Differential effects of dialysis and ultrafiltrate from individuals with CKD, with or without diabetes, on platelet phosphatidylserine externalization

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Submitted 19 June 2007; accepted in final form 30 July 2007

CARDIOVASCULAR DISEASE, in particular atherothrombosis, is a common cause of death in patients receiving dialysis, and comorbid conditions such as diabetes mellitus (DM) further influence the incidence of vascular events (13, 19, 20). Progression is associated with vascular endothelial cell injury, atherosclerotic plaque fissuring, and rupture (33). An early key event in such progression is increased adherence of monocytes to the damaged vascular endothelium, resulting in fatty streak/atheroma development, with platelets subsequently attaching firmly to the site of the lesion (29).

In the plasma membrane of resting platelets, the phospholipid phosphatidylserine (PS) resides in the inner leaflet (5), but, on activation of Scramblase by PKCδ, translocates to the outer leaflet of the plasma membrane (5, 15). It has become increasingly clear that chronically elevated or prolonged exposure of PS on the cell surface both increases vascular damage and results in the formation of a hypercoagulable environment by stimulating adherence of inflammatory cells to the vascular endothelium (e.g., Ref. 22) and providing a catalytic surface for assembly of the prothrombinase complex (29) that accelerates the generation of thrombin (32, 49).

Evidence now suggests that classic platelet agonists such as thrombin and ADP elicit PS externalization in a small subpopulation of these cells (45, 48) and that the size of this population correlates with prothrombinase activity (48). In addition, increased PS externalization in a subpopulation of platelets has been observed in individuals with chronic kidney disease (CKD) and DM (45), although the factor(s) responsible for this response is unknown.

Advanced glycation end products (AGE) arise by covalent modification of cellular and plasma proteins and form a series of heterogeneous compounds (21). AGE are substantially increased in individuals with either CKD or DM compared with the general population (25, 44) and may contribute to the development and progression of cardiovascular disease in these patient groups (35, 39).

In a recent study, we showed that human serum albumin-AGE (HSA-AGE), prepared in vitro, elicits PS externalization in a small subpopulation of human platelets. Moreover, this response was completely blocked by both the 5-hydroxytryptamine (5-HT)2A/2C receptor antagonist ritalisertin and the PKCδ inhibitor rottlerin (45).

To translate this work toward the in vivo situation, we have now explored, first, the effect of hemodialysis on the elevated PS externalization we observed in patients with CKD/DM and, second, the ability of ultrafiltrate from these individuals to elicit PS externalization in platelets from healthy controls and the mechanism(s) involved.

METHODS

Materials and reagents. Annexin V-FITC was purchased from Immunotech (Marseille, France), and the FluoroSpheres for FACScan calibration were obtained from DakoCytomation (Glostrup, Denmark). The fluorescence-activated cell-scanner CD61-PerCP and the Annexin V-FITC were purchased from Becton Dickinson (Cowley, Oxford, UK). Rottlerin and bisindolylmaleimide 1 were from Merck Biosciences (Beeston, Nottingham, UK). The 5-HT2A/2C receptor antagonist ritalisertin and all general chemicals were from Sigma-Aldrich (Poznan, Poland).

To examine the effect of UF from these individuals on PS externalization in platelets, we performed a fluorescence-activated cell sorter using annexin V in platelet-rich plasma. PS externalization was quantified by a fluorescence-activated cell-scanner CD61-PerCP and the FluoroSpheres for FACScan. The data suggest that uremic toxins present in UF mediate PS externalization in a small subpopulation of platelets from healthy controls. The effect of UF from individuals with CKD and DM was significantly greater than that from patients with CKD alone, and the response was partially inhibited by the protein kinase Cδ (PKCδ) inhibitor rottlerin and the 5-hydroxytryptamine (5-HT)2A/2C receptor antagonist ritalisertin. The data suggest that uremic toxins present in UF mediate PS externalization in a small subpopulation of platelets, at least in part, via the 5-HT2A/2C receptor and PKCδ and demonstrate that DM further enhances platelet PS externalization in CKD patients undergoing hemodialysis. This may explain, at least in part, the additional increase in vascular damage observed in CKD patients when DM is present.

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were obtained from Sigma-Aldrich (Poole, Dorset, UK). The low-flux Fresenius polysulfone hemodialysis membrane was purchased from Fresenius Medical Care (Bad Homburg, Germany), and the ENDOSAFE LAL Gel Clot Test was from Charles River (Charleston, SC).

**Patients.** Twenty-six stable individuals (age range 32–85 yr) who had been receiving low-flux conventional hemodialysis using a Fresenius polysulfone membrane three times per week for at least 3 mo participated in the study, which was submitted to and approved by the local research ethics committee. Of the patients participating, eight (6 men and 2 women) also had type 2 DM, while the remaining 18 (13 men and 5 women) did not.

Effect of a single, low-flux hemodialysis treatment on platelet PS externalization in individuals with either CKD or CKD and DM. Hemodialysis patients at Newcastle undergo three sessions a week for 4 h at a time. The individuals selected for this study were treated on Monday, Wednesday, and Friday. There was no preference for any particular day when the effect of a single treatment was examined.

A 2.5-ml blood sample was withdrawn predialysis and then 1, 2, and 3 h after the commencement of hemodialysis from patients with either CKD or CKD and DM using a standard 16-G hypodermic needle. This was then immediately added to a tube containing acid citrate dextrose (ACD) and centrifuged at 100 g for 15 min at 20°C (Beckman J6-MC). The upper phase containing the platelet-rich plasma (PRP) was removed, and the extent of PS externalization was determined as described below.

Effect of three low-flux hemodialysis treatments during a 7-day period on platelet PS externalization in individuals with either CKD or CKD and DM. Pre- and 3-h postdialysis blood samples (2.5 ml) were withdrawn on Monday, Wednesday, and Friday from patients with either CKD or CKD and DM using a standard 16-G hypodermic needle. PRP was then prepared as above, and the extent of PS externalization was determined as described below.

Collection of ultrafiltrate from individuals with either CKD or CKD and DM. Studies using urea as a candidate molecule to understand solute kinetics in hemodialysis have revealed that it is rapidly removed during the first 30 min but that its removal rate then gradually declines throughout the rest of dialysis (e.g., Ref. 24). We have therefore utilized two time points, 20 and 180 min, to investigate effects on PS externalization in these two different kinetic phases.

To collect pure ultrafiltrate, the dialysis fluid was disconnected from the dialyzer for a short period both 20 (UF_{20}) and 180 min (UF_{180}) after the start of treatment. The dialysis fluid pathway was evacuated, ultrafiltrate was allowed to flow for 2–3 min, and 3 ml of ultrafiltrate was collected into an endotoxin-free tube. The system was then reconnected, and the treatment was continued.

Effect of ultrafiltrate from individuals with CKD or CKD and DM on PS externalization. PRP was prepared from healthy controls as described above and then incubated with increasing volumes (2.5–20 μl) of either UF_{20} or UF_{180} from individuals with CKD or CKD and DM at 37°C for 10 min. Samples were immediately placed on ice, and PS externalization was determined as described below.

**Fluorescence Intensity (Annexin V)**

**Fluorescence Intensity (CD-61)**

**Fig. 1.** Effect of a single, low-flux hemodialysis treatment on phosphatidylserine (PS) externalization in a subpopulation of human platelets in individuals with chronic kidney disease (CKD). Platelet-rich plasma (PRP) was prepared from individuals with CKD either predialysis or 1, 2, and 3 h after the commencement of dialysis. PS externalization was then assessed using annexin V-FITC and CD61-PerCP with subsequent quantification by FACSscan. For comparison purposes, a healthy control is also shown, and data are presented as both a histogram (top) and a dot-plot (bottom).

**Dialysis Time**

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controls. ADP (100 nM) was then added for 1 min. The extent of PS externalization was then determined as described below.

Effect of inhibition of caspase 3 on PS externalization mediated by ultrafiltrate from individuals with CKD or CKD and DM. PRP prepared from healthy controls was preincubated with 20 μM Z-DEVD-FMK, a caspase 3 inhibitor, or Z-FAD-FMK, its negative control (17), for 30 min, and then 10 μl of either UF20 or UF180 from individuals with CKD or CKD and DM was added for 10 min at 37°C. The extent of PS externalization was determined as described below.

Effect of inhibition of PKC on PS externalization mediated by ultrafiltrate from individuals with CKD or CKD and DM. PRP prepared from healthy controls was preincubated with 10 μM rottlerin, a PKC inhibitor, for 5 min. This concentration has been reported to block PKC activity by ~80–90% while having no effect on representative examples of either PKCα (classic) or PKCζ (atypical) isoforms (15). Ten microliters of either UF20 or UF180 from individuals with CKD or CKD and DM was then added for 10 min at 37°C. The extent of PS externalization was determined as described below.

Effect of a 5-HT2A/2C receptor antagonist on PS externalization mediated by ultrafiltrate from individuals with CKD or CKD and DM. PRP prepared from healthy controls was preincubated with 1 μM ritanserin, a 5-HT2A/2C receptor antagonist (26). Ten microliters of either UF20 or UF180 from individuals with CKD or CKD and DM was then added for 10 min at 37°C. The extent of PS externalization was determined as described below.

Measurement of PS externalization in PRP. PS externalization was determined using the cell membrane-impermeant, PS-specific annexin V-FITC conjugate and CD61-PerCP as a platelet-specific marker, with subsequent quantification by a fluorescence-activated cell scanner (FACScan) supporting Lysis II software as described previously (45).

Statistical analysis. Statistical analysis was undertaken using Minitab 14 (Minitab, State College, PA). Studies with the caspase 3/PKC inhibitors and the 5-HT2A/2C receptor antagonist were analyzed by use of Student’s t-test and are presented as means ± SE with *P < 0.05, **P < 0.01, and ***P < 0.001 with respect to the relevant control. Serial measurements with ultrafiltrate from individuals with CKD or CKD and DM were analyzed as summary measures (27), i.e., the areas under the concentration or time curves. All experiments were performed on at least three occasions.

RESULTS

The analysis of PRP prepared from individuals with CKD or CKD and DM predialysis revealed a subpopulation of platelets (~3 and 10% of the total population, respectively) with increased PS externalization compared with healthy controls (Figs. 1 and 2). We have therefore characterized this small population in detail, and all subsequent data describing the effects of dialysis relate to this fraction.

The degree of predialysis PS externalization in platelets derived from individuals with CKD and DM was significantly higher than that from patients with CKD alone (161 ± 1 vs. 99 ± 5 × 10² total fluorescent binding sites, P < 0.001; n = 3). Analysis of PS externalization during low-flux (Fresenius polysulfone) dialysis for 3 h in individuals with CKD showed that, while still slightly elevated after 60 min, this parameter had returned to basal values after 2 h (Figs. 1 and 3). In

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Fig. 2. Effect of a single, low-flux hemodialysis treatment on PS externalization in a subpopulation of human platelets in individuals with CKD and diabetes mellitus (DM). PRP was prepared from individuals with CKD and DM either predialysis or 1, 2, and 3 h after the commencement of dialysis. PS externalization was then assessed using annexin V-FITC and CD61-PerCP with subsequent quantification by FACScan. For comparison purposes, a healthy control is also shown, and data are presented as both a histogram (top) and a dot-plot (bottom).
contrast, PS externalization in the platelet subpopulation derived from patients with CKD and DM, while significantly reduced, was still well above basal values (78 ± 1 vs. 30 ± 1 x 10^2 total fluorescent binding sites, P < 0.001; n = 3) after 3-h dialysis (Figs. 2 and 3). When patients undergo dialysis on Monday, Wednesday, and Friday, there is a 2-day interval between treatments, Monday-Wednesday and Wednesday-Friday, but a 3-day interval between Friday and Monday. We were therefore interested to establish whether the 3-day interval, with its likely greater accumulation of metabolites, resulted in increased PS externalization on a Monday compared with Wednesday/Friday. Analysis showed that the degree of predialysis PS externalization in patients with CKD or CKD and DM was significantly higher on Monday than the 2 other days of the week when dialysis took place (Fig. 4). Moreover, despite the increased PS externalization seen in CKD patients on a Monday, this had returned to basal values following dialysis treatment (Fig. 4A).

When increasing volumes of ultrafiltrate from individuals with either CKD or CKD and DM were added to PRP from healthy controls, statistically significant (P < 0.001 in all cases; n = 4) increases in PS externalization in a subpopulation of platelets (~3 and 5% of the total population, respectively) were observed (Fig. 5). In contrast, equivalent material prepared from healthy controls using a 30-kDa cutoff filter had no effect (data not shown). A small, but highly significant stimulation (P < 0.001, n = 4) was also seen when hemodialysis fluid, which had been used to rinse the dialyzer, was added to PRP (Fig. 5). We have therefore characterized this small population in detail, and all subsequent data relate to this fraction. Optimal responses were observed with 10 μl of ultrafiltrate (data not shown), and analysis demonstrated that the levels of endotoxin present were below the minimum value (0.03 EU/ml) detectable by the test. The effects of UF20 and UF180 taken from individuals with CKD and DM were significantly greater than the corresponding samples generated by patients with CKD alone and, interestingly, UF180 from both patient groups appeared as potent in eliciting PS externalization as UF20 (Fig. 5).

At this point in our investigation, it was not clear why, with the CKD data for example, PS externalization had returned to healthy controls.
basal values after 180-min dialysis (Figs. 1 and 2), yet the ultrafiltrate removed at the same time point elicited a substantial increase in PS externalization when added to PRP from healthy individuals (Fig. 5). To address this question, we first examined the ability of UF20 and UF180 from individuals with CKD to elicit PS externalization on PRP from both healthy controls and that taken from patients with CKD after 180-min hemodialysis. While UF20 and UF180 elicited substantial increases in PS externalization in PRP from healthy controls, they had no effect on that generated from individuals with CKD following 180-min dialysis (Fig. 6A). Similar data were obtained when ultrafiltrate and PRP from patients with CKD and DM were used (Fig. 6B). This insensitivity could be due to either the uremic status per se of the individuals and/or the hemodialysis process, which in some way desensitizes platelets to the active component(s) present in the ultrafiltrate.

To investigate these observations further, we then examined the ability of ADP, a classic physiological activator of human platelets, to elicit PS externalization in PRP from either healthy controls or individuals with CKD or CKD and DM after 20- or 180-min hemodialysis. In platelets obtained from patients with CKD after 20 min, the magnitude of the response to ADP was reduced (Fig. 7A) compared with healthy controls (although PS externalization in the untreated sample was still significantly elevated as reported in Fig. 3A). After 180-min hemodiagnosis, platelets from individuals with CKD were unresponsive to ADP (Fig. 7A). Platelets obtained from patients with CKD and DM were unresponsive to ADP at both time points, although, as we have shown in Fig. 3B, PS externalization in the untreated samples followed by 20- and 180-min hemodialysis had not returned to basal values (Fig. 7B). This suggests that hemodialysis results in both 1) the removal of one or more factors able to elicit PS externalization for at least 180 min after the commencement of treatment and 2) a time-dependent desensitization of the small platelet subpopulation to both physiological agonists such as ADP and uremic toxins.

In addition to its role in thrombosis, PS externalization is also implicated in apoptosis via caspase 3-mediated cleavage of PKCδ (11, 30). When PRP from healthy controls was preincubated for 30 min with the caspase 3 inhibitor Z-DVED-FMK (20 μM) and then subsequently challenged for 10 min with UF20 or UF180 from individuals with either CKD or CKD and DM, their ability to elicit PS externalization was unaffected (data not shown), suggesting that the pathway(s) employed by the active component(s) did not involve caspase 3. We have previously shown that when used under identical conditions Z-DVED-FMK completely abolished the effect of ADP (100 nM) added for 1 min while the negative control, Z-FAD-FMK (20 μM), had no effect (45).
The preincubation of PRP from healthy controls for 10 min with bisindolylmaleimide 1 (10 nM), an inhibitor of both the classic (\(\varepsilon\)) and novel (\(\varepsilon\)) isoforms of PKC (40, 46), resulted in a partial inhibition of the increase in PS externalization in the small platelet subpopulation elicited by the addition of either UF20 or UF180 from individuals with CKD or CKD and DM for 10 min (data not shown). Similarly, preincubation of PRP from healthy controls for 5 min with the PKC\(\varepsilon\) inhibitor rottlerin (10 \(\mu\)M) partially prevented (57–73% inhibition) the increase in PS externalization elicited by the addition of either UF20 or UF180 from both patient groups after 10-min incubation (Fig. 8), suggesting a role for PKC\(\varepsilon\) in this response. In all cases, the effects of ultrafiltrate and inhibitor were significantly different from inhibitor alone. This suggests that a significant fraction of the effects of ultrafiltrate from individuals with CKD or CKD and DM on PS externalization in the small subpopulation of platelets are mediated via 5-HT\(_{2A/2C}\) receptors.

**DISCUSSION**

Cardiovascular disease, particularly atherosclerosis, is a major cause of morbidity and mortality in patients undergoing current hemodialysis therapies (13, 19, 20). It is clear from our study that, with regard to platelet PS externalization, there is a distinct difference between the effect of hemodialysis on individuals with CKD and DM compared with CKD alone. In the former group, PS externalization in the platelet subpopulation had essentially reached a nadir after 3-h hemodialysis at a value well above that observed in healthy controls, while in the latter it had returned to normal values. It remains to be established whether the failure of PS externalization to return to normal values in individuals with comorbid conditions such...
as DM plays a role in the increased vascular damage observed in this patient group (13, 19, 20).

A primary cause of vascular disease in individuals undergoing hemodialysis is the inadequate removal of uremic toxins (3), and AGE, which are among the best studied of these toxins (42), may contribute to the development and progression of cardiovascular disease in these patients (35, 39). In support of this hypothesis, we have recently provided evidence suggesting that HSA-AGE elicits PS externalization, a key step in the generation of thrombin (32, 49), in a small subpopulation of human platelets via the 5-HT2A/2C receptor and PKC (41). Intriguingly, in preliminary experiments with dithiothreitol, which both reverses the increased PS externalization seen in the subpopulation of platelets either is, or becomes, desensitized to this hypothesis, we have recently provided evidence suggesting that nitrosative stress both activates Scramblase and inhibits AP LT, resulting in PS externalization (41). Intriguingly, in preliminary experiments with dithiothreitol, which both reduces disulfides and denitrosylates S-nitrosylated proteins in platelet factor-4 (PF4), and β-thromboglobulin (βTG), some of which do not return to basal values until 20 h after the end of the hemodialysis session (4, 8). It is possible that autocrine/paracrine loops play a role in our observations.

As well as a possible role for receptor-mediated pathways in the stimulation of PS externalization we report here, other mechanisms also require consideration. Protein-bound uremic solutes (42, 43) such as p-cresol and indoxyl sulfate, at concentrations commonly found in uremia, have been shown to have biological effects (9). The involvement of such agents is worthy of future study.

Perhaps the most interesting possibility involves a direct effect of one or more uremic toxins on the activity of Scramblase and/or aminophospholipid translocase (APLT), which opposites the role of Scramblase and is responsible for the inward translocation of aminophospholipids such as PS (6). There is increasing evidence that oxidative stress is an important complication in hemodialysis (12), and it is now clear that the activity of both Scramblase and APLT can be modified by alterations to critical sulfhydryl groups (7, 10); e.g., the activity of Scramblase is enhanced by oxidative modification of one or more sulfhydryl groups and is suppressed by the reducing agent dithiothreitol (23). In addition to oxidative stress, there is a growing interest in nitrative stress (14), and recent evidence suggests that this is present in patients undergoing hemodialysis (1, 28); e.g., 3-nitrotyrosine levels in plasma proteins have been reported to be elevated threefold before treatment (1). Moreover, a very recent investigation has shown that nitrative stress both activates Scramblase and inhibits APLT, resulting in PS externalization (41). In addition to uremic toxins, which do not return to basal values until 20 h after the end of the hemodialysis session (4, 8). It is possible that autocrine/paracrine loops play a role in our observations.

Regardless of the mechanism(s) involved, our data suggest that in both patient groups PS externalization in the small subpopulation of platelets either is, or becomes, desensitized to

**Fig. 9.** Effect of 5-hydroxytryptamine (5-HT2A/2C) receptor antagonist ritanerin (Rit) on PS externalization in a subpopulation of human platelets in response to UF20 or UF180 from individuals with either CKD or CKD and DM. PRP from healthy controls was preincubated with or without 1 μM ritanerin for 30 min at 37°C. UF20 or UF180 from individuals with CKD (A) or CKD and DM (B) was then added, and the incubation was continued for a further 10 min. PS externalization was determined using annexin V-FITC and CD61-PerCP with subsequent quantification by FACScan. Values are means ± SE of 3 independent experiments and are expressed as total fluorescent binding sites with statistical analysis by Student’s t-test. *P < 0.05, **P < 0.01, ***P < 0.001 vs. control.
uremic toxins/ADP during a 3-h hemodialysis session. A number of other studies have also observed decreased platelet activation following hemodialysis (2, 31, 36, 37). For example, individuals with CKD undergoing hemodialysis (with either a cellulose acetate or polysulfone membrane) had a lower percentage of platelets expressing the adhesion molecule P-selectin in response to ADP (0–1 μM) at the end of dialysis than at the start (2). In contrast, increased platelet activation following hemodialysis has also been reported (18). The mechanism through which PS externalization becomes desensitized is not yet clear, but a better understanding of these observations may potentially facilitate the development of novel treatments aimed at manipulating platelet responses in pathophysiological states.

We have previously reported that the effect of HSA-AGE on platelet PS externalization was independent of caspase 3 activity (45) and demonstrate similar findings here with UF20 and UF180 from individuals with CKD or CKD and DM. In nucleated cells, these observations correlate with the efficient preparation and control of coagulation and thrombosis (49), rather than a signal for apoptotic cell removal (11). Although recent studies have identified the presence of caspase activity (including caspase 3) in human platelets (34, 47), their precise role in these cells remains unclear.

In conclusion, we have demonstrated distinct differences both between 1) the effects of hemodialysis on PS externalization in a small subpopulation of platelets in individuals with CKD and those with CKD and DM and 2) responses of healthy platelets to ultrafiltrate derived from these two patient groups. The metabolites/mechanisms involved and their consequences in relation to the increased vascular damage observed in these individuals require further investigation.

GRANTS

This work was supported by scientific grants from the HOSPAL Cardiovascular Research Programme and the Northern Counties Kidney Research Fund.

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