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Intra-and Postdialytic Platelet Activation, Increased Platelet Phosphatidylserine Exposure and Ultrastructural Changes in Platelets in Children with Chronic Uremia

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The present research evaluated the inta-and postdialytic changes in PF4 and βTG plasma levels by ELISA method and platelet aggregation by ADP as well as flow cytometric percentage of annexin V-positive platelets as a measure of PS externalization and ultrastructural examination of platelets in 37 uremic patients on regular hemodialysis and 25 age and sex matched controls. PF4 plasma levels increased, remain consistently high during hemodialysis session (20.24±3.05 IU mL^{-1} after 30 min, $p<0.001$ and 23.67±3.68 IU mL^{-1} after 240 min, $p<0.001$) and returned to control values (6.10±1.54 IU mL^{-1}) only after 24 h following the end of the session. βTG showed a similar trend to PF4. Platelet aggregation by ADP showed reduced function in comparison to controls (69.32±12.37% versus 91.95±1.59%, $p<0.001$). Flow cytometric percentage of annexin V-positive platelet, was significantly elevated ($p<0.001$) in uremic patients when compared to normal controls. Ultrastructural studies of platelets 30 min after starting of dialysis showing degranulation of its granules and at 240 min showing complete degranulation, while in the postdialytic phase (12 h after the end of dialysis) refilled α granules started to appear. Positive correlations were found between platelet concentration and both PF4 and βTG plasma levels during and after dialysis ($p<0.001$). Positive correlations between PF4 and βTG plasma levels during and after dialysis ($p<0.001$) and annexin V-positive platelets percentage were positively correlated with platelet concentration and both PF4 and βTG plasma levels during and after dialysis ($p<0.001$). Conclusion, activated platelets were found in chronic hemodialysis patients, a finding that may explain why uremics often suffer from thrombotic accidents. The platelet activation is associated with exposure of PS on the platelet exterior. PF4 and βTG are released from platelets mainly as consequence of the blood-membrane contact during dialysis and they return only slowly to control values. Understanding of the mechanisms of platelet activation may be critical in limiting the severity of thromboembolic events in uremic patients.

Key words: PF4, βTG, platelet aggregation, phosphatidylserine, platelets, hemodialysis

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INTRODUCTION

Chronic Renal Failure (CRF) is a functional diagnosis that is present when sufficient nephrons have been destroyed with subsequent irreversible progression to end stage renal disease (Ruggenenti *et al*., 1998). As renal failure progresses to End Stage Renal Disease (ESRD), some form of renal replacement therapy such as dialysis is required (Itoh *et al*., 2006).

The incidence of end stage renal disease in children is twenty per million population in United States (Davenport, 2006). Several studies have demonstrated that patients with renal failure on Hemodialysis (HD) actually live in a state of chronic platelet activation related to both uremia and the dialysis procedure (Hakim and Schafer, 1985; Bonomini *et al*., 1997; Himmelfarb *et al*., 1997; Sirolli *et al*., 2001; Sabovic *et al*., 2005).

Though the consequences of chronic platelet activation in uremia remain to be definitely established, activated platelets may be involved in biological reactions of potential pathophysiologic significance (Sirolli *et al*., 2001; Davenport, 2006; Hemmendinger *et al*., 1989). Further, since alterations in the platelet reactivity state enable these activated cells to participate actively in the thrombotic process Bevers *et al*. (1991) activated platelets might contribute to the thrombophilic tendency of uremia (Lindsay, 1972; Andrassy and Ritz, 1985; Bertoli, 1984), which is at present a major problem in dialysis patients (Bloembergen *et al*., 1995; Locatelli *et al*., 1998; Davenport, 2006).

As is well known, contact of blood with artificial material during hemodialysis causes both platelet-dense granules (Adenosine Diphosphate (ADP) and seretonin) and the α granules to release their contents which contain platelet factor-4 (PF4, 358000 Daltons) and β-thromboglobulin (βTG, 35800 Daltons). Regarding the release reaction, there is general agreement that PF4 and βTG can be used as indices of platelet activation and of membrane biocompatability (Kaplan and Owen, 1981; Sabovic *et al*., 2005). Intradialytic administration of heparin into the extra corporeal circuit has been shown to stimulate platelet aggregation (Andrassy and Ritz, 1987; Cianciolo *et al*., 2001).

Recently, platelet dysfunction was addressed specifically by flow cytometric percentage of annexin V-positive platelets as a measure of phosphatidylserine (PS) externalization. Within the broader process of cell activation response to a variety of different stimuli, platelets expose negatively charged phosphatidylserine at their outer surface (Shattil *et al*., 1998; Heemskerk *et al*., 1997; Daniel *et al*., 2006).

PS is one of the four major phospholipids distributed asymmetrically in the bilayer of cell plasma membrane and is normally confined to the membrane's inner leaflet. The maintenance of this asymmetry is an energy-requiring process of major importance for cells since the appearance of PS at the cell surface is associated with several physiologic and pathologic phenomena (Devaux and Zachowski, 1994; Zwaal and Schroit, 1997; Kuypers, 1998; Bonomini *et al*., 2004). Transbilayer migration of PS to the outer membrane leaflet may serve as a signal that is recognized by macrophages and promotes cell phagocytosis (Fadok *et al*., 1998; Daniel *et al*., 2006).

In this study, we evaluate the intra-and post-dialytic changes in PF4 and βTG plasma levels during HD sessions using polysulfone membrane, in addition to platelet aggregation by ADP. We also examine the exposure of PS on the outer membrane leaflet of uremia platelets by flow cytometric percentage of annexin V-positive platelets and ultimately ultrastructural examination of platelets in patients with uremia on maintenance HD aiming to detect platelet activation in these patients and to explain why uremics often suffer from thrombotic accidents.

MATERIALS AND METHODS

Thirty-seven stable End-stage Renal Disease (ESRD) patients on regular HD therapy selected from Urology Department, Theodor Bilharz Research Institute were included in this study. The study was done in a period from January to July 2006. The examined patients were (20 (54.1%) males, 17 (45.9%) females; aged 10.04±3.18 years, range 2.75-16.5 years) were being treated with hemodialysis for 3-4 h thrice weekly with polysulfone membrane (mean time on dialysis 1.80±1.02 years, range 0.5-4 years), blood flow rate ranged from 80-180 mL min^{-1} according to body weight, dialysate flow rate was 500 mL min^{-1} and did not change and heparin was used as anticoagulant during HD.

Inclusion criteria included children on regular HD treatment for not less than 6 months, using bicarbonate dialysate and free from apparent acute illness. The etiology of renal failure was reflux nephropathy (n = 1(2.7%)), glomerular disease (n = 2 (5.41%)), hereditary causes (n = 4 (10.81%)), anatomic causes (n = 11(29.73%)) and unknown causes (n = 19 (51.35%)). Patients were maintained on medications as calcium (n = 37(100%), vitamin D (n = 37(100%)), folic acid (n = 37 (100%), Erythropoietin (EPO) (n = 37(100%), Iron (n = 37 (100%) and calcium channel blockers (n = 28 (75.67%)).

Exclusion criteria: Diabetic patients were excluded as diabetes may alter platelet Intracellular mechanism (Cohen *et al*., 2002). Other exclusion criteria included

acute infection or blood transfusion in the past 3 months, unstable clinical conditions including vascular and cardiac instability; unstabilized erythropoietin dosage and a history of malignancy. None of the patients was known to have a pre-existing hemostatic disorder unrelated to uremia and all had been free of medications known to affect platelet function for at least one month prior to the study. Twenty five (mean age, 6.44±3.16 years; range 2-16 years) and gender-matched healthy individuals (serum creatinine<1.5 mg dL^{-1}) (Fox, 1996) with normal platelet count were included as normal control subjects. Informed consent was obtained from parents of each participant in the study.

All patients were subjected to:

- Full history taking
- Thorough clinical examination
- Complete blood count
- Pre-and post-dialysis kidney function test
- Serum albumin
- Estimation of intra-and post-dialytic PF4 and βTG plasma levels by ELSA methods
- Platelet aggregation test using ADP by platelets lumiaggregometer.
- Assay of Annexin V expression on the platelets surface by Flow Cytometeric analysis.
- Ultrastructural examination of platelets by electron microscopy

Blood samples collection: Five peripheral blood samples were collected from each patients one before dialysis (for annexinV expression, platelet aggregation and ultrastructural studies) (S_1), 2 during dialysis one after 30 min (S_2) and the other after 240 min and 2 samples after dialysis, one after 12 h (S_4) and the other after 24 h (for PF4 and βTG assay and ultrastructural studies) (S_5). Blood samples (3 mL each) were collected on 0.109 M citrate anticoagulant containing theophylline, adenosine and dipyridamole (CTAD tubes) (Boehringer Mannheim). Blood collected was carefully handled to avoid release of βTG and PF4 from platelets and was immediately cooled for 15 min on ice. All data obtained were corrected for hemoconcentration (evaluated as variation in total serum protein concentration measured from peripheral blood) to avoid overestimation of the molecules released.

Separation of peripheral platelets and β-TG and PF-4 assay: Blood was centrifuged at 270 g and 4°C for 15 min and the supernatant Platelet-Rich Plasma (PRP) was aspirated carefully without disturbing the buffy coat or red cells. Then, PRP was centrifuged in a conical tube at 2000 g and 4°C for 30 min and one milliliter of the middle part of the supernatant Platelet-Poor Plasma (PPP) was collected and kept frozen at -80°C till examined. β-TG and PF-4 were assayed using ASSERCHRO βTG and PF4 ELISA kits (Boehringer Mannheim) (Amiral *et al*., 1985).

Measurement of platelet aggregation: After PRP was obtained, platelet aggregation was determined by the turbidimetric method of Born and Cross (1963) using lumiaggregometer.

Expression of annexin V on platelets: After Platelet Rich Plasma (PRP) was collected, it is centrifuged again at 1500 g for 10 min at room temperature. platelets resuspended in washing buffer according to manufacture guidance. Fifty microliter of platelets suspension was then incubated with 10 μL of the fluorescent-conjugated annexin V and propidium iodide (DAKO product No. K2350). As α negative control 10 μ of mouse Ig G FITC (DAKO product No. LS 191) were added to a tube containing 50 μL of the platelet suspension, Flow cytometric analysis was performed on MoflO High-Performance Cell Sorter (Bonomini *et al*., 2004).

Electron microscopic examination: Peripheral blood platelets: After centrifugation of PRP the pellet is re-suspend in fixative solution 4% glutaraldhyde with sodium cacodylate then fixed in 2% osmium tetraoxide, dehydrated with ascending concentration of alcohol and embedded in epoxy resin according to the technique of Grimaud *et al*. (1980). Semi-thin and ultra-thin section were cut with a Leika Ultramicrotome. Ultra-thin section were contrasted with uranyl acetate and lead citrate and examined by Phillips EM 208.

Statistical methods: SPSS (Statistical Package for Social Sciences) version 9 was used for data analysis. Mean and standard deviation described quantitative data. Sample student's t-test was used to determine statistical significance, the paired student's t-test was used to confirm the data obtained with the sample student's t-test. Pearson's correlation analysis predict association of platelet activation markers to different numerical variables, association between PF4 and βTG, intra-and post-dialytic plasma levels and ultimately association of annexin V-positive platelets to platelet activation markers and other numerical variables. p-value was significant at 0.05 level.

RESULTS

Demographic, clinical and laboratory data of the studied groups shown in (Table 1) with statistically significant difference in white blood cells count in patients compared with controls ($p<0.001$).

PF4 peripheral plasma levels showed an increase in the values during the whole session. In particular

the PF4 plasma levels showed a peak at 30 min (20.24±3.05 IU mL^{-1} versus 6.10±1.54 IU mL^{-1} in controls $p<0.001$) while at the end of the session, the PF4 plasma levels showed a 2nd peak at 240 min (23.68±13.88 IU mL^{-1} versus 6.10±1.54 IU mL^{-1} in controls $p<0.001$).

Post-dialytic evaluation showed a progressive decrease in PF4 plasma levels at 12 h (10.97±1.63 IU mL^{-1} versus 6.10±1.54 IU mL^{-1} in controls $p<0.001$) and returned to the control values 24 h after the end of the session (5.45±1.59 IU mL^{-1} versus 6.10±1.54 IU mL^{-1} in controls). βTG plasma levels showed an increase in the values during the whole session. In particular the βTG plasma levels showed a peak at 30 min (74.35±13.58 IU mL^{-1} versus 32.29±6.06 IU mL^{-1} in controls $p<0.001$) while at the end of the session, the β-TG plasma levels showed a 2nd peak at 240 min (90.37±7.29 IU mL^{-1} versus 32.29±6.06 IU mL^{-1} in controls $p<0.001$) (Table 2).

Post-dialytic evaluation showed a progressive decrease in βTG plasma levels at 12 h (51.40±9.02 IU mL^{-1} versus 32.29±6.06 IU mL^{-1} in controls $p<0.001$) and returned to the control values 24 h after the end of the session (34.67±4.01 IU mL^{-1} versus 32.29±6.06 IU mL^{-1} in controls) (Table 2).

There was a significant reduction in platelet aggregation caused by ADP in hemodialysis patients compared with the healthy control subjects (69.32±12.37% versus 91.95±1.59% $p<0.001$). The phosphatidylserine exposure in platelets as expressed by the mean percentage of annexin V-positive platelets was significantly high in hemodialysis patients than in healthy control subjects under resting condition (30.97±4.78% versus 2.26±0.63% in controls, $p<0.001$) (Table 3).

Table 1: Demographic, clinical and laboratory data of the studied groups

Parameters	Controls	HD patients
No. of subjects	25	37
Sex (male/female)	18/19 (48.6%/51.4%)	20/17(54.1%/45.9%)
Age (Years)	6.44±3.16	10.04±3.18
Duration of hemodialysis (Years)	-	1.8±1.02 (0.5-4)
BUN (mg dL^{-1})		
Predialysis	-	92.97±23.18
Postdialysis	-	26.67±8.62
Equilibrated KT/V	-	1.47±.28
HCT (%)	38.88±3.62	31.40±11.22
$WBC_s \times 10^3/mm^3$	3.57±1.42	6.05±1.71*
Nephropathies		
Anatomic		11 (29.73%)
Hereditary		4 (10.81%)
Glomerular		2 (5.41%)
Reflux nephropathy		1 (2.7%)
Unknown		19 (51.35%)
Drugs		
Erythropoietin	-	37 (100%)
Calcium blockers		28 (75.67%)

Data are means±SD, or number (%), or range, as applicable, *$p<0.001$ compared with controls. Significance was estimated using paired students t-tests

Table 2: PF4 and β TG plasma levels in control subjects and in patients during and after HD

Parameters	Control (N = 20)	Patients (N = 37)
Intradialytic PF4 (IU mL^{-1})		
30 min	(6.10±1.54)	20.24±3.05*
240 min		23.68±13.88*
Postdialytic PF4		
12		10.97±1.61*
24		5.45±1.59*
Intradialytic βTG (IU mL^{-1})		
30 min	(32.29±6.06)	74.35±13.58*
240 min		90.37±7.29*
Postdialytic βTG		
12		51.40±9.02*
24		34.67±4.01*

Data are reported as means±SD. p was significant if <0.001

Table 3: Platelet concentration and aggregation and annexin V percentage in control subjects and HD patients

Parameters	Control (N = 20)	Patients (N = 37)
Platelets$\times 10^3/mm^3$	269.45±84.02	235.10±122.23
Aggregation		
ADP (6 μM)	91.95±1.59	69.32±12.37*
Annexin V- positive platelets	2.26±10.63%	30.97±4.78%*

Platelets were stimulated with ADP. Data are reported as means±SD or percentage as applicable, p was significant if <0.001

Figure 1 shows the electron micrograph of normal platelet before dialysis.

Figure 2 shows the electron micrograph of three forms of activated platelets in the intra-dialytic phase (30 min after starting of dialysis), showing centralization of the organelles followed by extending of long thin filopodia (F) and finally degranulation of its granules.

Electron micrograph of activated platelets in the intra-dialytic phase (240 min after starting of dialysis)

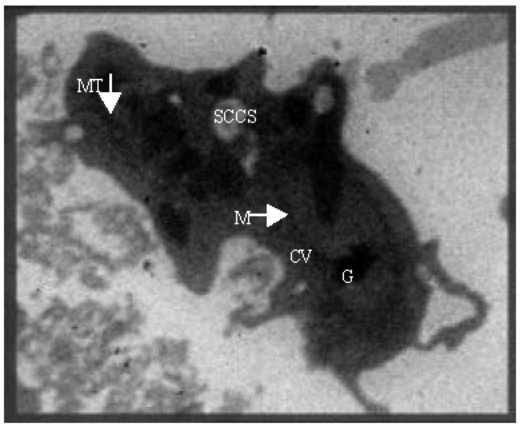

Fig. 1: Electron micrograph of normal platelet before dialysis. Membranous organelles including the surface-connected canalicular system (SCCS) and cytoplasmic organelles including mitochondria (M), a-granules (G) and coated vesicles (CV). Microtubules (MT) are present as cross-sectional and longitudinal profiles could be seen. (X 20000)

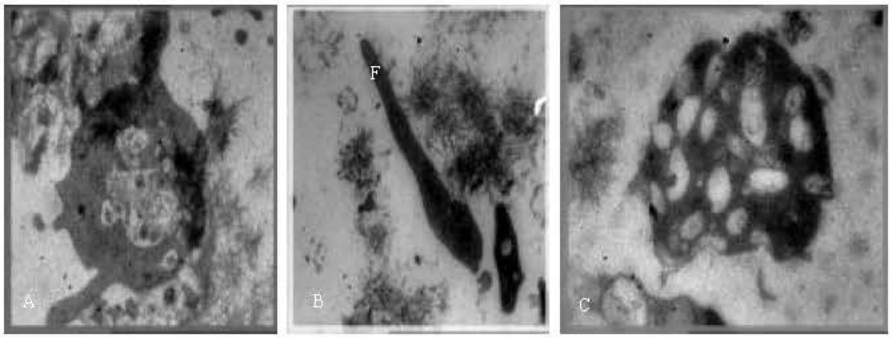

Fig. 2: Electron micrograph of three forms of activated platelets in the intradialytic phase (1/2 h after starting of dialysis). (A) showing centralization of the organelles. (B) showing extending of long thin filopodia (F) and (C) showing degranulation of its granules (X 18000)

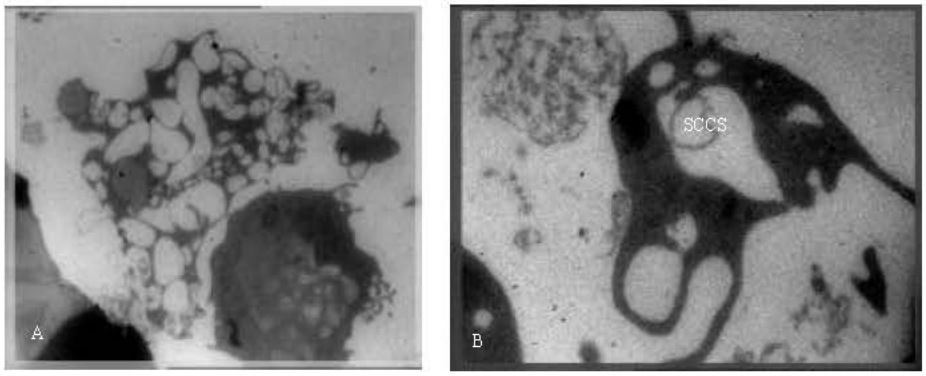

Fig. 3: Electron micrograph of activated platelets in the intradialytic phase (4 h after starting of dialysis). (A) showing complete degranulation and (B) showing dilatation of the surface-connected canalicular system (SCCS) (X 18000)

Table 4: Correlations between markers of platelet activation during and after HD and different parameters

	Intradialytic								Postdialytic				Postdialytic			
	30 min				240 min				12 h				24 h			
	PF4		β-TG		PF4		β-TG		PF4		β-TG		PF4		β-TG	
Parameters	r	p-value	r	p-value	r	p-value	r	p-value	r	p-value	r	p-value	r	p-value	r	p-value
Age	-0.29	NS	-0.34	0.03	-0.27	NS	-0.21	NS	-0.20	NS	-0.17	NS	-0.26	NS	-0.20	NS
HDD	-0.06	NS	-0.01	NS	0.02	NS	-0.06	NS	-0.01	NS	-0.02	NS	-0.06	NS	0.01	NS
KT/V	0.01	NS	-0.08	NS	-0.05	NS	-0.07	NS	0.11	NS	-0.08	NS	-0.11	NS	-0.04	NS
Platelets	0.66	0.001*	0.75	0.001*	0.71	0.001*	0.91	0.001*	0.74	0.001*	0.69	0.001*	0.69	0.001*	0.69	0.001*
P.agg	0.01	NS	-0.01	NS	-0.04	NS	0.09	NS	-0.05	NS	-0.01	NS	-0.02	NS	-0.01	NS

Correlations was performed by Pearsons analysis. Significant p = 0.001*. HDD = hemodialysis duration, PreDurea = predialysis urea, P.agg = platelet aggregation

showing complete degranulation and dilatation of the surface-connected canalicular system (SCCS) as shown in Fig. 3.

Electron micrograph of platelets in the post-dialytic phase (12 h after the end of the dialysis). As shown in Fig. 4. Refilled α granules (G) started to appear.

Pearson's correlation coefficients between platelet activation markers and different parameters. There was a statistically significant correlation between βTG plasma levels and the age of the patients (r = -0.34, p = 0.03). Intra and pos-dialytic plasma levels of PF4 and βTG significantly correlated with platelet concentration (p = 0.001) (Table 4).

Pearson's correlation coefficients between PF4 and βTG plasma levels during and after HD. PF4 plasma levels significantly correlated with βTG plasma levels during and after the dialysis session (p = 0.001) (Table 5).

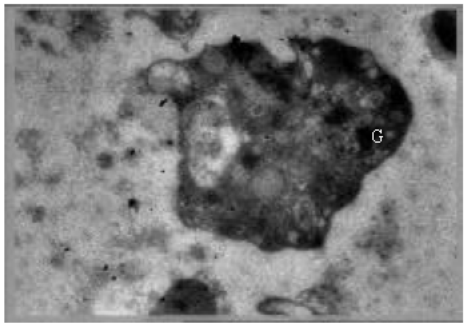

Fig. 4: Electron micrograph of platelet in the postdialytic phase (12 h after the end of dialysis). Refilled a granules (G) started to appear (X 20000)

Table 5: Correlation between annexin V positive platelets percentage and different parameters of patients

Parameters	r	p-value
Age	0.47	0.003*
Duration on hemodialysis-	0.26	NS
KT/V	0.17	NS
Platelets	0.51	0.001*
Platelet aggregation by ADP	0.06	NS
Intradialytic PF4		
30 min	0.72	0.001*
240 min	0.76	0.001*
Postdialytic PF4		
12 h	0.64	0.001*
24 h	0.68	0.001*
Intradialytic βTG		
30 min	0.77	0.001*
240 min	0.56	0.001*
Postdialytic βTG		
12 h	0.59	0.001*
24 h	0.56	0.001*

Correlations was performed by Pearson's analysis. Significant p = 0.001*

Table 6: Correlation between PF4 and βTG during and after HD

	Intradialytic PF4				Postdialytic PF4			
	30 min		240 min		12 h		24 h	
	r	p-value	r	p-value	r	p-value	r	p-value
Intradialytic βTG								
30 min	0.92	0.001*	0.89	0.001*	0.83	0.001*	0.87	0.01*
240 min	0.72	0.001*	0.73	0.001*	0.80	0.001*	0.74	0.001*
Postdialytic βTG								
12 h	0.78	0.001*	0.73	0.001*	0.87	0.001*	0.86	0.001*
24 h	0.76	0.001*	0.76	0.001*	0.81	0.001	0.86	0.001

Correlations was performed by Pearson's analysis. Significant p = 0.001

Pearson's correlation coefficients between annexin-V-positive platelets percentage and different parameters. Annexin V-positive platelets percentage correlated with platelet concentration (r = 0.51 p = 0.001) and platelet activation markers (p = 0.001) (Table 6).

DISCUSSION

In end-stage renal disease, in particularly when treated with haemodialysis, the function of platelets, coagulation and fibrinolytic systems can be disturbed, thus contributing to either thrombotic or bleeding complications (Sabovic *et al*., 2005). It is important to know whether the currently used haemodialysis procedure itself affects platelets, coagulation or fibrinolysis.

The results of the present study seem to confirm a state of chronic platelet activation in uremic patients as stated by Hakim and Schafer (1998), Cases *et al*. (1993), Himmelfarb *et al*. (1997), Sirolli *et al*. (2001) and Bonomini *et al*. (1997) in addition to Platelet activation and aggregation and coagulative activation during HD which are the earliest and most important phenomena that follow on from blood-membrane contact (Coli *et al*., 1995). Also Davenport (2006) found that The initiation of coagulation in the extracorporeal hemodialysis circuit is a manifestation of bioincompatibility, due to the activation of leukocytes, platelets and the coagulation cascades, rather than simple contact of intrinsic system coagulation proteins which the dialyser surface and plastic tubing leading to activation of the contact coagulation cascade.

After the protein layer has been adsorbed onto the membrane surface, the platelets adhere; lose their discoid shape, become. Irregularly spherical with a reduction of their mean platelet volume spread out and begin the release reaction (Andrassy *et al*., 1987).

The platelet release reaction is the secretary process following primary platelet aggregation where by the contents of the platelet granules are released into the blood. It is widely agreed that platelet activation and the consequent release of active biological molecules, are due mainly to platelet-membrane contact (Windus *et al*., 1996).

Present study showed statistically significant difference in white blood cell counts between patients and controls. Baumgartner *et al*. (1995) and Yoshida *et al*. (1995) found that platelet activation is affected by shear stress as well as other biological reactions triggered by the blood-membrane contact, including protein adsorption, complement, coagulative and leukocyte activation. Endo *et al*. (1981) found that blood factors such as RBC, WBC, platelets, fibrinogen, etc were elevated by about 20% during HD due to hemoconcentration and Itoh *et al*. (2006) suggests that platelets activated through interaction with hemodialysis membranes stimulate neutrophils to produce reactive oxygen species via P-selectin-mediated adhesion and that this property of adhesion to platelets

Present study confirms the finding that during dialysis sessions there is a considerable platelet activation. PF4 and βTG are released into the blood from first minutes of the dialysis session and they therefore be considered, along with other factors, as suitable markers of platelet activation.

For PF4, the initial peak observed at 30 min could presumably be related to the heparin-induced release of PF4 form heparin sulphate-binding sites in endothelial cells (Hoenich, 1993).

βTG peripheral levels present, in the intra-dialytic period, a trend similar to PF4. The post-dialytic levels of both PF4 and βTG decrease during the 24 h. following the end of the session. The fact that during this period PF4 and βTG plasma values decreases slowly could be induced by their longer half life (13 and 100 min, respectively) (Flicker *et al*., 1982) in addition to the persisting platelet activation. With regard to PF4 it is very interesting that this protein exerts a chemo tactic effect on neutrophils and monocytes with consequent further damage to the vascular wall (Stemerman, 1981). This results were in agreement with the results of Cianciolo *et al.* (2001) who reported that 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.

This prolonged platelet activation is probably a multi factorial phenomenon that could be caused mainly by: (i) the presence of younger and more reactive platelets, (ii) the progressive exhaustion of the heparin activity that involves a reduced neutralization of the activated coagulative factors (Matsuda, 1989). These results are in accordance with who found that PF4 and βTG plasma levels increased remained consistently high during HD session and returned to the basal values only after 20 h. following the end of the session (Cianciolo *et al*., 1999).

In this study we observed a significant reduction in platelet aggregation caused by ADP in these patients compared with the healthy control group. This result is not due to the differences in platelet concentrations of the two groups, since their mean values were not significantly different. Many chronic renal failure patients present a reduction in platelet aggregation (Sreedhara *et al*., 1996; Gralnick *et al*., 1988; Smits *et al*., 2000; Tan *et al*., 2000). However the mechanisms involved in this process have yet to be understood. Sreedhara *et al.* (1996) observed a reduction in the availability of GP IIb-IIIa membrane receptors in uremic patients. According to Gralnick *et al.* (1988) the reduced platelet aggregation could be ascribed to a reduction in von-willibr and factor levels.

In this study the primary aggregation response to ADP agonist dislayed normal levels ruling out the possibility of a problem at the receptor level. However the secondary aggregation response was significantly inhibited, suggesting *in vivo* activation in these patients with the consequent release of contents of α-granules so the platelets become completely degranulated with delayed platelet aggregation. This results is in accordance with the results of Neiva *et al.* (2002) who found that human platelets of HD patients showed reduced function when stimulated with collagen, adenosine diphosphate and epinephrine.

Our data showed that in HD patients the exposure of negatively charged aminophospholipid PS at the outer surface of platelets, a late platelet activation event, is significantly higher than in healthy controls. Loss of platelet membrane phospholipid asymmetry with increased PS exposure represents a new observation in chronic uremia and may cause a prothrombatic condition in a patient population at risk from thromboembolic events. This result in accordance with the result of Bonomini *et al.* (2004) who found that flow cytometric percentage of annexin V-positive platelets, a measure of PS externalization was significantly elevated in uremic patients when compared to normal controls under both unstimulated and agonist stimulated conditions.

When exposed on the outer membrane surface of activated platelets, PS causes coagulation and thrombosis by providing a suitable surface for assembly of the prothrombinase complex, which converts prothrombin to thrombin (Bevers *et al*., 1982; Zwaal *et al*., 1992; Bevers *et al*., 1991).

Activated platelets may also promote hypercoagulability through the shedding of lipid-asymmetric microvesicles from the cell surface, which usually accompanies loss of membrane phospholipid asymmetry (Zawaal *et al*., 1992).

Platelet-derived microparticles are able to accelerate thrombin generation (Zawaal *et al*., 1992; Walsh, 2001) and elevated circulating levels have been reported in association with several thrombotic disorders (Geiser *et al*., 1998; Gawaz *et al*., 1996; Nieuwland *et al*., 1997; Daniel *et al*., 2006). Raised levels of circulating platelet-derived annexin V-staining microparticles have recently been observed in uremic patients (Minoru *et al*., 2002; Daniel *et al*., 2006). The finding may have clinical significance, since levels were significantly higher in patients who had suffered from thrombotic events than in those without such events. Because exposure of PS on platelets seems to be required for microparticle release (Zawaal and Schroit, 1997) although we did not investigate circulating microparticle levels, our present findings of increased PS exposure in platelets from hemodialysis patients could explain the reported evidence of increased platelet-derived microparticle levels in uremia (Minoru *et al*., 2002; Daniel *et al*., 2006).

Besides controlling blood coagulation, the regulation of PS distribution in cell membranes may be critical in determining the survival of aged or damaged cells in

circulation, since surface-exposed PS facilitates the cell's inter`action with phogocytic cells (Schroit *et al*., 1985; Fadok *et al*., 1992; Fadok *et al*., 1998). A PS- recognition mechanism may cause uremic red blood cells to be susceptible to phagocytosis (Bonomini *et al*., 2001) and thus may be involved in the shortened erythrocyte life span of uremia. Platelet survival is also shortened in dialysis patients, as demonstrated by increased levels of circulating reticulated platelets (Himmelfarb *et al*., 1997) a measure of platelet turnover (Richard and Baglin, 1995). Though the death program that accounts for platelet deletion *in vivo* is still largely unknown, studies on different models of senescent cells suggest that a PS-mediated mechanism may play an important role in the removal of platelets from circulation (Pereira *et al*., 1999; Pereira *et al*., 2002). Thus, the increased turnover of platelets in uremia (Himmelfarb *et al*., 1997) which may contribute to the acquired platelet defect associated with renal failure as manifested by decreased platelet aggregation in this study, might be related to increased platelet PS exposure leading to a propensity of the cell to be recognized and subsequently removed by macrophages.

In present study ultrastructural examination of platelets in the intra-dialytic phase (30 min and 24 h after starting of the dialysis session) was in accordance with Kuzniewsk *et al*. (1990) found that during the course of HD, the platelets showed signs of activation manifested by increases in number and length of cytoplasmic processes and by a tendency to aggregate as revealed by scanning electron microscopy. Mason *et al*. (1980) and Coli *et al*. (1995) found that the intra-dialytic release reaction is induced either by surface factors (micro-macroscopic characteristics and the physiochemical status of the dialysis membrane) or by circulating factors, such as thrombin, heparin, ADP, thromboxane A_2, fibrinogen, von-will brand factors and others. Leither *et al*. (1980) found that in HD an interaction between platelets and dialysator membrane occurs and can be demonstrated by parietal deposition of platelets in the capillaries of the artificial kidney by scanning electron microscopy as well as in marred increase of reversible platelet microaggregates during the first phase of dialysis.

In this study ultrastructural examination of platelets in post-dialytic phase was supported by the finding of Windus *et al*. (1996) who proved that platelet activation and consequent release of the content of platelet α-granules are mainly due to platelet-membrane contacts during hemodialysis with complete regranulation of platelets in the post dialytic phase.

In this study we found positive correlations between intra-and post-dialytic βTG and PF4 plasma levels with platelet concentration. 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 blood stream a further heterogenous population of new (larger) and old (reduced) platelets. Thus during hemodialysis, assessment of platelet count in the study of thrombocyte response to the blood-membrane contact is important and is correlated with the contents of α-granules (Mohr *et al*., 1986).

In this study we found positive correlations between PF4 plasma levels and βTG plasma levels during and after HD. Sagripantieti *et al*. (1993) found that uremics, presented significantly higher levels of βTG, PF4, von-will braned factor and seretonin and the βTG plasma levels are correlated with PF4 plasma levels and both hemodialysis procedure and uremia-related factors are likely to contribute to the hemostatic derangement. However Endo *et al*. (1981) found that statistical correlation between βTG and PF4 was not found in uremic patients, the reason is thought to be due to difference in molecular weight and half life time and due to difficulty in calculating statistically the correlation because of the narrow distribution of PF4 levels, but in our study there was a wide distribution of PF4 levels so there was a positive correlation between PF4 and βTG.

In this study annexin V-positive platelets percentage positively correlated with platelet concentration and platelet activation markers. These results are supported by the result of Bonomini *et al*. (2004) who found a positive correlation between annexin V-positive platelets and P-Selectin which is platelet α-granule membrane protein that is rapidly translocated to the cell surface upon stimulation and is considered as a marker of platelet activation. Itoh *et al*. (2006) suggests that platelets activated through interaction with hemodialysis membranes stimulate neutrophils via P-selectin-mediated adhesion and that this property of adhesion to platelets.

CONCLUSION

Platelet activation was found in chronic hemodialysis patients, a finding that may help explain why uremics often suffer from thrombotic accidents. The thrombophilic susceptibility of uremic patients may be partly ascribed to increased PS exposure to the outer membrane leaflet of platelets. PF4 and βTG were released during dialysis due to a defect in α-granules as shown by electron microscopy mainly as consequence of the blood-membrane contact and returns only slowly to control

values. During hemodialysis the decrease of other platelet functions such as aggregation induced by ADP had occurred.

Both hemodialysis procedure and uremia-related factors are likely to contribute to the abnormal platelet function, as hemodialysis causes repeated platelet stress compromising the platelet function in uremia. Further studies of platelet signaling pathways are warranted to elucidate the exact mechanisms leading to loss of platelet membrane phospholipid asymmetry in uremia. Understanding of the mechanisms of platelet activation may be critical in limiting the severity of thrombo-embolic events in uremic patients.

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