Vol. 215, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 4, 1995 Pages 98-105

Relationship between Susceptibility to Apoptosis and Fas Expression in Peripheral Blood T Cells from Uremic Patients: A Possible Mechanism for Lymphopenia in Chronic Renal Failure

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Received August 29, 1995

SUMMARY: Chronic renal failure (CRF) is often complicated by lymphopenia, which may be partly responsible for immune deficiency. We hypothesized that lymphopenia in CRF might result from apoptosis of T cells in vivo. To elucidate the involvement of Fas antigen which mediates apoptosis, we analyzed Fas expression on peripheral blood T cells in uremic non-dialyzed (non-HD) patients and hemodialysis (HD) patients. T cells from both uremic groups expressed Fas with higher intensity than control T cells. When two uremic groups were compared, Fas intensity on T cells was significantly higher in non-HD patients than in patients on HD. Moreover, uremic T cells were shown to undergo accelerated apoptosis when cultured in vitro, in correlation with Fas expression. Our results suggest that T cells in CRF may undergo apoptosis by the Fas system and that hemodialysis treatment has beneficial effects in the light of the inhibition of T cell apoptosis.

Chronic renal failure induces a state of immunodeficiency that mainly involves T cell-mediated responses, manifested by increased susceptibility to intracellular pathogens such as mycobacteria, and to malignant tumors, together with cutaneous anergy and the altered response to influenza and hepatitis B vaccines. This immunodeficiency may be at least partly attributable to decreased T cell population. Many studies have revealed that end-stage renal diseases are often complicated by lymphopenia (1-3). Moreover, McKerrow et al. (4) found that the absolute numbers of total T cells and some lymphocyte subsets were decreased in non-dialyzed uremic patients, and that the decrease of T cell population is normalized to some

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0006-291X/95 $12.00

extent by hemodialysis treatment. However, nothing is known about the regulation of the lymphocyte number in uremia.

Peripheral blood lymphocytes (PBL) are produced and destroyed in vivo with a precision that implies the existence of homeostatic mechanisms. The stimuli for PBL production are becoming well characterized, but the mechanisms involved in eliminating these cells are poorly understood. One mechanism involved in cellular elimination is programmed cell death (PCD) or apoptosis triggered by ligation of the Fas molecule. PCD of T cells has been described in some diseases accompanied by lymphopenia, such as acquired immune deficiency syndrome (AIDS) and systemic lupus erythematosus (SLE) (5-7). Thus, it was of interest to explore the possibility that PCD may affect the T cell population in patients with chronic renal failure. In the present study, we investigated the occurrence of apoptosis of T cells and appearance of Fas on T cells in non-dialyzed uremic patients and patients on hemodialysis.

MATERIALS AND METHODS

Patients: Twenty patients presenting CRF were included in this study. Ten of these patients underwent hemodialysis three times a week for four hours using cuprammonium rayon membranes. This group included 5 men and 5 women whose ages ranged from 52 to 72 years old (mean ± SD: 60.4 ± 6.0 years). In these hemodialyzed patients CRF was secondary to chronic glomerulonephritis (7 patients) and diabetic glomerulopathy (3 patients). The other ten patients presented end-stage renal failure (creatinemia ranging from 9 to 15 mg/dl) and were studied before institution of hemodialysis. This group included 5 men and 5 women, aged 48 to 64 years (mean ± SD: 54.9 ± 7.5 years). In these patients CRF was secondary to chronic glomerulonephritis (7 patients) and diabetic glomerulopathy (3 patients). Patients with CRF secondary to immunologic diseases such as SLE were excluded. None of the patients received prior to or during the study any treatment known to interfere with the immune system (steroids or immunosuppressants, etc.), or presented clinical or biological signs of an evolving infectious process.
As a control population 10 normal volunteers (3 men and 7 women, aged 48 to 65 years (mean ± SD: 56.5 ± 8.7 years)) were studied.

Cell preparations: All blood samples were collected in heparine containing vacutainers. In the case of hemodialyzed patients blood samples were collected immediately before dialysis. Peripheral blood mononuclear cells (PBMC) were isolated by centrifugation at 400 x g on a Ficoll-Paque (Pharmacia, Uppsala, Sweden) gradient. PBMC were recovered at the interface and washed twice in Hanks' balanced salt solution (HBSS). For T cell enrichment, these cells were incubated on plastic tissue culture plates at 37°C for 1 h to allow monocytes to adhere and nonadherent cells were passed over the Cellect Human T Cell column (Biotex Laboratories Inc., Alberta, Canada). The column-passed cells contained >93% $CD3^+$ cells as assessed by immunofluorescence.

Immunofluorescence analysis: Monoclonal antibodies (mAbs) used for labeling assays were PE-conjugated anti-CD3 (IgG1, SK7) and FITC-conjugated anti-Fas (IgG1, UB2) which were purchased from Becton Dickinson & Co. (Mountain View, CA) and MBL (Nagoya, Japan), respectively. Freshly isolated

PBMC were incubated for 30 minutes in cold PBS with appropriate dilutions of these mAbs. Detection of bcl-2 protein was performed as described previously (8) with minor modifications. Briefly, cells were fixed in 4% paraformaldehyde, washed, and permealized with saponin 0.1% in PBS. FITC-conjugated bcl-2 from DAKOPATTS (Glostrug, Denmark) was stained as outlined above except that all washes contained 0.1% saponin. The fluorescence-positive cells were analyzed on a FACScan (Becton Dickinson & Co.).

Cell culture and quantification of cell death: Culture medium was RPMI 1640 supplemented with 10 mM Hepes, 5 x 10^{-5} M 2-ME, 100 U/ml penicillin, 100 μg/ml streptomycin, and 5% FCS. T cells were resuspended at a concentration of 2 x 10^6 cells/ml in medium and incubated for 48h in 24-well plates. After incubation, dead cells were stained by adding propidium iodide (PI) (Sigma Chemical Co., St. Louis, MO) at a final concentration of 5 μg/ml in PBS. Red fluorescence was analyzed on a FACScan.

DNA electrophoresis: Cells were incubated at 37°C for 48h, and then pelleted. DNA gel analysis was performed as described previously (9). After suspension in extraction buffer with proteinase K, the lysates were incubated. The fragmented DNA was phenol/chloroform extracted and ethanol precipitated. DNA was analyzed on a 2% agarose gel.

Statistical methods: Data were analyzed by using Student's *t*-test. Values of $P<0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

Fas expression on freshly obtained peripheral blood T cells: First, expression of Fas antigen on $CD3^+$ cells was examined in normal controls, uremic non-dialyzed (non-HD) patients and hemodialysis (HD) patients by two-color immunofluorescence analysis. We observed marked differences among three groups. Representative expression of Fas was shown in histograms after gating on the $CD3^+$ cells (Fig. 1a). The intensity of Fas expression per cell was assessed by the mean log fluorescence channel because the continuous Fas expression of T cells cannot delineate negative and positive fractions. As shown in Fig. 1b, both the intensity of Fas in non-HD patients and that in HD patients were significantly greater than in controls. When comparing the Fas intensity in non-HD and HD patients, the intensity in non-HD patients was noticeably greater than in HD patients.

Bcl-2 expression on freshly obtained peripheral blood T cells: Since bcl-2 is effective in inhibiting cell death in a number of different cell types (10-13), modulation of the endogenous level of bcl-2 may control the survival and death of uremic lymphocytes. To investigate the bcl-2 participation in Fas ligation-induced apoptosis in uremic T cells, we examined expression of bcl-2 in each three patients selected randomly from all groups. As can be seen in Fig. 2, the bcl-2 expression in uremic T cells from non-HD patients was reduced in comparison with that from HD

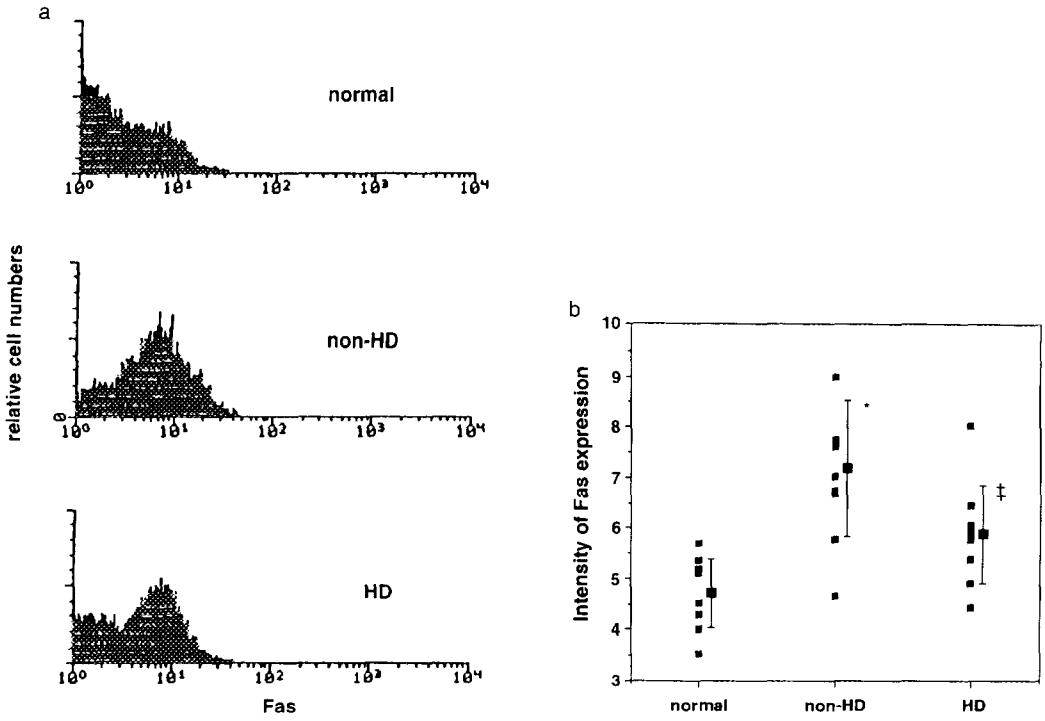

Fig. 1. Expression of Fas on peripheral blood T cells from controls, non-HD and HD patients. PBMC were isolated and labelled with PE-conjugated anti-CD3 mAb and FITC-conjugated anti-Fas mAb. (a) Representative Fas expression from three groups were shown in histograms after gating on the $CD3^+$ cells. (b) Intensity of Fas expression on T cells assessed by the mean log fluorescence channel. Data are means ± SD. $^*p < 0.05$, significantly different from values for HD groups. $^‡p < 0.01$, significantly different from values for control groups.

patients, whereas not much difference was apparent between controls and HD patients (data not shown). These data indicated that bcl-2 is downregulated in T cells at the stage of uremia before institution of hemodialysis, and provided the possibility that the diminished bcl-2 expression may facilitate apoptosis by signaling via the Fas in predialyzed uremic patients.

Apoptosis of T lymphocytes in culture: One could easily postulate that uremic T lymphocytes with these intrinsic abnormalities may undergo accelerated apoptosis when purified ex vivo. To quantificate cell death in culture, cell samples from three groups were cultured for 2d without stimulation and stained with PI. As shown in Fig. 3a, both T lymphocytes from non-HD and HD patients rapidly underwent cell death compared with normal T lymphocytes. When comparing the viability between two patients groups, T lymphocytes from non-HD patients showed significantly lower viability than T lymphocytes from HD patients. To determine whether such cell death is due to apoptosis, cell samples after culture were

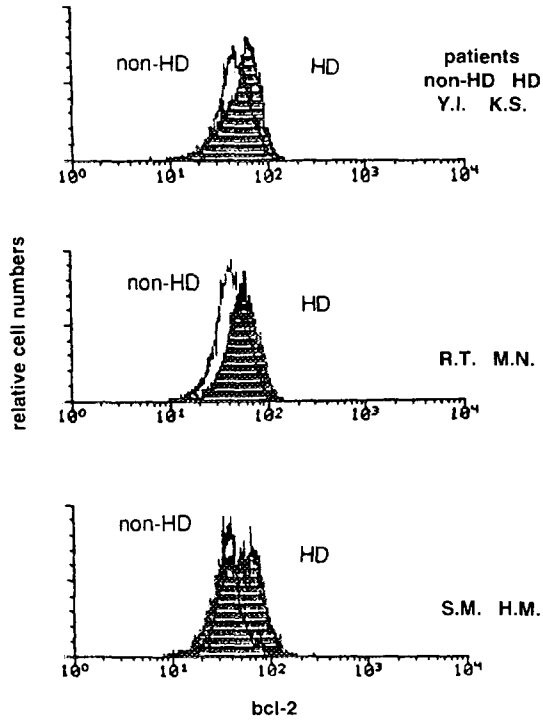

Fig. 2. Expression of bcl-2 protein in peripheral blood T cells from patients with CRF. PBMC were isolated and stained with PE-conjugated anti-CD3 mAb and FITC-conjugated anti-bcl-2 mAb as described in Materials and methods. Three pairs of non-HD (open histogram) and HD (hatched histogram) patients were selected at random. T cells in non-HD patients expressed low levels of bcl-2 compared with those in HD patients, whereas there were no significant differences between HD patients and controls (data not shown).

analyzed for DNA fragmentation, a putative marker of apoptosis. DNA fragmentation was detected clearly in cells from non-HD patients and not at significant levels in cells from HD patients, although control samples showed little or no DNA ladder (Fig. 3b). These results might account for the clinically fascinating data of McKerrow *et al.* (4).

T cells from uremic individuals have increased expression of such activation markers as IL-2-R (15) and LFA-1 (Y. M. et al., submitted), fail to proliferate in response to mitogen (16-18), and seem to die rapidly upon culture (Fig. 3, 4). These findings suggest that uremic T cells are made hyporesponsive by hyperactivatiion, and in this apparent anergic or hyperactivated state, T cells could be programmed for death.

So far, it has been shown that both $CD4^+$ and $CD8^+$ T cells from patients with AIDS are abnormally sensitive to activation-induced apoptosis, which may be partly

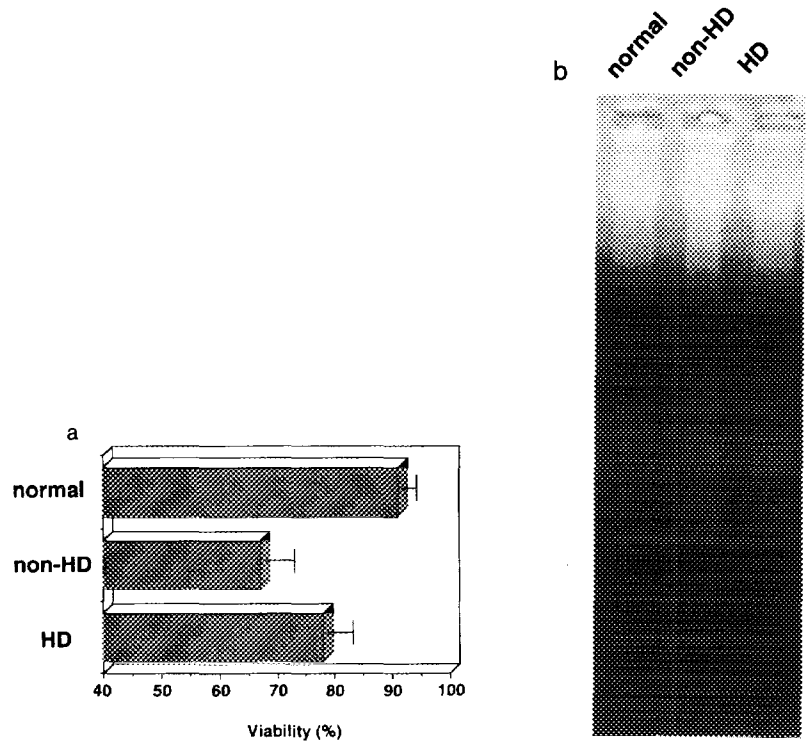

Fig. 3. (a) Quantification of cell death by PI staining in T lymphocytes cultured for 2d. T cells were isolated and cultured at 2 x 10^6/ml in RPMI medium. At 48 h, samples were removed and the viability was measured by PI staining. This figure shows the means ± SD of studies in four different samples per group. **(b)** Gel analysis of DNA from T lymphocytes cultured for 2d. T cells (2 x 10^6) were cultured in 1ml of RPMI medium. At 48 h, cells were harvested and DNA was analyzed for the nucleosomal ladders by gel electrophoresis as in Materials and methods. This figure represent cellular DNA from one individual per group, and similar results were obtained from two other studies.

responsible for lymphopenia in this disease (5, 6). Another study (7) in patients with active SLE has suggested that the increased expression of Fas results in enhanced susceptibility to apoptosis and subsequent lymphopenia in vivo, since the Fas pathway appears to be functional in SLE lymphocytes activated in vitro (7). Similarly, in CRF patients, abnormally activated Fas systems might play a role in lymphopenia. If so, where is the Fas-ligand (Fas-L) that induces an apoptotic signal by binding to Fas on uremic T cells? Recently, Suda et al. (19) have demonstrated that Fas-L is expressed not only in activated splenocytes and thymocytes but several non-lymphoid tissues such as lung and small intestine. As cell-autonomous Fas/Fas-L interaction in murine T cell hybridomas mediates activation-induced apoptosis (20), human peripheral T cells in uremia may express up-regulated Fas-L

as well as Fas. Alternatively, we cannot rule out the possibility that the soluble form of Fas-L is present in the serum of individuals suffering from CRF, because TCR-induced apoptosis in malignant Jurkat cells can occur through an autocrine suicide mediated by Fas-L as a soluble cytokine (21). It will be of interest to examine whether the induction of Fas-L in HIV infection, SLE, CRF and other disease is an important means of regulating lymphocyte survival.

One might speculate whether cell death is activated in a random fashion, or some T cell populations might be susceptible to apoptosis in chronic renal failure. In human Epstein-Barr virus infection, dying cells were reported to be confined to the $CD45RO^+$ population (22), and in early HIV infection, functional and phenotypical evidence have revealed the specific loss of memory T cell function (23, 24). These findings suggest that apoptosis in the memory T cell population residing within the activated $CD45RO^+$ T cell pool may contribute to lymphopenia in uremia. Our recent findings indicate that peripheral blood $\gamma\delta$ T cells from hemodialyzed patients are markedly reduced compared to healthy controls (Y. M. et al., submitted). This supports our notion, because the majority of $\gamma\delta$ T cells seem to be "primed" memory population (25, 26).

In this report, we suggested that T cells under uremic environment may undergo apoptosis by the Fas system, gave a possible explanation for the ability of bcl-2 to synergize with Fas-mediated pathway, and approached the mechanisms whereby uremic patients, especially those with end-stage CRF before institution of hemodialysis, are complicated with lymphopenia. Further evaluation will define the cellular regulatory mechanisms and biochemical processes involved in uremia. Such knowledge may provide clues to understanding T cell pathology in chronic renal failure and may also lead to new therapeutic approaches for treating many of the complications experienced by patients with uremia.

ACKNOWLEDGMENT

We thank Emi Ito for technical and secretarial assistance.

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