Effects of uraemia and haemodialysis on neutrophil apoptosis and expression of apoptosis-related proteins

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Abstract

Background. In haemodialysis (HD) patients, it is unclear whether increased apoptosis of neutrophils is due to uraemia or HD itself. The purpose of the current study was to assess the effect of uraemia and HD on the rate of apoptosis and apoptosis-related protein expression in whole blood neutrophils.

Methods. We employed a whole-blood micromethod to test spontaneous apoptosis and expression of apoptosis-regulating proteins in cultured neutrophils from uraemic patients (pre-HD), HD patients and healthy controls. Blood samples were drawn before, after 20 min and after 4 h of haemodialysis, and were then cultured for 20 h. We evaluated the rate of apoptosis from annexin V and propidium iodide staining, and examined bcl-2, Fas/Apo-1 and p53 expression in the cultured neutrophils.

Results. Fas/APO-1 expression and total percentage of apoptotic whole blood neutrophils of pre-HD and HD patients before HD were significantly higher than controls. There was a transient but significant decrease in the percentage of apoptotic neutrophils and Fas/APO-1 expression after 20 min of dialysis. The expression of bcl-2 protein was significantly lower from neutrophils in HD patients compared with controls, and HD significantly downregulated bcl-2 expression. The p53 protein content in HD patients before HD was significantly higher than in pre-HD patients.

Conclusions. These findings suggest that uraemia accelerates neutrophil apoptosis by increasing Fas/Apo-1, and that HD does not affect neutrophil apoptosis more than uraemia. In addition, HD produces only in a transient sequestration of potentially apoptotic neutrophils.

Keywords: apoptosis; bcl-2; Fas/Apo-1; haemodialysis; neutrophil; uraemia

Introduction

Among haemodialysis (HD) patients, impaired function of polymorphonuclear leukocyte neutrophils (PMNs) results in bacterial infections that are the main cause of hospitalization and mortality [1,2]. During bacterial infections, PMNs are the first-line cells of the non-specific defence system. A normal resolution of inflammation depends on the timely removal of cells from the site of inflammation, which is related to their ability to undergo apoptosis [3]. Any dysfunction in this ability may lead to weakening of host defence systems.

In vitro studies have shown that neutrophils from uraemic patients undergo accelerated apoptosis [4], and uraemic plasma obtained from dialysed patients accelerates apoptosis in PMNs from healthy donors [5]. During dialysis sessions, PMN activation and apoptosis acceleration are mainly triggered through the generation of complement components following contact of neutrophils with dialysis membranes [6].

The process of apoptosis is highly regulated, and involves the Fas/Apo-1 and p53 genes which act to induce apoptosis [7] as well as bcl-2 which is a survival-related gene [8]. Although the relationship between activation of these genes and the presence of gene products has been well described for lymphoid cells, the genetic mechanism of neutrophil apoptosis is still poorly understood.
Neutrophil apoptosis in uraemic patients

Thus far, all experiments in this field have focused on influences of HD treatment or uraemic plasma on apoptosis in neutrophils isolated from their natural environment. Therefore, it has been difficult to distinguish between effects of HD or uraemia per se on apoptosis in neutrophils.

In HD patients, the rate of neutrophil apoptosis is affected by both death and survival factors present in normal blood that are generated as a consequence of uraemia and HD. Therefore, by testing the influence of HD on neutrophil apoptosis in whole blood, we avoid the loss of potentially apoptotic neutrophils and avoid downregulation of cell viability during their isolation from whole blood. Moreover, cytokines and many other soluble uraemic toxins are not eliminated, all of which can modulate neutrophil apoptosis.

In the present study, we employed a micromethod [9] to test neutrophil apoptosis and apoptosis-regulating protein expression. We further examined the influence of long-term HD on the ability of cultured whole blood neutrophils to undergo spontaneous apoptosis and determined whether this apoptosis is related to Fas/Apo-1, p53 and bcl-2 protein expression.

Subjects and methods

Patient population

These studies were performed in 16 patients (four females and 12 males) with end-stage renal disease (aged 49 ± 9 years) due to glomerulonephritis (n = 10), interstitial nephritis (n = 1), nephroangiosclerosis (n = 1), polycystic kidney diseases (n = 2) and undetermined nephropathy (n = 2). Their endogenous creatinine clearances were < 5 ml/min/1.73 m². The patients were on regular HD treatment with cuprophane membranes for 2–21 years (average 6.3 ± 3.8 years). There were no symptoms of infection or aluminium toxicity in any of the patients. None of the patients suffered from symptomatic hyperparathyroidism; their blood serum calcium and phosphate concentrations were effectively controlled with calcium carbonate and by elimination of phosphate-abundant diets as well as adequate efficacy of HD. The patients were not given nitrates, arachidonic acid cyclooxygenase inhibitors, calcium antagonists, angiotensin-converting enzyme inhibitors, antiplatelet drugs or any other drugs known to affect immune functions. All of the patients received recombinant human erythropoietin (rHuEpo) (Eprex; Cilag AG, Zug, Switzerland), given in a dose of 4000 U once a week or as 3000 U weekly in pre-HD patients with haemoglobin levels < 11 g/l to support the haematocrit level of ~30%. Iron supplementation was not given during rHuEpo treatment and none of the patients required blood transfusion. Twelve healthy volunteers (five females, seven males; aged 41 ± 12 years) and nine uraemic patients (three females, six males; aged 37 ± 10 years) not yet dialysed (pre-HD patients) served as controls. The causes of the chronic renal failure in pre-HD patients were chronic glomerulonephritis (n = 5), polycystic kidney disease (n = 1) and undiagnosed nephropathy (n = 3). Endogenous creatinine clearances were 20.3 ± 7.5 ml/min/1.73 m². Patients with diabetes or other severe systemic diseases were excluded from the study. Informed consent was obtained from the patients and healthy volunteers, and the protocol was approved by the Regional Commission on studies for Ethics in Research.

Whole blood neutrophil culture

Blood samples were drawn from HD patients before (time 0), at 20 min into dialysis and immediately after HD sessions (4 h), and were placed in sterile heparinized tubes. The samples were diluted 1:10 in 20% fetal calf serum (FCS)-supplemented RPMI 1640 and were cultured in a 95% humidified atmosphere with 5% CO₂ at 37°C. After 20 h, blood suspensions were drawn from the culture, washed with cold phosphate-buffered saline (PBS) several times and lysed by use of hypotonic NH₄Cl solution.

The mean percentage of PMNs in blood samples was 58.3 ± 6.1%, and neutrophils represented 98.2 ± 1.8% of all PMNs found in blood smears.

PMN identification

Quantities of lymphocytes, monocytes and granulocytes in fresh blood can be identified by analysis of physical parameters such as forward scatter (FSC) and side scatter (SSC), which indicate cell size, granularity and complexity [10].

When blood is cultured for 20 h, dot plots reveal typical regions for neutrophils, lymphocytes and monocytes identified before culture, and a new population of cells showing diminutions in cell volume and granularity, which CD15–fluorescein isothiocyanate (FITC) monoclonal antibodies (mouse anti-human IgM, Dako, Glostrup, Denmark) helped to identify as neutrophils (for more details see [9]). Percentages of apoptotic neutrophils and Fas/Apo-1 expression were determined in cells in which localization on the FSC/SSC dot plot was confirmed by CD15 antibody binding. p53 and bcl-2 protein expression was then assessed in CD15-positive cells (double staining).

Measurement of PMN apoptosis by flow cytometry

The percentage of apoptotic neutrophils was assessed from annexin V–FITC protein binding according to the manufacturer’s specifications (Annexin V/FITC Kit, Bender MedSystems Diagnostics GmbH, Vienna, Austria). A 5 μl aliquot of annexin V–FITC and 10 μl of propidium iodide (PI; final concentration 1 μg/ml) were added to each cell suspension and these were incubated for 25 min in the dark at room temperature. Annexin V fluorescence emission was detected with the FL-1 channel, and PI was detected at room temperature. Annexin V fluorescence emission was detected with the FL-1 channel, and PI was detected with the FL-2 channel. Cell populations which were annexin V+/PI– were considered to be alive, those which were annexin V+/PI– were considered as an early apoptotic population, and those which were annexin V+/PI+ were in the late stage apoptotic stage or were necrotic [11].

Light microscopy has revealed that various stages of apoptosis are present in neutrophils by showing morphological changes typical for apoptotic cells, such as chromatin condensation, formation of rounded nuclear profiles, cell shrinking and the presence of cytoplasmic vacuolization [12].
Quantification of apoptosis-related molecules in PMN by flow cytometry

We used whole blood samples to assess apoptosis-related molecules, using a modification of the methods reported by Hsieh [13] and Filippini [14]. For detection of cytoplasmic proteins such as p53 and bcl-2, cells underwent hypotonic lysis, and $1 \times 10^6$ leukocytes were fixed in 0.5% paraformaldehyde solution for 10–15 min at 4–8°C. The leukocytes were then washed twice in cold PBS and incubated for 30 min at room temperature with mouse anti-human p53 IgG2b monoclonal antibody (clone DO-7, Dako) or with mouse anti-human bcl-2 IgG1-RPE-conjugated monoclonal antibody (clone 100, Becton Dickinson, Mountain View, CA) which was diluted 1:5 in 100 µl of 0.1% saponin (Sigma Chemical Co., St Louis, MO) solution. After three washes in PBS, the second step antibody for p53, a RPE-conjugated rabbit anti-mouse F(ab)2 fragment, was added for 30 min at room temperature, and finally, after additional washes, we added 10 µl of mouse anti-human CD15 IgM–FITC-conjugated antibodies (clone C3D-1, Dako). An isotype-matched IgG2b (Dako) and IgG1-RPE monoclonal antibodies (Becton Dickinson) were used in every experiment as negative controls for p53 and bcl-2, respectively.

Cell surface expression of Fas/Apo-1 was assayed by direct immunofluorescence using a saturating concentration of monoclonal mouse anti-human IgG3 to Fas/Apo-1 (human)–FITC (APO-1-3, Alexis Biochemicals, San Diego, CA). A simultaneous negative control staining reaction was performed with anti-human IgG3–FITC monoclonal antibody (mouse IgG3, clone J606, PharMingen, San Diego, CA). Staining of the cells with monoclonal antibodies was performed in the dark.

The percentage and/or median fluorescence intensity (MFI) of the positively stained cells was detected by flow cytometry.

Statistical analysis

Evaluation of statistical significance was performed by Wilcoxon’s signed rank tests for paired data and by Mann–Whitney tests for unpaired data. Values of $P < 0.05$ were considered significant. Data are presented as means ± SD.

Results

Early apoptosis was observed in $41.3 \pm 15.9\%$ of cultured whole blood neutrophils drawn from HD patients at time 0, which was different from $34 \pm 18.1\%$ in uraemic patients (pre-HD patients) or $36.6 \pm 9.7\%$ in healthy subjects (Figure 1). The total percentage of apoptotic whole blood neutrophils (early and late stage) of pre-HD ($69 \pm 6.4\%$, $P < 0.01$) and HD patients ($61 \pm 13.8\%$, $P < 0.03$) was significantly greater than controls ($44 \pm 10.6\%$) (Figure 2). There was a significant decrease in the percentage of early ($24 \pm 8.9\%$, $P < 0.007$) and total apoptotic neutrophils ($44 \pm 13.3\%$, $P < 0.02$) at 20 min of dialysis compared with levels found before dialysis (time 0) (Figures 1 and 2). By 4 h of HD, the values returned to baseline.

The surface expression of Fas/Apo-1 molecules on cultured neutrophils was significantly greater in both pre-HD ($69.5 \pm 25.4$ MFI, $P < 0.005$) and HD patients ($41.1 \pm 19.9$ MFI, $P < 0.05$) compared with healthy controls ($27.7 \pm 13.9$ MFI). Fas/Apo-1 expression did not differ between pre-HD and HD patients (Figure 3). We observed a transient but significant ($P < 0.01$) decrease in Fas/Apo-1 expression to $34.3 \pm 17.4$ MFI at 20 min of dialysis.

**Fig. 1.** Changes in percentage of early apoptotic neutrophils during HD. The percentage of early apoptotic neutrophils significantly decreased by 20 min of the procedure ($P < 0.007$) but returned to initial values at the end of the HD session. The percentage of early apoptotic neutrophils was not different between HD patients at time 0 and pre-HD patients and healthy donors (control).
Intracellular expression of bcl-2 protein was significantly lower in CD15$^+$ whole blood cultured cells in HD patients (182 ± 385 MFI, $P < 0.03$) compared with expression in healthy donors (265 ± 168 MFI). Expression of bcl-2 was significantly downregulated at 20 min and 4 h of HD (Figure 4).

Expression of p53 protein in CD15$^+$ cells from HD patients before HD was significantly higher (235 ± 147 MFI, $P < 0.04$) than in pre-HD patients (83 ± 34 MFI) (Figure 5). After 20 min of HD, intracellular expression of p53 transiently decreased to 99 ± 68 MFI.
Discussion

Increases in neutrophil apoptosis, which have been observed in uraemic patients on long-term HD programmes, may lead to disturbances in immune responses.

In the present study, the proportion of early apoptotic neutrophils was not different in HD patients before HD, in pre-HD patients or in healthy donors. However, the total percentage of apoptotic whole blood neutrophils (in both early and late stage) from pre-HD and HD patients was significantly greater than...
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indicating that neutrophils possess cytoplasmic bcl-2 remain largely unexplored. Our previous data [9], processes, the mechanisms of neutrophil apoptosis associated Fas.

uraemic patients increased the expression of neutrophil-creatinine clearance. They also found that serum from Fas/Apo-1 expression was strongly correlated with renal failure than in control subjects, and that phils was significantly higher in patients with chronic who showed that Fas/Apo-1 expression on neutro-

are compatible, in part, with those of Jaber Fas/Apo-1 expression than on live cells. Our results or potentially apoptotic neutrophils having higher HD causes a transient sequestration of early apoptotic apoptosis by increasing Fas/Apo-1 expression whereas pre-HD and HD patients than in controls. Fas/Apo-1 expression was better associated with uraemia than in early stages of apoptosis [9]. Here, we found that Fas/Apo-1 expression was higher in both pre-HD and HD patients compared with live cells and that this expression was higher on neutrophils in advanced than in early stages of apoptosis [9]. Here, we found that Fas/Apo-1 expression was higher in both pre-HD and HD patients than in controls. Fas/Apo-1 expression decreased after 20 min of HD but remained statistically greater than in healthy donors. These results suggest that uraemia accelerates neutrophil apoptosis by increasing Fas/Apo-1 expression whereas HD causes a transient sequestration of early apoptotic or potentially apoptotic neutrophils having higher Fas/Apo-1 expression than on live cells. Our results are compatible, in part, with those of Jaber et al. [18], who showed that Fas/Apo-1 expression on neutrophils was significantly higher in patients with chronic renal failure than in control subjects, and that Fas/Apo-1 expression was strongly correlated with creatinine clearance. They also found that serum from uraemic patients increased the expression of neutrophil-associated Fas.

Although apoptosis is highly regulated by genetic processes, the mechanisms of neutrophil apoptosis remain largely unexplored. Our previous data [9], indicating that neutrophils possess cytoplasmic bcl-2 expression, are compatible with those of Hsieh et al. [13], but are opposite to the findings of Delia et al. [19] and Iwai et al. [20]. Buemi and colleagues [21] observed that concentrations of bcl-2 in blood were reduced in patients undergoing HD. Some authors suggested that bcl-2 may be involved in acceleration of neutrophil apoptosis during oxidative stress in the uraemic environment or in HD, even though bcl-2 is poorly expressed in mature neutrophils [22]. In the present study, we found that intracellular bcl-2 protein expression was significantly lower in cultured neutrophils harvested from patients on HD treatment (at time 0) compared with expression in healthy donors but not in pre-HD patients. Thus, HD results in diminished bcl-2 expression, which may be a factor responsible for the increased susceptibility of cultured neutrophils to undergo apoptosis in the HD patients.

Although the Fas (Apo-1)/Fas ligand system and the members of the bcl-2 gene family are believed to be key regulators of the apoptotic process, p53 belongs to the major apoptosis-inducing gene family [7]. Neutrophils constitutively express p53 protein [9,13,20] and this expression is upregulated when cells undergo spontaneous apoptosis [9]. Some studies have shown that Fas/Apo-1 levels are elevated in response to DNA damage, and a potential contribution of this system to p53-mediated apoptosis has been suggested. It has been reported that overexpression of p53 is followed by increases in surface expression of Fas/Apo-1 recep-
tor and that p53 may sensitize cells to Fas/Apo-1-mediated apoptosis [23]. p53 can also induce or potentiate apoptosis by regulating the expression of bcl-2 through mechanisms independent of transcription. In the present study, we found higher intracellular expression of p53 protein in cultured neutrophils from haemodialysed patients before their HD sessions (at time 0) compared with expression in pre-HD patients. p53 expression was not different in cultured neutrophils from controls and from both HD and pre-HD patients.

Even though bcl-2 and p53 proteins were present in the tested neutrophils and were probably exerting their normal control on apoptosis, the changes in Fas/Apo-1 expression were better correlated with the susceptibility of the cells to undergo apoptosis in both pre-HD and HD patients.

In summary, we found that acceleration of neutrophil apoptosis was better associated with uraemia than with HD, and that this acceleration is genetically regulated and probably depends on changes in Fas/Apo-1 expression. Even though HD generates pro-apoptotic factors, our results suggest that the procedure causes only a transient sequestration of potentially apoptotic neutrophils.

Acknowledgements. This study was supported by a grant from the State Committee for Scientific Research (KBN Poland) 4p05a 056 14 and Military Medical Academy grant BW 029/99/.

Conflict of interest statement. None declared.
References


