Zn^{2+} Metabolism Affects Apoptosis Rate and Proliferative Responsiveness of PBMC From Patients on Chronic Hemodialysis

J. Weissgarten, S. Berman, D. Modai, R. Rosenberg, M. Rapoport, M. Cohen, and Z. Averbukh

Patients with end-stage renal failure suffer from severe plasma trace metal deficiency that is not corrected by dialysis. Trace metals, including Zn^{2+}, are critical for cell differentiation and replication. Zn^{2+}also plays important role in cell apoptosis. Both processes are known to be impaired in uremia. The present study was undertaken to evaluate the effect of Zn^{2+} supplementation on apoptosis of cultured peripheral blood mononuclear cells (PBMC) from patients on chronic hemodialysis versus those from healthy control subjects, concomitantly with assessment of mitogen-induced cell proliferation. The results showed that (1) basal total cell-associated Zn^{2+} was elevated in uremic PBMC, compared to normal controls (23.9 ± 5.64 *v* 10.5 ± 2.64 μmol/L/mg protein). The gap persisted following incubation in Zn^{2+}-enriched medium (63.3 ± 26.12 *v* 81.6 ± 13.4 μmol/L/mg protein, *P* < .005). (2) Basal proliferative response to phytohemagglutinin (PHA) was significantly decreased in uremic PBMC compared to normal controls (12,000 ± 1,560 cpm *v* 16,600 ± 1,460 cpm, *P* < .01). Incubation of uremic PBMC in Zn^{2+}-enriched medium improved their proliferative response to PHA, yielding counts per minute significantly higher compared to their normal counterparts (37,000 ± 7,500 cpm *v* 22,000 ± 3,000 cpm, *P* < .001). (3) Basal apoptosis rate in uremic PBMC was significantly elevated compared to normal control cells (7.6% *v* 2.6%, *P* < .05). Following incubation in Zn^{2+}-enriched medium, apoptosis was increased both in normal and uremic PBMC. Percent apoptosis of uremic PBMC remained significantly elevated compared to control cells (11.7% *v* 5.7%). We conclude that uremic PBMC are more responsive to exogenous Zn^{2+} in culture than their normal counterparts. This, among other abnormalities, might reflect an abnormal regulation of Zn^{2+} transport by uremic mononuclear cell menbranes. The resultant increase in total cell-associated Zn^{2+} content improves poor proliferative responsiveness of uremic PBMC. On the other hand, increased total cell-associated Zn^{2+} stimulates enhanced apoptosis in uremic PBMC, which, probably by eliminating defective cells, contributes to the functional capability of the population as a whole. The net effect of the 2 processes is still augmentation of cell proliferation.

ACCORDING TO current concepts, apoptosis, or programmed cell death, plays a role in a variety of physiologic processes. Thus, it has been shown that the rate of apoptosis increases in parallel with enhanced cell proliferation.[1-5] It is plausible that in such situations apoptosis eliminates defective cells, therefore contributing to the total functional quality of the relevant cell populations.[1-5]

Uremic immunodeficiency is a multifacet phenomenon composed of both humoral and cellular defects.[6-11] Thus, it has repeatedly been shown that mitogen-induced proliferative responses of peripheral blood mononuclear cells (PBMC) from uremic patients are impaired.[6-11] This is probably the end result of a number of factors associated with uremia, such as aberrations in cytokine production in response to various immunologic insults.[12]

It has been shown that patients with end-stage renal failure demonstrate considerably reduced plasma Zn^{2+} concentrations.[13-17] Plasma and intracellular Zn^{2+} deficiency are known to be associated with a number of defective cell functions, including cell proliferation, cell membrane permeability, cytokine production, and apoptosis.[18-21] Finally, as a logical consequence, oral or, in some cases, peritoneal dialysis-mediated Zn^{2+} supplementation has been shown to improve various immunologic responses of uremic patients.[13-17,21]

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In a previous report we showed that mitogen-induced proliferation of normal and, to a greater extent, uremic PBMC is significantly enhanced by incubation in Zn^{2+}-enriched medium.[22] The present investigation was undertaken to evaluate the effect of Zn^{2+}-supplemented medium on apoptosis of uremic PBMC.

MATERIALS AND METHODS

Twelve patients on chronic hemodialysis participated in this investigation. Care was taken to include in the study only the patients who were treated by chronic hemodialysis for more than 2 years and were not suffering from any intercurrent infections or immune system disorders, or taking any immunoregulatory drugs at the time of the study. Twelve age- and sex-matched healthy volunteers served as a control group.

For PBMC procurement, 20 mL of heparinized blood was drawn in the morning before starting the dialysis session. The cells were isolated on Ficoll-Hypaque (Pharmacia, Upsala, Sweden). Cell viability was assessed by 0.1% eosin exclusion, and only cultures with viability exceeding 98% were included in the study. Cell count was performed using Turk solution (4% glacial acetic acid).

Experimental Design

Each cell sample was considered a separate experiment (n = 12 in either control or patient group).Each cell sample was divided, in 6 equal aliquots, into U-bottomed tissue culture test tubes, so that every experimental variable was composed of 3 equal pairs.

The cells were incubated in 1 mL RPMI1640 cell culture medium supplemented with 10% fetal calf serum (FCS) for 72 hours in a humid incubator with 5% CO_2. The experimental variables, each composed of 3 equal pairs, were seeded in 24-well tissue culture plates as follows: (1) baseline—unstimulated PBMC, no additions to the culture medium; (2) mitogen-stimulated PBMC—10 μg/mL phytohemagglutinin (PHA) added to the medium; (3) unstimulated PBMC, with $ZnCl_2$ added to the medium (final Zn^{2+} concentration, 80 μmol/L); (4) mitogen-stimulated PBMC—10 μg/mL PHA and 80 μmol/L Zn^{2+} added to the culture medium.

Following 72 hours of incubation, the cell cultures were terminated. The

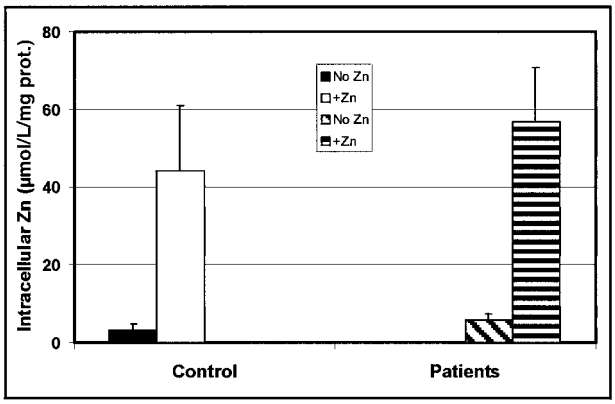

Fig 1. Intracellular Zn^{2+} in PBMC from patients on chronic hemodialysis *v* normal control PBMC. No Zn = intracellular Zn^{2+} concentrations (μmol/L/mg protein) in PBMC; + Zn = intracellular Zn^{2+} content of PBMC cultured in medium enriched with 80 μmol/L Zn^{2+}.

first duplicate of each experimental variable was used to determine total cell-associated Zn^{2+}, the second for apoptosis assay, and the third for evaluation of proliferation rate by ^{3}H-thymidine incorporation. In the latter, for each pair of the 4 experimental variables, the magnitude of mitogenic response to PHA was calculated by subtracting the radioactive counts of experiments in the absence of PHA from the corresponding results obtained in the presence of PHA (ie, 2 − 1 and 4 − 3). Total intracellular Zn^{2+} was determined on atomic absorption spectrometer (Varian, Mulgrave, Australia) by methods described in our previous report.[22] Total protein of the samples was assessed by Bradford's assay,[23] and the results of total cell-associated Zn^{2+} were presented as μmol/L/mg protein. Proliferative rate of PBMC was evaluated by ^{3}H-thymidine incorporation as described elsewere.[22] In brief, 1 μCi of ^{3}H-thymidine was added to the wells 48 hours before terminating the cell cultures. Subsequently, the cells were harvested on fiber glass filters and their radioactivity counted in a β-counter (Packard, USA).

For apoptosis assays, the cells were transferred onto regular microscope glass slides by 10-minute centrifugation in a Cytospin-3 device (Shandon, Pittsburgh, PA) at 2,000 rpm. Apoptosis was assessed by in situ dUTP-biotin nick-end labeling (TUNEL) procedure using an Oncor Apoptag kit (Intergen, New York, NY) designed to label the fragmented apoptotic DNA termini. In brief, the DNA fragments were labeled with digoxygenin nucleotides using terminal deoxynucleotydic transferase (TdT). Subsequently, they were allowed to bind to an antidigoxygenin antibody conjugated to horseradish peroxydase. Counterstaining was performed using Mayer hematoxylin dye. Percent apoptosis was established by differential counting of peroxidase-stained apoptotic cells per total 1,000 cells of each slide.

Statistical Analysis

Data are presented as means ± SD of 12 experiments.

Because it is extremely difficult to assume that the population distribution has any particular form (eg, normal distribution) in experiments performed on such a diverse population as uremic patients on chronic hemodialysis, nonparametric Kruskal-Wallis analysis of variances in which no assumptions concerning the population distribution are made, was used to evaluate the statistical differences between the results. A P value less than .05 was considered statistically significant.

RESULTS

Figure 1 presents total cell-associated Zn^{2+} concentrations in PBMC from hemodialysis patients or healthy controls following incubation in regular versus Zn^{2+}-enriched culture media. As can be seen, in a regular medium the basal cell-associated Zn^{2+} of PBMC from uremic patients was significantly increased compared to healthy controls (5.8 ± 1.40 *v* 3.3 ± 1.80 μmol/L/mg protein, $P < .05$).

Following 72 hours incubation in Zn^{2+}-supplemented medium, total cell-associated Zn^{2+} increased in both uremic and normal PBMC (56.15 ± 14.4 *v* 5.8 ± 1.4 μmol/L/mg protein and 44.01 ± 17.59 *v* 3.25 ± 1.8 μmol/L/mg protein, respectively; $P < .0001$ in each comparison), However, in uremic PBMC the final total cell-associated Zn^{2+} concentration at the end of the 72-hour incubation period was significantly higher than that of normal controls (56.15 ± 14.4 *v* 44.01 ± 17.59 μmol/L/mg protein, $P < .05$).

Figure 2 depicts percent apoptosis in parallel cultures of uremic versus normal PBMC. As can be seen, basal values of percent apoptosis in PBMC from uremic patients were significantly increased compared to those from healthy subjects (7.64% ± 2.18% *v* 3.69% ± 1.98%, $P < 0.05$). Seventy-two hours incubation of PBMC from healthy controls in Zn^{2+}-enriched medium resulted in an increase of percent apoptosis, which did not reach statistical significance (5.72% ± 2.49% *v* 3.69% ± 1.98%, P = not significant [NS]), while the augmentation was statistically significant in uremic patients (7.64% ± 2.18% *v* 11.7% ± 2.08%, $P < .05$). The apoptosis rate of Zn^{2+}-supplemented uremic PBMC remained significantly elevated compared to control cells (11.7% ± 2.08% *v* 5.72% ± 2.49%, respectively).

Figure 3 shows proliferation rates of uremic versus healthy control PBMC cultured in a medium that did or did not contain 80 μmol/L Zn^{2+}. As expected, basal proliferative rate of uremic PBMC was decreased compared to control cells (12,000 ± 1,560 cpm *v* 15,500 ± 1,464 cpm, $P < .05$). Both control and uremic PBMC demonstrated augmented proliferation following incubation in Zn^{2+}-supplemented medium. However, proliferative rate of uremic PBMC was significantly higher than that of their normal counterparts (37,000 ± 7,500 cpm *v* 22,000 ± 3,600 cpm, $P < .001$).

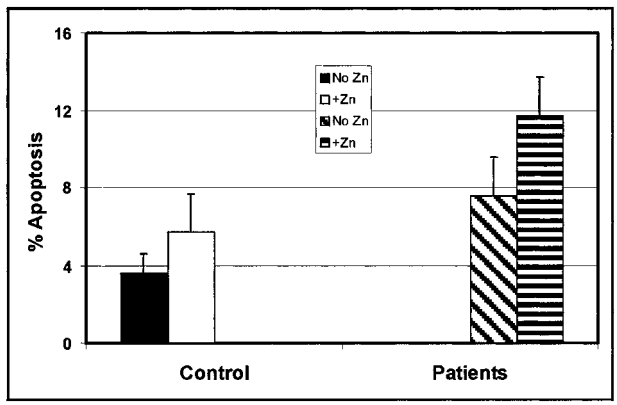

Fig 2. Percent apoptosis in PBMC of patients on chronic hemodialysis *v* normal control PBMC, cultured in medium supplemented or not with 80 μmol/L Zn^{2+}.

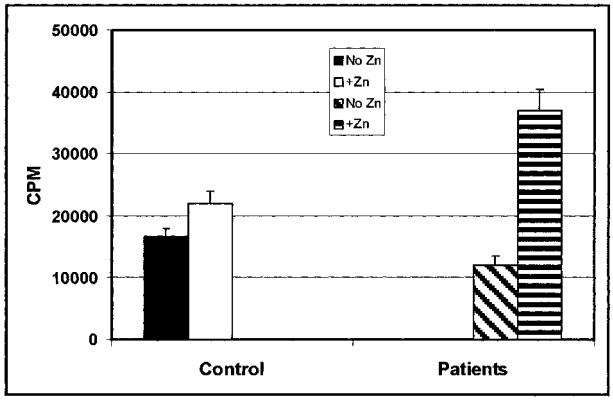

Fig 3. Proliferative rates of PBMC from patients on chronic hemodialysis *v* normal controls, cultured with or without addition of 80 μmol/L Zn^{2+} to the medium.

DISCUSSION

A number of metabolic processes and functions have been shown to be affected by Zn^{2+} status. Zn^{2+} proved to contribute an essential part of at least 300 enzymes.[24,25] Fifty important biochemical reactions are catalyzed by Zn^{2+} metalloenzymes.[24,25] Moreover, it has been demonstrated that in Zn^{2+} deficiency, the function of a number of enzymes is impaired[18] and can be restored by Zn^{2+} supplementation.[24,27] It has been demonstrated that Zn^{2+} deficiency is associated with impaired activity of various enzymes, including DNA polymerase, thymidine-kinase, and DNA-dependent RNA polymerase.[18,36] These aberrations eventually result in inhibition of DNA synthesis.[18,36] Moreover, Zn^{2+} has been shown to play an important role in the maintenance of stability, and thus physiologic permeability, of cell membranes.[37] Zn^{2+} proved to be essential for a number of metabolic events in relevant cells. Various discrete immunologic functions have been shown to be impaired in Zn^{2+}-deficient humans and experimental animals. These include delayed hypersensivity reaction, T-helper cell activity, natural killer cell function, and mitogen-induced cell proliferation.[19,20,28,38-42]

The responsiveness of T lymphocytes to antigens, as well as primary and secondary antibodies production by β lymphocytes, are decreased in the Zn^{2+} deficiency state.[28,29] Consequently, increased susceptibility to fungal, bacterial, and viral infections, as well as diarrhea and mucocutaneous lesions have been described in patients suffering from diseases associated with Zn^{2+} deficiency.[24,40]

These disorders proved correctable on restoration of immune cell activity by Zn^{2+} supplementation.[24-27] However, Zn^{2+} supplementation has been shown to enhance various immunologic functions in situations not necessarily associated with Zn^{2+} deficiency. Thus, Reardon and Lucas have demonstrated that normal splenic and lymph node lymphocytes respond to enrichment of culture medium with Zn^{2+} by augmented cell proliferation.[41]

Addition of Zn^{2+} to the medium of lymphocytes was shown to promote cell proliferation of T cells, simultaneously with enhancement of interleukin-2 (IL-2) production and IL-2 receptor expression.[42] Furthermore, non–IL-2–dependent B-cell proliferation was also shown to be induced by Zn^{2+} addition.[42]

In a different study, normal human PBMC cultured in Zn^{2+}-enriched medium were shown to produce enhanced amounts of cytokines, such as IL-2, IL-1, and tumor necrosis factor-beta.[30-32] The enhanced cytokine production, accompanied by elevation of free intracellular Zn^{2+}, proved to be regulated via tyrosine kinase cyclic adeninen monophosphate (cAMP)/cyclic guanosine monophosphate (cGMP)-dependent protein kinase pathways.[33] In addition, in a number of disease states not necessarily associated with Zn^{2+} deficiency, addition of Zn^{2+} to the medium has been shown to promote various immune cell functions (leprosy, human immunodeficiency virus [HIV]).[34,35] Thus, PHA-induced proliferation of PBMC from HIV patients was significantly enhanced by enrichment of culture medium by Zn^{2+}.[34] Cultured lymphocytes from leprosy patients were shown to release exaggerated amounts of IL-2 upon Zn^{2+} addition to the medium.[35]

Uremic immune deficiency, first described in 1957,[43] consists of a variety of defects, including both humoral and cell-mediated immune functions.[6-12,27] Thus, lymphopenia, or defective T-cell proliferation, associated with impaired IL-2 and interferon gamma production was demonstrated in uremia and confirmed at the gene expression level. In addition, a number of defects of β-cell function have been demonstrated in the uremic state.[6,44,46-48] At the clinical level, they result in impaired ability of uremic patients to mount antibody responses to various antigens, such as *Haemophilus* influenza or hepatitis B.[44,47,48] The similarity between the profile of discrete defective immune functions in uremia, as compared to Zn^{2+} deficiency, is intriguing.

Apoptosis and cell proliferation have been shown to be similarly modulated in various cell types.[1-5] It has been postulated that apoptosis plays an important regulatory role by eliminating defective cells. Thus, apoptosis contributes to the amelioration of the functional quality of the remaining cell populations. With respect to freshly procured as well as cultured PBMC from uremic patients, enhanced apoptosis has been demonstrated, both in dialytic and predialytic states.[49,50] Consequently, it has been postulated that this enhanced apoptosis contributes to the lymphopenia and absence of distinct lymphocyte subsets observed in uremia.[49,51]

The present results confirm that basal apoptosis rate of PBMC from uremic patients is augmented compared to control cells. Moreover, exposure of these cells to medium highly enriched with Zn^{2+} results in a significant rise in apoptosis rate. These results should be examined in the light of the parallel data concerning extracellular and cell-associated Zn^{2+} content. In the basal situation, Zn^{2+} concentration in sera procured from patients on chronic hemodialysis is significantly reduced compared to normal counterparts. On the other hand, their total cell-associated content of Zn^{2+} is significantly greater than in normal PBMC.[22] This would suggest, among other possible abnormalities, a functional impairment in uremic PBMC cell membrane, which may have resulted in inability to maintain a normal Zn^{2+} gradient. However, a further rise in total cell-associated uremic PBMC Zn^{2+} content triggered enhancement of apoptosis, as well as of PHA-induced proliferative response. Moreover, Zn^{2+}-stimulated enhancement of both apoptosis and

cell proliferation was significantly greater in uremic as compared to normal PBMC. Notwithstanding, net proliferative rate (proliferation minus apoptosis), produced by Zn^{2+} enrichment of the culture medium was significantly greater in uremic PBMC compared to normal controls.

The interpretation of these observations is not yet clear. As mentioned earlier, apoptosis, by eliminating defective cells, contributes to the functional capability of the population as a whole. If one accepts this premise, then one may expect that Zn^{2+} supplementation might have significant clinical implications by contributing considerably to a variety of uremic PBMC functions by simultaneously ameliorating performance and enhancing proliferative capability of the entire cell population.

Impaired immune responses, growth and puberty retardation, increased incidence of infections, impotence, morbidity, and other disorders closely associated with Zn^{2+} deficiency have been amply demonstrated.[24-44] Oral Zn^{2+} supplementation proved extremely successful in reducing Zn^{2+}-associated illnesses and decreasing death rates. With respect to the present investigation, all of the described complications are abundant in uremia.[6-17,21,22,27,46-51] The possibility of nutritional manipulation of the immune system by oral Zn^{2+} supplementation might serve as a powerful tool for reducing illnesses and deaths caused by Zn^{2+}-associated impaired immunity of hemodialysis patients.

On the cellular level, the nature of responses to Zn^{2+} enrichment by distinct uremic cell subtypes is still obscure. Elucidation of these responses may shed light on the eventual effects of Zn^{2+} supplementation on discrete metabolic and immunologic dysfunctions in uremia.

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