanism exerted on the platelets, and a reduced release reaction as a consequence; secondly, a greater removal of this growth factor may occur following an overexpression of specific receptors by the target cells [7].

The further increase in PDGF-AB peripheral blood levels observed in the second half of the dialysis session could be related to further platelet activation, which was probably due to: (i) a progressive increase in fibrinogen content within the protein layer on the membrane surface, which causes a more marked platelet activation over the dialysis membrane; (ii) the progressive appearance of new and larger platelets in the blood circulation (MPV higher than basal value) which are more reactive to proaggregating factors (thrombin, txA2); (iii) intradialytic haemoconcentration with a relative increase in activated coagulative factors. Younger and larger platelets indeed show a higher reactivity (adhesion and aggregation) to contact with synthetic surfaces than do older platelets with a lower MPV.

The presence of exhausted degranulated platelets was confirmed by the nadir in platelet size which generally occurred at 120 min during the extracorporeal session: size usually increased after the second hour with the arrival of new platelets in the blood stream. This may be deduced from the progressive increase in MPV observed in the second half of the dialysis sessions or immediately thereafter [13]. In our previous paper [9] we described a different PDGF-AB behaviour, characterized by a sizeable release reaction during haemodialysis with a cuprophane (CU) membrane. These high PDGF levels could result from the lower CU biocompatibility as well as from the ethylene dioxide sterilization and the acetate buffer employed. In the present study we used membranes that were more biocompatible than CU, we used a bicarbonate buffer, and we avoided ethylene dioxide sterilization. Nonetheless, CDA caused a permanent stimulus of platelets, even at 120 min, whereas the activating effect of PS seemed to be less powerful over the same period.

The marked reduction in PDGF-AB observed could be due to a prevalent negative feedback mechanism exerted by PDGF-AB itself on platelets. Post-dialytic evaluation shows a slow and progressive decrease in PDGF-AB peripheral levels, which return to basal values only 20 h after the end of the session. After CDA sessions, PDGF-AB values were significantly higher than after PS. This post-dialytic trend may reflect persistent platelet activation and release after the end of the session and one may hypothesize that PDGF itself causes PDGF-AB production and release from its target cells by cascade activation, and that this occurs specifically in vascular smooth-muscle cells, vascular endothelial cells, and fibroblasts [7]. It is interesting to note that platelets are generally activated at a rate that depends on thrombogenic stimuli. Only

when a stimulus exceeds a certain threshold does the process become irreversible and the platelets degranulate and irreversible shape changes occur. It is therefore conceivable that in both intra-dialytic and post-dialytic phases some platelets underwent minimal activation (adhesion phase) inside the extracorporeal circuit, aggregating and giving rise to a release reaction only when a further stimulus arose, such as proaggregating factors or contact with pre-existing vascular lesions.

Concerning the other target molecules, βTG peripheral levels showed a trend similar to PDGF-AB during the intra-dialytic period, with an increase during the first phase of dialysis that was significantly lower with the PS membrane than with the CDA, and an additional and larger peak at the end of the session. For PF4, the initial peak observed at 15 min may be related to the heparin-induced release of PF4 from heparan-sulphate binding sites in endothelial cells [6]. This appears to be confirmed by the analogous increase that we noted 15 min after the heparin bolus injection [9].

The smaller intradialytic increases in βTG and PF4, as compared with PDGF-AB, could be explained by neutralization of the anticoagulant activities of heparin, because it is well known that these two molecules (PF4 in particular) have a heparin-binding capacity. The post-dialytic levels of both βTG and PF4 decreased during the 20 h following the end of the session, partly owing to the progressive decrease in the heparin-binding effect. The slower decreases in βTG and PF4 compared with PDGF-AB could also be due to their longer half-life (respectively 100 and 13 min).

Analysis of the behaviours of PDGF-AB, βTG, and PF4 would thus seem to point to the existence of a prolonged platelet activation (outside the extracorporeal circuit and after the dialysis period). Intradialytic heparinization may also have some impact on platelet activation. The pathway for this effect is not yet known but could be mediated by an inhibition of platelet adenylate cyclase regardless of any alteration in ADP action or by changes in prostaglandin metabolism [9]. These mechanisms could also be influenced by the interaction (absorption or immobilization) between heparin and the dialysis membrane, and by inter-individual variation in the effect of heparin on platelet activation [16]. However, our results do not show correlations between aPTT and PDGF, PF4, and βTG. This does not negate the heparin effect on platelet activation since this correlation prevented us from measuring the effective heparin action on platelets and does not show PF4 and βTG heparin-binding effects.

Our results show that both membranes induce platelet activation, but to differing degrees. We observed greater activation with CDA than with PS, as has been reported by others [14–16].

The existence of intra-dialytic PDGF-AB release seems established and one can speculate as to the possible clinical implications.

Interestingly, PDGF has recently been shown to play a key role in the development of advanced atherosclerotic lesions by stimulating the migration and proliferation of vascular smooth-muscle cells [17]. PDGF-AB levels are also significantly higher in post-myocardial infarction [18]. PDGF-AB, released after coronary angioplasty, promotes the proliferation of the smooth-muscle cells [19]. The inhibition of the PDGF receptor (PDGFR) by a specific PDGFR tyrosine kinase inhibitor (CGP 53716) prevents smooth-muscle cell migration and proliferation *in vitro*, and to a lesser extent the proliferation of these cells after balloon injury *in vivo* [20].

In conclusion, our results show that PDGF-AB, PF4, and βTG, may be considered as indexes of intra- and post-dialytic platelet activation, and their plasma levels could significantly depend on membrane biocompatibility. Considering the duration of dialysis therapy, the biocompatibility of the dialysis treatment, the cyclical release of PDGF, and above all its slow return to basal values, the role of PDGF-AB as an atherosclerotic risk-factor in uraemic patients merits further investigation. A full understanding of its role would require analysis of correlations with other atherogenic and anti-atherogenic growth factors in dialysis, and prospective clinical studies in which PDGF-AB is assessed alongside other recognized risk factors for cardiovascular disease in dialysis patients.

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