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Effects of hemodialysis on platelet-derived thrombospondin

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Effects of hemodialysis on platelet-derived thrombospondin. The effects on platelet-derived thrombospondin (TSP) of hemodialysis with a cellulose membrane were studied in patients during routine hemodialysis and in normal subjects using an ex vivo model. Plasma and platelet-bound TSP were determined pre- and post-dialysis, in blood entering and leaving the dialyzer after 1, 3, 5, 15, and 30 minutes of dialysis, and in blood leaving the ex vivo module after 5, 10, 15, 20, and 25 minutes of perfusion. Plasma concentrations of β-thromboglobulin (βTG) and thromboxane B_2 (TxB_2), and platelet membrane expression of the α-granule protein GMP-140, were also measured. Significant increases in plasma concentrations of TSP and βTG occurred between the inlet and outlet of the dialyzer after 5, 15, and 30 minutes of dialysis, accompanied by a slow, but significant, increase in their arterial plasma concentrations. In contrast, initiation of dialysis was associated with an immediate increase in plasma TxB_2 concentration between the inlet and outlet of the dialyzer and an abrupt increase in arterial plasma TxB_2 concentration which plateaued at 250% of the pre-dialysis value after five minutes. Transit of platelets through the dialyzer had no effect on platelet-membrane-associated TSP or GMP-140. Plasma TSP and βTG concentrations at the outlet of the ex vivo module also increased significantly during perfusion, but plasma TSP concentrations were twofold greater than those during hemodialysis. In vitro stimulation of platelets with thrombin and immunoblotting studies of platelet release proteins showed reduced TSP release by platelets of hemodialysis patients. These data demonstrate that dialyzer-induced platelet activation results in TSP release, suggesting that TSP can be a useful marker of platelet activation during hemodialysis. However, platelets of hemodialysis patients have an impaired ability to release TSP, most probably due to a TSP storage pool deficiency.

Bleeding disorders are a well known complication of uremia [1]. Although these disorders appear to be multifactorial in origin, alterations in platelet function and defective platelet-platelet or platelet-subendothelium interactions have been suggested to play a role in their pathophysiology [2–4]. Hemodialysis has been reported to improve uremic platelet dysfunction [4, 5].

Thrombospondin (TSP) is a multifunctional, trimeric 440 kD glycoprotein that is secreted during the release reaction of human platelets [6–8]. Along with other constituents such as β-thromboglobulin (βTG) and platelet factor 4, TSP is a major component of platelet alpha granules [8], but also has been found in a variety of other cell types, including fibroblasts, endothelial cells, and monocytes [6].

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Although the pathophysiological role of TSP is still poorly understood, it is a protein of remarkable functional diversity. TSP has been reported to bind to heparin, fibrinogen, fibronectin, plasminogen, collagen, and histidine-rich protein [8]. Recently, TSP has received considerable attention because of its putative role as an adhesive glycoprotein in platelet aggregation [9] and cytoadhesion [10]. To date, only a limited number of studies have evaluated plasma TSP concentrations in disease states. Significantly increased plasma levels of TSP have been described in patients with acute myocardial infarction [11], acute [12] and chronic [13] liver failure, and chronic renal failure [13], although, with the exception of acute liver failure, these increases were modest.

Since TSP may play an important role in platelet aggregation and adhesion [9, 10], and because platelet function appears to be modulated by hemodialysis [14, 15], we examined the impact of hemodialysis on platelet TSP in vivo and using an ex vivo model [16, 17]. Dialysis-induced changes in plasma TSP were compared with those of two other markers of platelet activation, βTG and thromboxane B_2 (TxB_2). In addition, flow cytometric techniques were used to evaluate changes in cell surface receptor binding of TSP and another alpha granule protein, GMP-140, that becomes combined with the plasma membrane during the degranulation process [18].

Methods

Patients and control subjects

Changes in TSP during dialysis were evaluated in nine patients with end-stage renal failure being treated with hemodialysis for four hours thrice weekly. Details of the patients are presented in Table 1. None of the patients was known to have a hemostatic disorder and all had been free of infection for at least one month prior to study. A careful history was obtained from each subject to exclude use of aspirin-containing medications during the two weeks preceding the study. All patients received aluminum hydroxide or calcium carbonate, vitamin B_{12}, and ferrous sulfate. Five of the patients were being treated with recombinant human erythropoietin (1000 to 3000 U, three times a week) for correction of anemia. Nine other, randomly selected patients [5 male, 4 female; age 51 ± 3 years; etiology of renal failure: glomerulonephritis (2), polycystic kidney disease (2), diabetic nephropathy (2), hypertensive nephrosclerosis (1), and unknown (2); length of time on dialysis 40 ± 13 months] were studied before and after hemodialysis. Fifteen healthy individuals (9 male, 6 female; age: 22 to 51 years) with normal platelet count and hematocrit were included as normal control

Table 1. Demographic details of patients participating in the hemodialysis studies

Patient	Etiology of renal failure	Age *years*	Gender	Time on dialysis *months*	Prior hemostatic disorders[a]	Hct %	Platelet count $10^3/\mu liter$
1	HTN	66	F	22	None	30	141
2	Unknown	63	M	37	None	32	195
3	GN	38	M	14	None	28	198
4	PCKD	50	F	3	GI bleeding (2)	29	313
5	Unknown	74	M	7	None	31	160
6	DN	44	M	44	None	29	244
7	Unknown	52	F	17	Epistaxis (28)	31	209
8	DN	56	F	128	Clotted shunt (18)	28	151
9	Unknown	41	M	20	None	32	296

Abbreviations are: GN, glomerulonephritis; DN, diabetic nephrosclerosis; HTN, hypertensive nephrosclerosis; PCKD, polycystic kidney disease.

[a] Numbers in parentheses are months elapsed between occurrence of hemostatic disorder and this study

subjects. The study was approved by the Human Studies Committee of the University of Louisville and informed consent was obtained from each subject prior to the study.

In vivo study

All patients enrolled in this study underwent regular hemodialysis treatment with dialyzers containing 1.5 m^2 of hollow fiber cellulose membrane (AM 300M, Asahi Medical, Tokyo, Japan). Although all patients routinely reused their dialyzers, only new dialyzers were used for this study. Anticoagulation was achieved by means of a loading dose and constant infusion of heparin (Elkins-Sinn, Cherry Hill, New Jersey, USA). For each patient, a pharmacokinetic model [19] was used to calculate the dose of heparin required to produce a twofold increase in the whole blood partial thromboplastin time during dialysis. The mean loading dose of heparin was 2056 IU (range: 1000 to 3500 IU) and the mean infusion rate 1472 IU/hr (range: 1000 to 2250 IU/hr). All treatments were performed using SPS 550 delivery systems (Baxter Healthcare, Deerfield, Illinois, USA) and dialysate containing bicarbonate as the base repletion agent. Blood flows were maintained at 250 ml/min. Predialysis blood samples were drawn from the access needle prior to administration of heparin and after withdrawal of 3 ml of blood to clear the needle of any plasma or tissue components activated by needle insertion. Further blood samples were obtained after 1, 3, 5, 15, and 30 minutes of dialysis from the inlet and outlet blood lines of the dialyzer and from the arterial blood line, post-dialysis (240 min). Blood for platelet counts and hematocrit was collected in sodium EDTA tubes and analyzed on a model STKS Coulter Counter (Coulter Electronics, Hialeah, Florida, USA). Blood samples for determination of TSP, βTG, and TxB_2 were drawn into precooled plastic syringes containing an anti-platelet activation cocktail composed of 0.9 mM EDTA, 10 mM adenosine, 1 mM theophylline, and 1 mM indomethacin in Tyrode's buffer, pH 7.5 (blood:anticoagulant = 7:1), and cooled on ice for 30 minutes before centrifugation. In preliminary experiments, this cocktail was found to inhibit in vitro platelet activation effectively. For flow cytometric studies of spontaneous receptor expression, an aliquot of the blood collected in the anti-platelet activation cocktail was immediately added to 2% paraformaldehyde in Tyrode's buffer, pH 7.5, (blood:fixative = 1:1) and fixed at 4°C for one hour. For experiments with thrombin stimulation of platelets, blood was collected using ACD (NIH formula) as the anticoagulant.

Ex vivo study

The effect of cellulosic hollow fiber membranes on whole blood platelet activation was evaluated using a previously described ex vivo model which closely mimics hemodialysis [16, 17]. Briefly, blood from healthy volunteers was used to simultaneously perfuse modules containing 250 cm^2 of cellulosic membrane (Cuprophan, AKZO, Wuppertal, Germany) and a tubing control at a rate of 5 ml/min. Under these conditions, shear rates are similar to those found in routine hemodialysis. Blood was anticoagulated by continuous infusion of heparin at the tip of the access needle. The rate of heparin infusion was set to achieve a concentration of 0.5 IU/ml, similar to that found in hemodialysis. Samples were collected from the outlet of the module and the tubing control into ice-cold polypropylene tubes containing the anti-platelet activation cocktail after 5, 10, 15, 20 and 25 minutes of perfusion.

In vitro studies

Blood was collected from patients just before hemodialysis and from normal controls into plastic syringes containing ACD (ACD:blood = 1:7). Aliquots of 2.5 ml were immediately distributed into 5 ml polypropylene tubes preloaded with thrombin (Sigma Chemical Co., St. Louis, Missouri, USA) in PBS (200 μl) at final concentrations of 0.005 and 0.1 U/ml. 1 U of hirudin (Sigma) in PBS was added to one tube as a baseline control. All tubes were inverted once and platelet activation allowed to proceed by incubating the tubes at 37°C for 10 minutes without agitation. Activation was stopped by adding 200 μl of ice-cold anti-platelet activation cocktail containing 1 U hirudin. The tubes were then placed on crushed ice for a further 10 minutes before separating the plasma by centrifugation.

Measurement of thrombospondin, β-thromboglobulin, thromboxane B_2

Platelet-poor plasma was prepared by centrifuging pre-cooled blood samples at 4°C for 30 minutes at 1500 × g. The upper two thirds of the supernatant was then removed carefully to avoid disturbing the surface lipid layer. Platelet-rich plasma was obtained by centrifugation at 150 × g for 10 minutes. To effect platelet lysis, Triton X-100 was added at a final concentration of 0.5% and incubated at room temperature for 10 minutes. All samples of platelet-poor plasma and lysed platelet-rich plasma were frozen at −70°C and assayed within 30 days. TSP and βTG

were determined in duplicate using commercially available enzyme immunoassays (Diagnostica Stago, Asnières, France) with a sensitivity for TSP of 4 ng/ml and for βTG of 10 ng/ml. TxB_2 levels were determined by radioimmunoassay (Amersham, Arlington Heights, Illinois, USA) after extraction and purification on C18-silica columns [20] (recovery 81 ± 5%, mean ± SD).

Flow cytometry

The following murine monoclonal antibodies were used: fluorescein-(FITC)-conjugated anti-CD41 (Gentrak Inc., Plymouth Meeting, Pennsylvania, USA), activation independent and specific for the GPIIb-IIIa surface membrane complex; phycoerythrin-(PE)-conjugated S12, directed against α-granule protein GMP-140 (CD62) [18]; and, TSP-B7 (Sigma), a monoclonal antibody specific for platelet TSP. TSP-B7 was further purified by protein A affinity chromatography and biotinylated with N-hydroxysuccimide-biotin-ester using standard methods. An IgG_1 monoclonal antibody (Sigma) that recognizes an epitope on the CD5 molecule was biotinylated and used as control for non-specific binding. PE-IgG control and PE-streptavidin were purchased from Becton Dickinson Immunocytometry Systems (San Jose, California, USA).

To determine TSP and GMP-140 associated with the surface of non-stimulated platelets, fixed whole blood was centrifuged at 150 × g for five minutes at 4°C. The platelet-rich plasma was decanted and the platelets pelleted by centrifugation at 1500 × g for five minutes. After washing off the fixative, platelets were resuspended in Tyrodes's buffer (10^7 platelets/ml). To determine cell surface-associated TSP and GMP-140 following thrombin activation, platelets were isolated from ACD-anticoagulated blood by differential centrifugation and resuspended in Tyrode's buffer, containing divalent cations (1 mM $CaCl_2$, 0.5 mM $MgCl_2$) at approximately 10^7 platelets/ml. Thrombin (0.1 U/ml) was added and the platelets incubated at 37°C for 20 minutes without agitation. Activation was stopped by adding an equal amount of 2% paraformaldehyde in Tyrode's buffer and the cells allowed to fix for one hour at 4°C. The cells were then washed and resuspended in the original volume of Tyrode's buffer.

Saturating concentrations of biotin-conjugated TSP-B7, PE-S12, or control antibody with 20 μl of the platelet suspension in 0.5 ml polypropylene tubes was for 30 minutes at 25°C. After washing, PE-streptavidin and FITC-CD41 were added and the incubation continued for a further 20 minutes. Platelets were analyzed by means of a two-color method on a Cytofluorograph IIs (Ortho Diagnostic Systems, Westwood, Massachusetts, USA). To distinguish platelets from other cells in the light scatter profile, the gate was set around those cells exhibiting a high degree of green fluorescence, indicating the presence of the platelet-specific CD41 complex (Fig. 1; >90% positive cells); this platelet population was then analyzed for red fluorescence. The instrument was calibrated daily using 2 μm beads (Becton Dickinson Immunocytometry Systems). Light scatter and fluorescence data were obtained with gain settings in the logarithmic mode, and the data were analyzed with a 2151 Data Handling System (Ortho Diagnostic Systems). The mean channel number of fluorescence intensity (mcf) was used a quantitative measure of antibody binding. The flow cytometric assays were established with the help of Dr. Samuel R. Wellhausen.

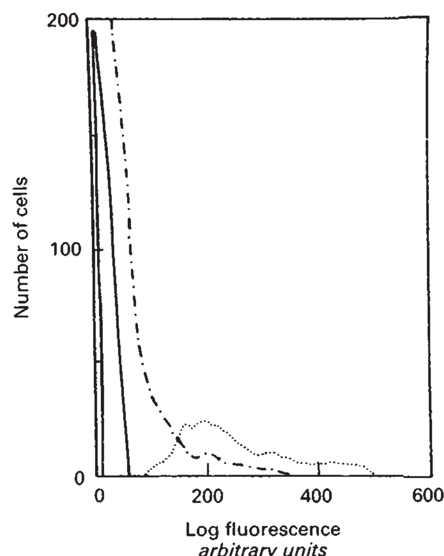

Fig. 1. *Effect of stimulation with thrombin (0.1 U/ml for 20 min) on platelet surface-bound thrombospondin as assessed by binding of anti-TSP monoclonal antibody.* Symbols are (—) control Ab; anti-TSP binding to non-stimulated (–·–); thrombin stimulated (· · ·) platelets.

SDS-polyacrylamide gel-electrophoresis (SDS-PAGE) and immunoblotting

Samples from uremic and control platelets were prepared by exposing platelets suspended in Tyrode's buffer, pH 7.5, containing divalent cations (10^7 platelets/ml) to 0.5 U/ml of bovine thrombin (Sigma) for three minutes at 37°C. Phenylmethanesulfonylfluoride (PMSF) (1 mM) was then added and samples were spun at 8000 × g for five minutes. Platelet polypeptides were fractionated by SDS-PAGE [21]. Washed platelets were solubilized with 3% SDS containing 1 mM PMSF and heated for three minutes at 80°C in the presence of β-mercaptoethanol. Proteins were electrophoretically transferred to nitrocellulose paper at 12 volts for one hour at 25°C (Idea Scientific Company, Corvallis, Oregon, USA) in pH 8.3 buffer containing 25 mmol/liter of Tris and 192 mmol/liter of glycine. Immunodecoration was performed with a monospecific polyclonal TSP-antibody (Diagnostica Stago) according to standard methods.

Data analysis

Platelet counts and plasma concentrations of TSP and βTG obtained in the hemodialysis studies were corrected for changes in plasma volume arising from ultrafiltration using concurrent hematocrit values. This correction was not used for TxB_2 because its small size (approximately 370 D) would result in it being freely filtered by the dialysis membrane and, therefore, not concentrated by ultrafiltration. Student's t-test for unpaired data was used to test for differences between normal controls and patients, predialysis. Analysis of variance for repeated measures was used to test whether or not a given parameter changed with time during hemodialysis or ex vivo perfusion time. The significance of the change in a parameter between the dialyzer outlet and inlet was determined by Student's t-test for paired data. Two-way analysis of variance was used to compare TSP and βTG release by normal and uremic platelets. Where a significant difference was found ($P < 0.05$), differences between

Table 2. Plasma thrombospondin and β-thromboglobulin, platelet count, surface-bound thrombospondin, and GMP-140 expression in normal subjects and hemodialysis patients, pre-dialysis

	Hemodialysis patients[a]		Normal controls	
Plasma thrombospondin *ng/ml*	5.0 ± 0.4	(N = 16)[b]	10.1 ± 2.8	(N = 15)
Plasma β-thromboglobulin *ng/ml*	172 ± 22	(N = 16)[c]	75 ± 14	(N = 15)
Platelet count $10^3/\mu l$	243 ± 19	(N = 13)	209 ± 18	(N = 9)
Platelet surface-bound thrombospondin *mcf*[d]				
Non-stimulated	176 ± 27	(N = 12)	130 ± 17	(N = 7)
Thrombin-stimulated	342 ± 28		320 ± 67	
Platelet GMP-140 expression *mcf*[d]				
Non-stimulated	73 ± 3	(N = 7)	69 ± 1	(N = 4)
Thrombin-stimulated	164 ± 29		182 ± 20	

[a] Plasma solute concentrations for the hemodialysis patients are presented as measured. No correction has been made for the difference in hematocrit between the patients and the normal controls.
[b] Significantly less than normal controls, P = 0.042
[c] Significantly greater than normal controls, P = 0.001
[d] mcf, Mean channel number of fluorescence intensity (**Methods**)

individual pairs of samples were tested using the Student-Newman-Keuls procedure. Many baseline plasma TSP concentrations were at the lower limit of detection (4 ng/ml), giving rise to data that was not normally distributed. In these instances, appropriate non-parametric tests (Mann-Whitney U test, Wilcoxon signed rank test) were used. All statistical testing was performed using the SPSS/PC+ statistical package (SPSS Inc, Chicago, Illinois, USA). Data are presented as mean ± SEM for N observations.

Results

Baseline plasma thrombospondin and β-thromboglobulin, platelet surface-bound thrombospondin, and platelet GMP-140 (CD62) expression

In comparison to normal controls, plasma TSP concentrations were significantly lower in the hemodialysis patients, pre-dialysis (P = 0.047, Table 2). In contrast, plasma βTG concentrations were significantly elevated in the hemodialysis patients compared to the normal controls (P = 0.001, Table 2). There was no correlation between TSP and βTG in either group.

Platelet counts in the hemodialysis group were not different from those in the control group (Table 2). As shown in Figure 1, non-stimulated control platelets demonstrated essentially no surface-bound TSP. Activation with thrombin (0.1 U/ml) for 20 minutes at 37°C resulted in a marked increase in surface-bound TSP (Fig. 1, Table 2). Comparable results were obtained for platelets from hemodialysis patients (Table 2). The pattern of platelet GMP-140 expression was similar to that of surface-bound TSP; again, there were no differences between platelets from hemodialysis patients and controls (Table 2).

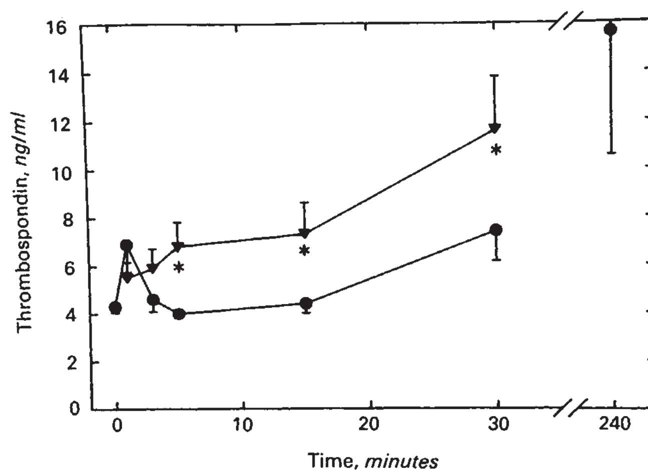

Fig. 2. *Changes in plasma thrombospondin concentrations in blood entering (●) and leaving (▼) the dialyzer during hemodialysis.* Data are presented as mean ± SEM for 9 experiments. A significant increase in thrombospondin concentration between the dialyzer outlet and inlet is indicated by *.

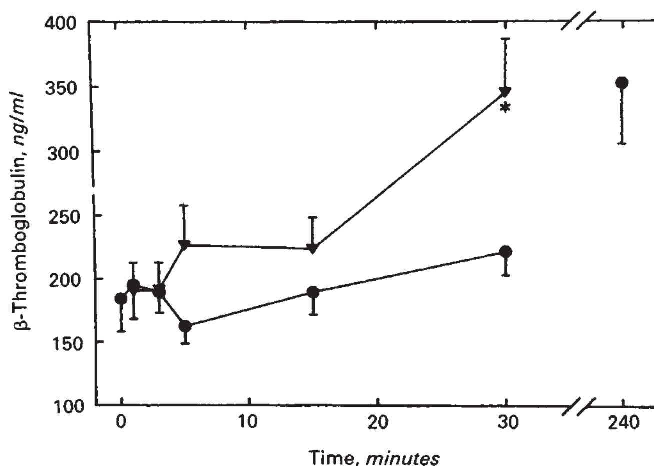

Fig. 3. *Changes in plasma β-thromboglobulin concentrations in blood entering (●) and leaving (▼) the dialyzer during hemodialysis.* Data are presented as mean ± SEM for 9 experiments. A significant increase in β-thromboglobulin concentration between the dialyzer outlet and inlet is indicated by *.

Hemodialysis studies

Administration of heparin did not affect the plasma concentrations of either TSP or βTG in the hemodialysis patients (data not shown). Arterial plasma TSP concentrations increased transiently during the first three minutes of dialysis and then returned to pre-dialysis levels (Fig. 2). After 15 minutes, arterial TSP concentrations again increased and were significantly greater than pre-dialysis values after 240 minutes (P = 0.004, Fig. 2). Plasma TSP concentrations leaving the dialyzer were greater than arterial values at all time points after five minutes. A similar pattern was observed for changes in plasma βTG during dialysis (Fig. 3). Arterial plasma βTG concentrations increased significantly during dialysis (P = 0.001), with the dialyzer inlet to outlet concentration difference achieving significance after 30 minutes of dialysis. In contrast to both TSP

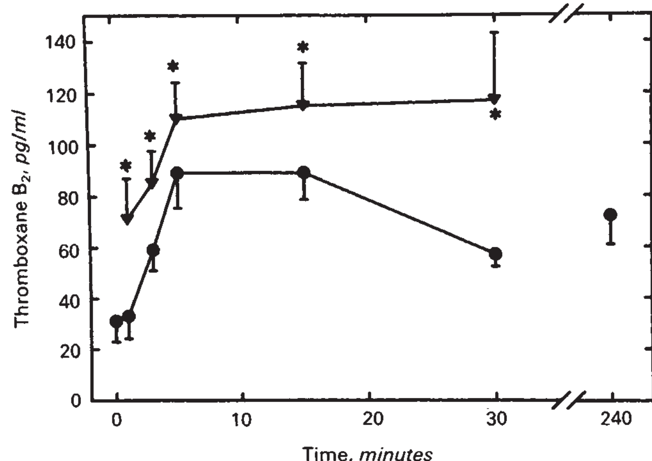

Fig. 4. *Changes in plasma thromboxane B_2 concentrations in blood entering (●) and leaving (▼) the dialyzer during hemodialysis.* Data are presented as mean ± SEM for 6 experiments. A significant increase in thromboxane B_2 concentration between the dialyzer outlet and inlet is indicated by *.

and βTG, arterial TxB_2 concentrations increased promptly following initiation of dialysis ($P = 0.001$), plateauing at approximately 250% of the pre-dialysis value after five minutes of dialysis (Fig. 4). Dialyzer outlet concentrations of TxB_2 were significantly greater than arterial concentrations at all sampling times.

Arterial platelet counts changed significantly ($P = 0.01$) with time of dialysis. The magnitude of the change was small, however, being no greater than 5% at any sampling time (Table 3). There was no change in platelet count between the inlet and outlet of the dialyzer at any sampling time. Administration of heparin did not affect the amount of platelet surface-bound TSP; for example, the level of surface-bound TSP following thrombin stimulation was 335 ± 48 mcf in the absence of heparin and 278 ± 60 mcf following heparin administration ($N = 7$, NS). The amount of surface-bound TSP on platelets from arterial blood remained unchanged throughout dialysis, both for non-stimulated and thrombin-stimulated cells (Table 3). The thrombin-induced increase in surface-bound TSP, however, did show a small, but significant ($P = 0.048$) dependence on dialysis time, increasing from 123 ± 12 channels, pre-dialysis, to 200 ± 35 channels by five minutes and remaining at that level, thereafter. The major contribution to the change in the thrombin-induced increase in surface-bound TSP appeared to arise from a decrease in the non-stimulated level, although this decrease was not significant. Transit of platelets through the dialyzer had no effect on the level of surface-bound TSP in either non-stimulated or thrombin-stimulated cells. Platelet GMP-140 expression on both non-stimulated and thrombin-stimulated platelets was unaffected by dialysis (Table 3).

Ex vivo studies

Plasma TSP and βTG levels during ex vivo blood perfusion of mini-modules containing cellulose membranes are shown in Figure 5. Plasma TSP concentrations were increased over baseline levels at the outlet of both the tubing control and the mini-module. This increase in concentration was evident after five minutes and its magnitude was similar in both the mini-module and tubing control. Plasma βTG concentrations were also increased significantly over baseline controls; however, unlike TSP, the increase in βTG depended significantly on both time and the presence of the membrane ($P = 0.047$). Surface-bound TSP on non-stimulated and thrombin-stimulated platelets exposed to both the cellulose membrane and the control tubing remained unchanged throughout the ex vivo perfusion (Table 4).

In vitro studies

Because of the apparent difference in the magnitude of TSP release by normal and uremic platelets following exposure to a cellulosic dialysis membrane (Fig. 2 and 5), the release of TSP by normal and uremic platelets in response to thrombin stimulation was examined in vitro. In the control group, thrombin activation significantly increased plasma levels of TSP and βTG (Table 5). The release of TSP from the platelets of hemodialysis patients was significantly less than that from normal platelets ($P = 0.034$), and essentially no increase in plasma βTG was seen (Table 5). Lysis of platelets in PRP by Triton X-100 revealed that hemodialysis patients tended to have lower total platelet TSP stores than normal, although this difference was not significant ($P = 0.149$). A reduction in TSP release by platelets from hemodialysis patients was also suggested by the results of the immunoblotting studies. As shown in Figure 6, immunoblotting of identical amounts of SDS-PAGE-separated release proteins from platelets obtained from the hemodialysis patients showed a distinctly weaker immunoreaction than release proteins from normal platelets. Probing of total platelet proteins for TSP antibody binding revealed an increased number of immunoreactive bands with greater molecular mobility for the hemodialysis patients compared to the normal controls (Fig. 6).

Discussion

Multiple, complex changes occur in hemostatic mechanisms during hemodialysis. Since TSP is suggested to play an important role in platelet aggregation and primary hemostasis [6–10], changes in its plasma concentration may be a useful indicator of thrombogenic events occurring during hemodialysis.

In agreement with previous reports [22, 23], βTG concentrations were significantly increased in hemodialysis patients, pre-dialysis, compared to normal controls (Table 2). In contrast, plasma concentrations of TSP were significantly decreased in hemodialysis patients, pre-dialysis, when compared to normal subjects (Table 2). Baseline values of plasma TSP obtained in this study were less than those reported in earlier studies [11–13]. The reasons for this discrepancy are not known, but may include differences in the antisera or TSP standards used previously as has been reported for the analysis of other α-granule proteins [24]. The amount of surface-bound TSP and GMP-140, α-granule proteins released and expressed on the platelet plasma membrane following activation, did not differ between the two groups, either for non-stimulated or thrombin-stimulated platelets (Table 2). The latter finding suggests that the α-granule release reaction is not altered in the platelets of hemodialysis patients.

Increased production of TxB_2 [25–27] and release of the platelet specific α-granule protein, βTG, [22, 28, 29] have been repeatedly reported to occur during hemodialysis. Our studies

Table 3. Changes in platelet count, surface-bound thrombospondin, and GMP-140 expression during hemodialysis

	Pre-HD	Time of dialysis *min*				
		3	5	15	30	240
Platelet count $10^3/\mu l$ (N = 9)						
Inlet (arterial)	212 ± 19	221 ± 16	218 ± 18	199 ± 17	201 ± 16	217 ± 22
Outlet		217 ± 20	219 ± 24	202 ± 20	191 ± 18	
Platelet surface-bound thrombospondin *mcf*[a] (N = 8)						
Non-stimulated						
Inlet (arterial)	200 ± 37	134 ± 32	140 ± 35	144 ± 49	192 ± 28	123 ± 28
Outlet		168 ± 25	141 ± 25	170 ± 35	160 ± 29	
Thrombin-stimulated						
Inlet (arterial)	323 ± 40	311 ± 45	351 ± 44	317 ± 59	335 ± 61	345 ± 45
Outlet		296 ± 41	329 ± 39	377 ± 40	289 ± 50	
Platelet GMP-140 expression *mcf*[a] (N = 5)						
Non-stimulated						
Inlet (arterial)	77 ± 4			74 ± 3	74 ± 3	70 ± 3
Outlet				82 ± 1	77 ± 3	
Thrombin-stimulated						
Inlet (arterial)	208 ± 39			195 ± 12	210 ± 23	204 ± 33
Outlet				190 ± 26	192 ± 33	

[a] mcf, Mean channel of fluorescence intensity (**Methods**)

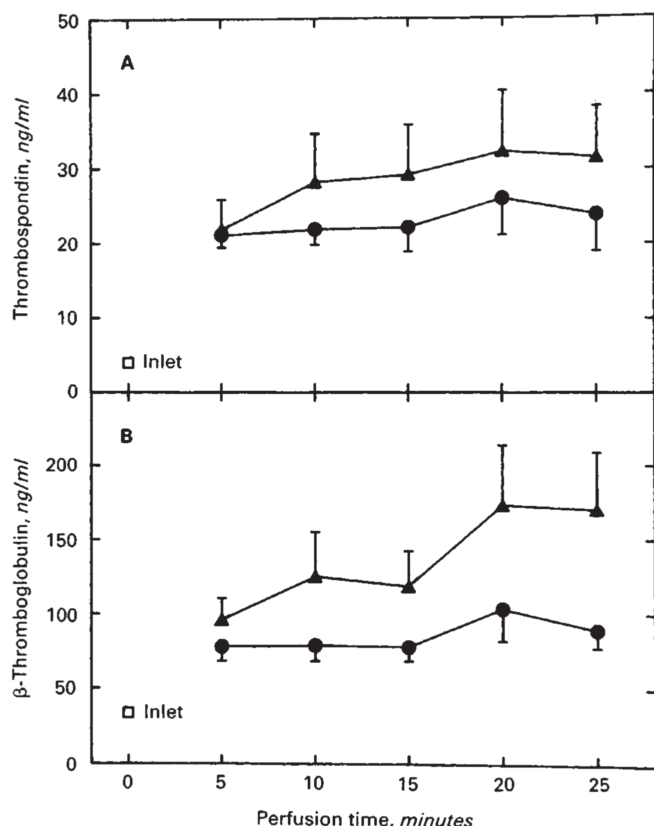

Fig. 5. *Changes in plasma thrombospondin* (**A**) *and β-thromboglobulin* (**B**) *concentrations leaving the membrane module* (▲) *and tubing control* (●) *following ex vivo perfusion with normal blood.* Data are presented as mean ± SEM for 5 experiments.

Table 4. Surface-bound thrombospondin on platelets following ex vivo perfusion of mini-modules containing cellulose membrane or tubing control

	Surface-bound thrombospondin *mcf*[a] (N = 3)			
	Tubing control		Membrane module	
	Non-stimulated	Activated	Non-stimulated	Activated
Baseline	167 ± 9	361 ± 55		
Perfusion time *minutes*				
3	136 ± 10	312 ± 7	174 ± 12	521 ± 88
5	138 ± 10	280 ± 15	185 ± 32	325 ± 36
10	131 ± 14	367 ± 55	148 ± 12	398 ± 50
20	134 ± 18	377 ± 57	166 ± 18	379 ± 72
30	124 ± 14	383 ± 21	173 ± 55	338 ± 25

[a] mcf, Mean channel of fluorescence intensity (**Methods**)

Table 5. Effect of thrombin stimulation on thrombospondin and β-thromboglobulin release from platelets of normal controls and hemodialysis patients, pre-dialysis

	Thrombin concentration *U/ml*			Total platelet content
	0	0.005	0.1	
Thrombospondin *ng/10⁸ platelets* (N = 4)				
Normal	2.7 ± 0.5	5.6 ± 1.4	6.1 ± 1.2	5580 ± 1017
Pre-dialysis	2.5 ± 0.3	2.9 ± 0.4	3.8 ± 0.5	3475 ± 617
β-thromboglobulin *ng/10⁸ platelets* (N = 4)				
Normal	20 ± 5	67 ± 23	72 ± 20	9978 ± 3196
Pre-dialysis	65 ± 8	60 ± 9	66 ± 5	12531 ± 3336

confirm these findings and, in addition, show significant increases in arterial plasma TSP concentrations, and in plasma TSP concentrations between the inlet and outlet of the dialyzer, in the first 30 minutes of dialysis (Fig. 2). The concurrent increases in TSP and βTG across the dialyzer suggest that dialyzer-induced platelet activation is the most likely mechanism for the increases in plasma TSP concentration. Although monocytes may also serve as a source of TSP [6], release of TSP by activated monocytes seems unlikely to account for the increases in plasma TSP seen in this study. Firstly, after 15 and 30 minutes of dialysis, when the increases in plasma TSP concentration between the inlet and outlet of the dialyzer were greatest (Fig. 2), there were very few circulating monocytes as

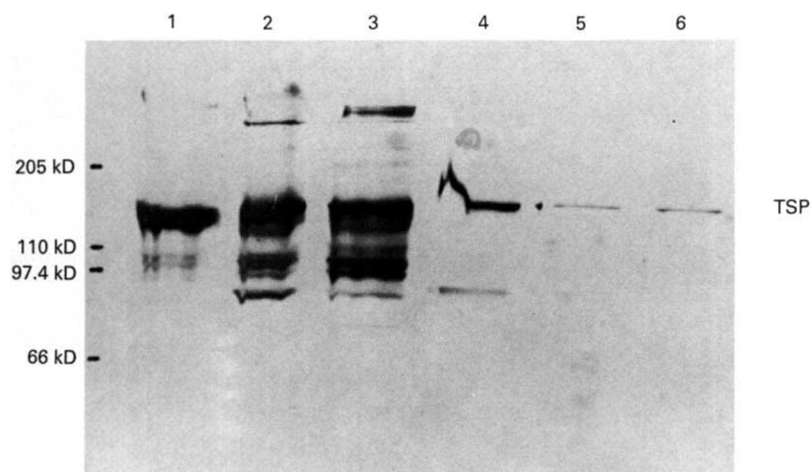

Fig. 6. *Immunoblot of total proteins and release proteins from platelets of a normal control (total proteins: lane 1, release proteins: lane 4), patient 4 (total proteins: lane 2, release proteins: lane 5) and patient 9 (total proteins: lane 3, release proteins: lane 6).* Samples were run under reducing conditions and each lane of the blotted gel contained equal amounts of total platelet protein (30 μg) or release proteins (20 μg). Standard molecular weight markers were: myosin (205 kD), β-galactosidase (116 kD), phosphorylase B (97.4 kD), and bovine albumin (66 kD).

a consequence of the complement-induced pulmonary sequestration of leukocytes which occurs during dialysis with cellulosic membranes [30]. Blood monocyte counts at the inlet to the dialyzer were 99 ± 18 and 149 ± 42 cells/μl after 15 and 30 minutes of dialysis, respectively, compared to platelet counts of 199,000 ± 17,000 and 201,000 ± 16,000 cells/μl, respectively. Secondly, Jaffe, Ruggiero and Falcone [31] have shown that, while monocytes are able to synthesize and secrete TSP, they do not store TSP, either intracellularly or in an extracellular matrix. Thus, TSP release by monocytes depends on protein synthesis which could not occur in the 20 seconds it takes a monocyte to pass through the dialyzer. Further, it is unlikely that the increase in plasma TSP concentrations resulted from an improvement in uremia, since plasma TSP concentrations also increased during passage of blood through the mini-modules of the ex vivo system; a circumstance free from the effects of uremia.

Plasma concentrations of TSP and βTG increased transiently during the first five minutes of dialysis, followed by a more sustained increase after 15 minutes (Fig. 2 and 3). While the initial increases did not reach statistical significance, they were consistently seen and suggest that the platelet response to extracorporeal circulation may be biphasic. At present, we can only speculate on the mechanisms of such a biphasic response. However, a transient release of platelet activating factor (PAF) in the first minutes of dialysis has been described [32], suggesting a role for this strong agonist in the initial phase of platelet degranulation. The rapid appearance of TxB_2 (Fig. 4), followed later by increases in TSP and βTG, suggests that the platelet α-granule release occurring after 15 minutes of dialysis may be mediated by thromboxane A_2, the active precursor of TxB_2 [33].

Since a significant fraction of TSP released during platelet activation is immediately bound to the platelet plasma membrane, surface-bound TSP has been suggested to be a better marker of platelet activation than plasma concentrations [13]. Accordingly, we used flow cytometry to measure intra-dialytic changes in platelet surface-associated TSP and GMP-140, another α-granule protein which becomes surface expressed following platelet activation [18]. We could not detect any change in the amount of surface-bound TSP or GMP-140 on platelets from arterial or venous blood samples during dialysis (Table 3), either on non-stimulated or on thrombin-stimulated cells.

The lack of an apparent increase in surface-bound TSP and GMP-140 during hemodialysis in the face of increased plasma concentrations of TSP and βTG is not easily explained. Similar results were found in the ex vivo studies (Fig. 5, Table 4), suggesting that the lack of TSP expression during dialysis was not the result of uremia. An increase in plasma levels of α-granule proteins without a significant increase in platelet-bound TSP has also been reported for cardiopulmonary bypass [34]. Heparin, which is used as an anticoagulant in both hemodialysis and cardiopulmonary bypass, binds to TSP and might either mask the epitope recognized by the anti-TSP monoclonal antibody or interfere with TSP binding to its receptor. However, preliminary results [35] argue against this mechanism. Alternatively, platelets with increased surface-bound TSP or GMP-140 may have aggregated and adhered to the dialysis membrane, thereby leaving the circulation and escaping detection. This explanation is consistent with the suggestion of Musial et al [36] that release of α-granule constituents does not occur until after activated platelets have adhered to the surface of the extracorporeal circuit. Recovery and analysis of platelets adhering to the membrane will be needed to examine this possibility.

We used an ex vivo model of hemodialysis [16, 17] to further characterize TSP release during blood flow through cellulose dialysis membranes. This model closely simulates hemodialysis, but uses normal blood. Plasma TSP increased immediately following initiation of blood perfusion (Fig. 5). Strikingly, plasma TSP levels obtained in the ex vivo experiments were about twofold greater than those observed during hemodialysis. This difference cannot be explained by the greater plasma volume associated with the anemia of chronic renal failure. Although hematocrits were greater in the ex vivo experiments than in the hemodialysis studies (40.1 ± 2.1 vs. 29.7 ± 0.5), this difference could only lead to a 17% difference in TSP concentrations following an equivalent degree of TSP release. In addition, plasma concentrations of βTG also increased to a similar extent during ex vivo perfusion and hemodialysis (Figs. 3 and 5). Further, since there was a significant correlation between TSP and βTG concentrations in the ex vivo experi-

Table 6. Increases in plasma concentrations of thrombospondin and β-thromboglobulin between dialyzer inlet and outlet in patients receiving and not receiving rHuEPO

	Time of dialysis *min*				
	1	3	5	15	30
Thrombospondin *ng/ml*					
With rHuEPO	−1.0 ± 1.4	1.8 ± 1.2	4.8 ± 1.5	3.1 ± 1.3	4.3 ± 1.9
Without rHuEPO	−2.0 ± 2.0	0.3 ± 0.4	0.0 ± 0.0	2.4 ± 2.4	4.0 ± 2.6
β-thromboglobulin *ng/ml*					
With rHuEPO	−4 ± 39	1 ± 34	111 ± 46	55 ± 43	154 ± 39
Without rHuEPO	−7 ± 16	2 ± 5	4 ± 2	5 ± 13	87 ± 55

Data are presented as mean ± SEM for $N = 5$ (with rHuEPO) or $N = 4$ (without rHuEPO).

ments (Pearson correlation coefficient = 0.7863, $P = 0.001$), it seems unlikely that the higher plasma TSP concentrations seen in the ex vivo experiments were due to TSP release from other blood cells, such as monocytes [6]. Therefore, we conclude that the platelets of hemodialysis patients may have a reduced ability to release TSP, as has been suggested previously for βTG [37].

To further investigate TSP release from platelets of hemodialysis patients, whole blood was stimulated with thrombin in vitro and the resulting plasma levels of TSP and βTG determined. The results of these experiments (Table 5) showed a blunted response to thrombin stimulation by the platelets of hemodialysis patients compared to those of normal subjects, confirming the results of the dialysis studies. Surface-bound TSP on activated platelets did not differ between the two groups (Table 2), suggesting that the decreased TSP release by platelets of hemodialysis patients resulted from a reduction of TSP stores, rather than an impaired release reaction. A reduction in TSP stores is also supported by the immunoblots (Fig. 6), which show a diminished immunoreactive band for TSP in the releasate from platelets of hemodialysis patients compared to normal subjects. Interestingly, total platelet proteins from the dialysis patients contained a number of TSP immunoreactive bands with greater molecular mobility (Fig. 6). The presence of these bands could be due to increased intra-platelet protease activity or impaired TSP biosynthesis, as has been suggested to occur in patients with thrombocythemia [38].

It has been demonstrated that treatment with recombinant human erythropoietin (rHuEPO) ameliorates the hemostatic disorders associated with uremia [39]. Comparison of the increases in plasma TSP and βTG concentrations between the dialyzer outlet and inlet for patients receiving and not receiving rHuEPO showed them to be greater for the patients receiving rHuEPO (Table 6), although the differences did not quite reach statistical significance ($P = 0.057$ and $P = 0.114$ for βTG and TSP, respectively). Why rHuEPO should improve platelet function is unclear. On the one hand, preliminary studies suggest that rHuEPO may impact directly on platelets. Sakaguchi et al [40] have shown that rHuEPO increases megakarypoiesis in vitro, while Van Geet et al [41], studying uremic children treated with rHuEPO, found improved aggregation and higher concentrations of intracellular calcium following thrombin stimulation of isolated platelets. On the other hand, are reports suggesting the normalization of prolonged bleeding times following correction of anemia is mediated by the increase in erythrocyte numbers, either through hemodynamic mechanisms [42] or transcellular metabolism involving erythrocyte effects on eicosanoid release by platelets [43]. The mechanisms by which rHuEPO affects platelet function, and whether or not improved function results in platelets being more easily activated during hemodialysis, remain unknown. However, rHuEPO therapy is now a common part of the treatment of end-stage renal disease and, if confirmed, any rHuEPO-induced improvement in platelet function could pose problems in terms of dialyzer blood loss and blood access thrombosis.

The clinical significance of these apparent abnormalities in TSP release remains unknown. Further studies are currently underway to extend our observations to determine whether the observed decrease in TSP release results from uremia, per se, or is a consequence of repeated hemodialysis. Also, since the plasma concentration of TSP is independent of renal catabolism and heparin infusion [7, 12], conditions which affect plasma levels of the α-granule proteins, βTG and platelet factor 4, respectively [11, 12], TSP may be a useful marker of platelet α-granule release in renal failure and hemodialysis.

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