Effects of uraemia and dialysis modality on polymorphonuclear cell apoptosis and function

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Abstract

Background. Previous studies have reported that incubation of polymorphonuclear cells (PMN) in uraemic plasma or with different haemodialysis membranes and peritoneal dialysis solutions increases apoptosis in this cell type. In addition, PMN harvested from uraemic patients show a reduced ability to generate superoxide in response to stimuli as well as impaired phagocytosis, chemotaxis and degranulation. The aim of the current study was to investigate the effect of uraemia and dialysis modality on apoptosis and function in freshly harvested non-incubated PMN.

Methods. Polymorphonuclear cells were harvested from 14 chronic haemodialysis (HD) patients, from 14 continuous peritoneal dialysis patients (CAPD), 28 chronic kidney disease (CKD), pre-dialysis patients and from 14 healthy subjects (Controls). In these in vivo experiments, PMN apoptosis was studied by means of flow cytometric analysis of annexin V binding to freshly isolated cells. Polymorphonuclear cell phagocytosis and production of reactive oxygen species by unstimulated or stimulated (S.aureus, fMLP, PMA) cells were also studied by flow cytometry using whole blood.

Results. We observed increased PMN apoptosis in CKD patients. CAPD and HD patients displayed PMN apoptosis rates similar to controls. In the HD group, PMN exhibited decreased phagocytosis rates. In contrast, phagocytosis rates in PMN from CAPD were not significantly different from controls. In the CKD and HD groups, apoptosis was inversely correlated with respiratory burst activity and phagocytosis.

Conclusion. Our results suggest that both uraemia and treatment modality may interfere with PMN apoptosis and function. Dialysis appears to normalize the increased PMN apoptosis rates observed in CKD patients.

Keywords: apoptosis; dialysis; immune dysfunction; polymorphonuclear cells; uraemia

Introduction

A growing body of evidence supports the notion that uraemia is associated with an acquired immune deficiency which involves both cellular and humoral immunity [1]. Polymorphonuclear leukocytes (PMN) are the first line of defense against foreign invaders and constitute the major cell type involved in certain types of acute and chronic inflammatory diseases [2]. The high frequency of bacterial infections in end stage renal disease (ESRD) patients suggests that PMN dysfunction may be involved in the immune deficiency observed in this population [3].

The factors related to PMN dysfunction are not completely understood and have been ascribed to malnutrition, iron overload, uraemic toxins, elevated levels of intracellular calcium, zinc deficiency and dialysis therapy per se [1,4]. Recently, Rao and coworkers [5] showed in a subgroup of haemodialysis patients using polysulphone membranes that the reuse process itself influenced the oxidative response of PMN, whereas the type of bleach–germicide combination during reuse was associated with the PMN phagocytosis index.

In peritoneal dialysis, the effects of various peritoneal dialysis solutions on resident peritoneal cells and on peripheral PMN after in vitro incubation have been studied; however, the systemic effects of this therapy on immune system function have not been completely elucidated [6].
The process of apoptosis, a physiologic mode of cell death, is related to PMN function. Studies by Whyte and coworkers [7] have demonstrated a direct relationship between apoptosis and loss of cytoskeletal functions, phagocytosis, degranulation and respiratory burst in PMN aged for 24 h in culture. PMN leukocytes from healthy subjects incubated with uremic plasma exhibit higher apoptosis rates and a dysfunctional pattern compared with those incubated with normal plasma [8].

At present, it is still unknown whether this relationship between PMN apoptosis and function is present in pre-dialysis, haemodialysis and peritoneal dialysis patients. In fact, increased PMN apoptosis in ESRD patients has only been demonstrated in PMN incubated in vitro. In the present study, we investigated the effect of uremia and dialysis modality on both apoptosis and function of freshly harvested PMN.

Subjects and methods

Patient population

Blood samples were collected from 28 pre-dialysis patients with chronic kidney disease (CKD group): 14 with creatinine clearance between 30 and 48 ml/min/1.73 m², and 14 with creatinine clearance equal or less than 30 ml/min/1.73 m². Because analysis revealed that these two groups were similar in most of the analysed parameters, they were pooled into a single group.

Of 28 additional patients included in the study, 14 were on long-term haemodialysis (HD group) and 14 were on long-term continuous ambulatory peritoneal dialysis (CAPD group). The control group included 14 healthy volunteers. All patients were recruited at the Hospital do Rim e Hipertensão – Escola Paulista de Medicina – Federal University of São Paulo, UNIFESP.

The haemodialysis patients were dialyzed for 3.5 to 4 h thrice weekly with polysulfone dialyzers (Fresenius Medical Care, Bad Homburg, Germany). Dialyzers were reprocessed with peracetic acid and reused for up to 12 times. The water for haemodialysis was treated in a system composed of a softener and a reverse osmosis unit (IPABRAS Inc., Rio de Janeiro, RJ, Brazil). During the study, the water quality for haemodialysis was treated in a system composed of a softener and a reverse osmosis unit (IPABRAS Inc., Rio de Janeiro, RJ, Brazil). During the study, the water quality was within the standards proposed by the American Association for the Advancement of Medical Instruments (AAMI). CAPD patients were dialyzed with 3 to 5 daily exchanges of glucose, lactate-based solutions (Dianeal®, Baxter, São Paulo, SP, Brazil) that usually included one 4.25% glucose solution exchange per day (0 to 2). Patients with diabetes, acute infection or blood transfusion in the past month, chronic infections (hepatitis B, hepatitis C, human immunodeficiency virus, osteomyelitis), active immunological disease (systemic lupus erythematosus, rheumatoid arthritis), immunosuppressive therapy, previous transplantation or a history of malignancy were excluded from the study. The patients on peritoneal dialysis were free from peritonitis for at least 3 months. All of the patients on haemodialysis had a permanent vascular access (arteriovenous fistula).

The study was approved by the Ethics Committee on Research of the Federal University of São Paulo. Only patients who signed an informed consent were enrolled in the study.

Clinical and laboratory data

Upon inclusion in the study, baseline demographic and clinical data were abstracted from patients’ hospital records. These included age, gender, body mass index (BMI), cause of renal failure, time on dialysis therapy and current medication. Laboratory data included haemoglobin levels, white blood cell and differential counts, as well as serum levels of urea, creatinine, and albumin.

Dialysis adequacy was assessed by \( Kt/V \). In the HD group, we used the arithmetic mean of the last three single-pool \( Kt/V \) values before entry into the study. In the CAPD group, we used the arithmetic mean of the last two weekly \( Kt/V \) values. In this group, \( Kt/V \) was measured routinely every 4 months. For CKD patients, creatinine clearance was estimated from serum creatinine levels, body weight, age, and gender using the Cockroft–Gault equation [9].

Patients in haemodialysis and peritoneal dialysis received intravenous iron and subcutaneous erythropoietin according to the NKF-K/DOQI guidelines [10]. Only patients on dialysis received 1.25 dihydroxycholecalciferol (CKD: 0/28, HD: 2/14, CAPD: 3/14; \( P = 0.052 \)). For the treatment of hypertension, all patient groups received angiotensin-converting enzyme inhibitors (CKD: 8/28, HD: 4/14, CAPD: 3/14; \( P = 0.8 \)). The CAPD and CKD groups received calcium-channel blockers (CKD: 15/28, HD: 0/14, CAPD: 5/14; \( P = 0.003 \)). No participant in the study, including the healthy volunteers, had been taking vitamin C and/or vitamin E.

In the HD group, the mean single-pool \( Kt/V \) was 1.35 ± 0.23. In the CAPD group, the mean weekly \( Kt/V \) was 1.72 ± 0.37.

Blood sample collection

In patients on HD, heparinized whole blood (10 IU/ml) was drawn from the fistula needle immediately before the second dialysis session of the week (Wednesday or Thursday). In healthy volunteers, and in CKD and CAPD patients, blood was drawn from a peripheral vein. For CKD and CAPD patients, blood was drawn along with the blood drawn for routine laboratory tests. In all instances, a 10 ml blood sample was obtained from each donor; 1 ml was used for the study of leukocyte reactive oxygen species (ROS) production and phagocytosis assay, and the remaining 9 ml were used to harvest PMN for the study of apoptosis.

PMN isolation

In brief, leukocyte preparations containing 95-98% PMN were isolated by Ficoll-Hypaque (Sigma Chemical Co., St. Louis, MO, NY, USA) density gradient centrifugation and dextran sedimentation [11]. Residual erythrocytes were lysed with hypotonic saline and the cells (1 × 10^6/ml) were suspended in phosphate buffered saline (PBS, Sigma, St Louis, MO, USA).

Cell apoptosis

Cell apoptosis was measured by Annexin V staining. One of the cell-membrane changes during the early and
Phagocytosis and ROS production

Phagocytosis was evaluated using heat-killed *S.aureus*, strain ATCC 25923 (Difco, Detroit, MI, USA), labelled with propidium iodide (PI) at 5%, 2.4 × 10^5 colony-forming units/ml. We evaluated PMN ROS production by examining the ability of PMN to respond to an appropriate stimulus. To do this, cells were incubated with PI-labelled *S.aureus*, 4-β phorbol 12-β-myristate 13-α-acetate (PMA, 100 ng/ml, Sigma, St. Louis, MO, USA), and N-formyl methionyl-leucyl-phenylalanine (fMLP, 10^{-5} M/ml, Sigma, St Louis, MO, USA). A mixture of 100 μl of heparinized whole blood, 100 μl of PI-labelled *S.aureus* and 900 μl of PBS was prepared in a plastic tube in order to evaluate phagocytosis. To study unstimulated and stimulated ROS production, a mixture of 100 ml of heparinized whole blood, 200 ml of 0.3 mM 2′,7′-dichlorofluorescein diacetate (DCFH-DA, Sigma, St Louis, MO, USA) within 1 h [12]. Cells staining positive for PI were considered as dead cells (necrosis or late apoptosis), cells staining positive only for annexin V were considered as apoptotic, and cells negative for both were considered as viable.

Flow cytometry analysis

Intracellular DCFH fluorescence of PMN was determined by flow cytometry (FACScalibur analyzer; Becton Dickinson Immunocytometry Systems, CA, USA). In the final suspension, monocytes, lymphocytes, a few contaminating erythrocytes, aggregated cells and debris were excluded from analysis using a gate analysis method based on forward light scatter and side scatter (linear 90° light scatter). Histograms of the fluorescence intensity were constructed for each tube and the geometric mean of the fluorescence intensity (mean fluorescence intensity – MFI) of that population of cells was determined. For each experimental condition, we subtracted the MFI of the control tube from the MFI of the test tube. For the unstimulated cells and cells exposed to PMA, the control tube contained cells and buffer, without DCFH-DA. For the *S.aureus*-stimulated cells, control tubes contained PI-stained *S.aureus*.

Results

Clinical and laboratory data

The demographic, clinical parameters, and the laboratory data are shown in Table 1. Controls were different from the other groups with respect to age and gender. We used multivariate analysis of variance and co-variance (ANCOVA) to control for imbalance between the groups. These differences did not account for the differences in PMN function between the groups (Table 2). Significant differences were also observed for white blood cell counts, serum urea, creatinine and albumin levels (lowest in the CAPD group). Creatinine clearance in the CKD group was 29 ± 19 ml/min/1.73 m^2, whereas all patients in the HD group were anuric, and only five patients in the CAPD group had a residual creatinine clearance (below 5 ml/min/1.73 m^2 in all patients).

ROS production and phagocytosis

Phagocytosis was significantly lower in the HD group compared with the other groups (Table 2). *S.aureus* and fMLP-stimulated ROS production was significantly...
higher in the CAPD group than in the other groups.
Unstimulated ROS production was also significantly higher in the CAPD group than in the HD and CKD groups (Table 2). There was no difference between the groups with respect to PMA-stimulated ROS production by neutrophils.

Apoptosis and viability

Apoptosis was significantly higher in the CRF group compared with the other groups (Table 3). The groups were not different with respect to the rates of viability.

Correlations between PMN apoptosis, PMN function and clinical parameters

Table 4 shows the correlations between apoptosis and PMN function. As expected, we observed positive correlations between the different functional parameters (ROS production and phagocytosis). We also found a negative correlation between apoptosis and Staphylococcus aureus as well as PMA-stimulated ROS production.

In the CKD group, we found a negative correlation between apoptosis and Staphylococcus aureus-stimulated ROS production by PMN ($r = -0.47$, $P = 0.02$). In the HD group, we also found a negative correlation between apoptosis and Staphylococcus aureus-stimulated ROS production by PMN ($r = -0.68$, $P = 0.03$).

Correlations between PMN viability and PMN function

Viability correlated positively with phagocytosis ($r = 0.21$, $P = 0.03$). In the HD group, we found positive correlations between viability and phagocytosis ($r = 0.82$, $P = 0.003$) and Staphylococcus aureus stimulated ROS production ($r = 0.64$, $P = 0.05$). In the CKD group, we also found positive correlations between viability and Staphylococcus aureus and PMA stimulated ROS production ($r = 0.53$, $P = 0.005$ and $r = 0.41$, $P = 0.05$ respectively).

Discussion

In the present study, we found that PMN from pre-dialysis CKD patients had increased apoptosis rates, whereas CAPD and HD patients displayed PMN...
The leading candidate for a molecular trigger of spontaneous apoptosis in PMN is the Fas protein (Apo-1; CD95). Fas protein is expressed on PMN plasma membranes, and Fas ligand (FasL) induces apoptosis in PMN. Polymorphonuclear cells constitutively release FasL, thus providing an autocrine/paracrine pathway for PMN to mediate their own programmed cell death [17]. Jaber et al. [18] showed that Fas expression was significantly higher among patients with chronic renal failure compared with control subjects, haemodialysis patients, and peritoneal dialysis patients. They also found that uraemic serum increased the expression of neutrophil-associated Fas and FasL protein, and that Fas-stimulated apoptosis strongly correlated with creatinine clearance. These results are in accordance with those found by Majewska and coworkers [19]. They studied apoptosis in cultured PMN and found that Fas expression and total percentage of apoptotic PMN from pre-dialysis patients was significantly greater than the percentage observed in healthy controls, suggesting that uraemia accelerates apoptosis by increasing Fas expression [19].

While examining PMN function, we found that the CAPD group had an enhanced ROS production from both unstimulated and stimulated PMN. In the HD group, PMN exhibited the lowest phagocytosis rates. Studies investigating phagocytosis and ROS production in dialysis patients have produced conflicting results. These disparities can be attributed to different methods used for the measurement of PMN function and to different procedures used to isolate and incubate these cells. Importantly, in the present study, PMN function was assessed by flow cytometry using whole blood, and not from isolated and incubated cells. We found that peritoneal dialysis patients displayed phagocytosis rates that were similar to controls. In agreement, Daniels et al. [20] observed a normalization of PMN function after patients were started on peritoneal dialysis therapy. The continuous and efficient removal of uraemic solutes from the blood in the peritoneal effluent may have advantages over intermittent therapies and this may contribute to the superior performance in the CAPD group.

In the present study, we also found negative correlations between S. aureus-stimulated ROS production and apoptosis in the CKD and HD groups. As previously discussed, Whyte et al. [7] in a seminal work published in 1993 demonstrated a direct relationship between apoptosis and loss of cytoskeletal functions, phagocytosis, degranulation and respiratory burst in PMN aged for 24h in culture. According to these findings, polymorphonuclear apoptosis and function are closely linked events. Phagocytosis and ROS production are able to trigger PMN apoptosis, whereas apoptotic PMNs are no longer capable of phagocytosis, degranulation, or respiratory burst activity [7,17]. Our results, however, were unable to prove causality between PMN apoptosis and (dys)function.
In conclusion, we found increased apoptosis rates in freshly isolated PMN from pre-dialysis patients, whereas HD and PD patients had PMN apoptosis rates that did not differ from controls. We speculate that each uraemic toxin has distinct effects on PMN viability and function. The mixture of various toxins in uraemic serum appears to result in increased PMN apoptosis, and the dialysis process may slow this acceleration in apoptosis rates.

The current study had a few limitations. First, the groups were not age- and sex-matched. Although multivariate analysis of variance and co-variance (ANCOVA) were used to control for imbalance between the groups, we cannot exclude an effect of these variables on the results. Interestingly, Rao and coworkers [5] found no correlations between PMN function and age and gender. As a second limitation, we did not study Fas expression or modulation in freshly isolated PMN from pre-dialysis patients, and that circulating PMN can also display increased apoptosis and dysfunction in pre-dialysis and dialysis patients.

Further studies will be needed to elucidate the relationship between PMN apoptosis and function in ESRD patients. Nevertheless, the present study demonstrated that this is not just an in vitro phenomenon, and that circulating PMN can also display increased apoptosis and dysfunction in pre-dialysis and dialysis patients.

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References


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