Long-term therapy with recombinant human erythropoietin increases CD8⁺ T-cell apoptosis in haemodialysis patients

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Abstract

Background. We intended to assess the intensity of apoptosis in the CD4⁺ and CD8⁺ T-lymphocytes of haemodialysis (HD) patients on recombinant human erythropoietin (rHuEpo).

Methods. The expression of Fas, tumour necrosis factor-α receptors (TNFRI and TNFRII) and the CD28 molecule on lymphocytes was evaluated in 15 HD patients before and during treatment with rHuEpo. In cultures of peripheral blood mononuclear cells (PBMCs) stimulated with rHuEpo, phytohaemagglutinin and camptothecin, our measures of apoptosis were the percentages of cells with subdiploid DNA content and of annexin V-stained cells.

Results. Therapy with rHuEpo did not affect CD4⁺ T cells but decreased the percentage of CD8⁺ T cells in peripheral blood. The intensity of apoptosis in both CD4⁺ and CD8⁺ T cells at baseline was lower in HD patients than in healthy volunteers, and increased in those treated with rHuEpo. In vitro, rHuEpo did not induce apoptosis in PBMCs. The percentage of CD8⁺ Fas⁺ T cells was constant, while that of CD8⁺ TNFRI⁺ cells declined during follow-up. There was an increase in the percentage of CD28⁺ T cells, mainly in the CD8⁺ compartment, as early as 1 month after the introduction of rHuEpo.

Conclusions. Treatment with rHuEpo caused a decline of CD8⁺ T cells in HD patients, which most probably was mediated via the TNFRI-related apoptotic pathway and was independent of Fas expression. Apoptosis in vitro was not directly influenced by rHuEpo, suggesting that the process in vivo was only initiated by rHuEpo supplementation.

Keywords: apoptosis; erythropoietin; haemodialysis

Introduction

Patients who have chronic renal insufficiency are more susceptible to infections, autoimmune diseases and neoplasms than healthy individuals [1]. It is commonly accepted that most of those disease processes are related to a deterioration of the immune response. Applying haemodialysis (HD) for those patients exaggerates the immunodeficiency, since it stimulates the proinflammatory responses of mononuclear leukocytes [2]. Recombinant human erythropoietin (rHuEpo) is believed to reduce those harmful changes by acting as an immunomodulatory agent [3].

In our previous study [3], we found that long-term therapy with rHuEpo in HD patients decreased peripheral blood CD8⁺ T cells, mainly those expressing the CD152 antigen. Since it is known that cross-linking of the CD152 receptor on activated T lymphocytes leads to programmed cell death, terminating the life of previously stimulated T cells [4], we were interested in finding out if the decrease of peripheral blood CD8⁺ T cells in HD patients treated with rHuEpo was connected with apoptosis. Data concerning apoptotic activity caused by rHuEpo in HD patients are scarce. It is believed that apoptosis might be induced by the contact of peripheral blood mononuclear cells (PBMCs) with the cellulose membranes of dialysers [5]. Another study suggests that PBMCs from HD patients are prematurely senescent due to their repeated HD-induced activation; thus, those cells are characterized by prolonged survival in the peripheral blood [6]. These data are apparently inconsistent—increased apoptosis should be concomitant with a short survival, but we confirmed in our previous work that senescent cells may exhibit an increased intensity of early apoptotic features that does not end up in cell death, therefore causing senescent cells to endure [7].

While PBMCs are very useful in mimicking conditions occurring in vivo, the interpretation of results based on PBMCs should be done with caution, because...
PBMCs are a mixed population consisting of monocytes and lymphocytes. Indeed, while we confirmed the findings of another group [8], that the apoptosis of monocytes and granulocytes is more intense in HD patients than in healthy subjects, this process is not so obvious in lymphocytes from HD patients. Although there is evidence that γδ T lymphocytes are depleted from the circulation of HD patients [9], those cells are a very small subpopulation of T lymphocytes. There also are no data on the influence of rHuEpo treatment on lymphocyte apoptosis in HD patients.

The reason why apoptosis may be involved in lymphocyte survival in HD patients treated with rHuEpo is that this hormone acts in many more ways than was previously recognized. Apart from rHuEpo’s well-known protective action, rescuing erythroid cells from apoptosis, it is also known to protect cardiac muscle and brain, contributing to apoptotic pathways during vascular remodelling in them [10] or, in the case of pancreatic islets and brain, through the erythropoietin receptors [11]. It is possible that erythropoietin treatment may influence lymphocytes because erythropoietin receptors have also been detected on lymphocyte-derived cell lines [12].

The aim of our current study was to assess the intensity and mechanisms of apoptosis in CD4⁺ and CD8⁺ T lymphocytes from the blood of HD patients treated with rHuEpo.

**Subjects and methods**

**Subjects**

This study was performed to confirm the findings of our previous study, and its inclusion criteria were similar to those previously applied [3]. Briefly, the study was performed on 15 HD patients (52 ± 13 years old) who required rHuEpo treatment during the first year of their treatment, six HD patients (53 ± 7 years old) who did not and 21 healthy volunteers (49 ± 14 years old). HD patients were on HD with a synthetic membrane polysulfone dialyser and bicarbonate dialysate thrice weekly; and the HD recipe did not change during the follow-up (Table 1). All HD patients had been on dialysis for at least 6 months before entering the study, and all suffered from chronic renal insufficiency as a result of primary glomerulonephritis. Severe anaemia [haemoglobin (Hb) concentration < 9 g/dl] was the main reason for initiating the treatments with rHuEpo (Eprex, Janssen-Cilag, Switzerland), which was administered three times a week as a subcutaneous (s.c.) injection, with a starting dose of 2000 IU (mean 95 ± 44 IU/kg/week) per injection adjusted according to Hb values to keep its level between 10 and 12 g/dl. None of the patients received blood transfusions, or any other treatment known to interfere with the immune system, from 6 months before the beginning of the study to its end. The indices of iron metabolism did not differ between HD patients who received rHuEpo and those who did not. Those indices were monitored, and intravenous (i.v.) iron sucrose was administered to keep them at their appropriate levels. None of the patients had chronic or acute infections or neoplasms during the study.

Healthy control subjects were chosen based upon physical examinations, carefully taken histories and laboratory data. Chronic or acute inflammatory diseases, neoplasms, the taking of drugs known to affect the immune system or abnormal laboratory data in the 6 months before the start of the study through to its end excluded the volunteers from the control group. The control subjects did not receive rHuEpo; only the cells harvested from their blood were exposed to this hormone.

To make the study more informative, we add here results related to the kinetics of CD28⁺ T lymphocytes. This aspect was implied by our earlier results; and this part of the study was carried out later than the rest and with a separate group of HD patients (n = 15, 57 ± 12 years old). The same inclusion criteria and follow-up protocol were applied to both phases of the study. However, the patients in the second cohort received rHuEpo i.v., when the s.c. administration of the drug was already contraindicated. Mindful of the coherence of the study, we determined and recorded all relevant parameters, such as total blood counts, iron status, etc., and did not find any differences when we compared the two groups with different routes of rHuEpo administration.

The patients and healthy volunteers were informed about the purpose of the study and gave written consent. The study was approved by The Ethical Committee of The Medical University of Gdańsk.

**Table 1. Design of the study**

<table>
<thead>
<tr>
<th>Patient groups</th>
<th>HD patients treated with rHuEpo</th>
<th>HD patients not treated with rHuEpo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time of sampling</td>
<td>Before = beginning of the study, before the first dose of rHuEpo in HD patients receiving rHuEpo</td>
<td>1 month = 1 month after the first dose of rHuEpo in HD patients receiving rHuEpo</td>
</tr>
<tr>
<td></td>
<td>6 months = 6 months after the first dose of rHuEpo in HD patients receiving rHuEpo</td>
<td>1 year = 1 year after the first dose of rHuEpo in HD patients receiving rHuEpo</td>
</tr>
<tr>
<td>Source of cells during analysis</td>
<td>Ex vivo = PBMCs analysed immediately after separation from peripheral blood</td>
<td>PHA = PBMCs separated and cultured in the presence of PHA for 24 h before the analysis</td>
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<tr>
<td></td>
<td>Non-stimulated = PBMCs separated and cultured without stimulants for 24 h before the analysis</td>
<td>PHA + camptothecin = PBMCs separated and cultured in the presence of PHA and camptothecin for 24 h before the analysis</td>
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<td></td>
<td>0.05 rHuEpo = PBMCs separated and cultured in the presence of 0.05 IU/ml rHuEpo for 24 h before the analysis</td>
<td>1.0 rHuEpo = PBMCs separated and cultured in the presence of 1.0 IU/ml rHuEpo for 24 h before the analysis</td>
</tr>
</tbody>
</table>
All measurements below were performed during every sampling. Each patient was evaluated using the complete panel of measurements during every sampling.

Specimen collection and preparation

Fasting venous blood samples (15 ml) were collected before the HD sessions into heparinized tubes (Becton Dickinson Company, USA) for direct analysis by flow cytometry and for the PBMC cultures. Blood samples from HD patients receiving rHuEpo were collected at the following time-intervals: before the beginning of rHuEpo treatment, and at 1, 6 and 12 months after the beginning of the therapy. Similar time intervals were applied in HD patients without rHuEpo and in healthy subjects.

Reagents and antibodies

NUNC, Denmark, was the supplier of single-use plastic equipment; RPMI 1640 medium, inactivated fetal calf serum (FCS) and other media were purchased from Gibco, Life Technologies Inc., USA. We used rHuEpo (Eprex, Janssen-Cilag, Switzerland), phytohaemagglutinin (PHA) (Pharmacia, Sweden) and camptothecin (Sigma, USA) for in vitro stimulation.

The following monoclonal antibodies (mAbs) were used in the flow cytometric studies: anti-CD3 [IgG1, fluorescein isothiocyanate (FITC) or CyChrome mAb, clone: UCHT1], anti-CD5 (IgG1, CyChrome mAb, clone: RPA-T8), anti-CD14 [IgG1, phycoerythrin (PE) mAb, clone: M5E2], anti-CD45 (IgG1, FITC mAb, clone: H130), anti-Fas (IgG1, PE mAb, clone: DX2) anti-CD28 (IgG1, FITC mAb, clone: CD28.2) and surface isotype controls (IgG1, FITC, PE, CyChrome mAbs, clone: MOPC-21; IgG1, PE mAb, clone: G155-178) (all from BD Bioscience, Pharmingen, Germany); anti-CD4 (IgG1, ECD mAb, clone: SFC112T/4D11) and appropriate surface isotype controls (IgG1, ECD mAb, clone: Cyto-Stat) (both from Immunotech, USA); anti-tumour necrosis factor-α receptor I (TNFR1) (IgG1, PE mAb, clone: 16803.1), anti-TNFRII (IgG1, PE mAb, clone: 22235.311) and surface isotype control (IgG1, PE, clone: 11711.11) (from R&D Systems, USA); and annexin V, FITC conjugated (purchased from Biosource, Belgium).

Isolation of PBMCs and cell cultures

PBMCs were isolated from the samples of peripheral blood, by Ficoll-Paque gradient centrifugation. The population of PBMCs, i.e. lymphocytes and monocytes together, was chosen to mimic conditions occurring in vivo, including possible cell–cell interactions of different PBMC subpopulations and various patterns of cytokine secretion by them. After two washes with phosphate-buffered saline (PBS), PBMCs were suspended in the culture medium RPMI 1640 supplemented with 5% FCS, and then 1 × 10⁶ cells were diluted with 1 ml of medium (RPMI 1640 + 5% FBS) and cultured in triplicate on plastic 24-well plates. The cultures were stimulated with 1 µg/ml of PHA at rHuEpo in low (0.05 IU/ml, 0.05 IU/ml rHuEpo) or high (1.0 IU/ml, 1.0 IU/ml rHuEpo) concentrations [5]. Thus, rHuEpo used in the cultures was the additional dose of rHuEpo used on the cells from patients already receiving it in vivo. Maximal apoptotic response, which illustrated the apoptotic potential of the examined cells, was obtained by the addition of 20 µg/ml of camptothecin to the cultures pre-stimulated with PHA. All the cultures were incubated for 24 h in a humidified atmosphere containing 5% CO₂. A portion of cells, control ex vivo samples, was stained and analysed immediately after the separation without culturing and stimulation. The other controls were non-stimulated cultures made without any stimuli in order to estimate the amplitude of response to applied stimuli or, in the case of annexin V, to correct for the non-specific binding of the reagent.

DNA gel electrophoresis

To confirm features of apoptosis in dying cells and to distinguish them from possible necrotic cells, we analysed all the PBMCs, i.e. lymphocytes and monocytes together, by DNA gel electrophoresis and by measuring the sub-G₁ peaks. PBMCs from the cultures were harvested by centrifugation, fixed with 70% ethanol and stored in the fixative at −20°C for 24–72 h. The cells were then centrifuged at 800 g for 5 min and the cell pellets (2 × 10⁶ cells) were resuspended in phosphate-citrate (PC) buffer and kept at room temperature for at least 30 min after re-centrifugation at 1000 g for 5 min. Then, the supernatant was transferred to the new tubes and concentrated by vacuum in a SpeedVac concentrator (Labtech, Finland). As the next step, 0.25% NP-40 (Sigma, USA) and 1 mg/ml RNase A (Sigma, USA) were added into the tubes, which were incubated subsequently at 37°C for 30 min. Then, 1 mg/ml of proteinase K (Sigma, USA) was added and the extract was incubated for an additional 30 min at 37°C. The loading buffer (MBI Fermentas, USA) was added after the incubation, and the content of the tube was transferred onto a 1% agarose gel. Electrophoresis was performed at 5 V/cm for 5 h. The DNA in the gels was visualized under UV light after staining with 5 µg/ml ethidium bromide.

Staining and flow cytometry analysis of the sub-G₁ DNA apoptotic peak

The pellets of cells removed from the PC buffer (described above) were resuspended in 800 µl of PBS. Then 100 µl of RNase A and 100 µl of propidium iodide were added. Flow cytometry analysis of the sub-G₁ peaks was performed after 30 min of incubation on an EPICS XL flow cytometer (Coulter, USA). The data obtained at this stage were analysed using CycIcRed, version 1.2 (software provided by Terry Hoy).

Staining and analysis of CD4⁺ and CD8⁺ T cells with annexin V

Aliquots of PBMCs from the cultures were put into 12 × 75 mm tubes (1 × 10⁶ cells per tube), washed twice with cold PBS, stained with anti-CD4-PE, anti-CD8-CyChrome mAb and the appropriate isotype control antibodies (20 µg/test) and were incubated for 15 min in the dark at room temperature. Then, the cells were washed with PBS and resuspended in a solution containing 10 mM HEPES, 140 mM NaCl and 2.5 mM CaCl₂. As the next step, 2 µl of annexin V–FITC was added to the appropriate samples and the tubes were incubated for 15 min in the dark. To acquire flow cytometry data after incubation, we used
the EPICS XL flow cytometer (Coulter, USA). Analysis of these data was done using the WinList 5.0 software (Verity, USA). Two approaches were applied: the first used forward scatter (FSC) and side scatter (SSC) dot plots to establish the lymphocyte gate containing all lymphocytes (Figure 3A), the second used dot plots of anti-CD4 (or anti-CD8) vs SSC containing only the examined populations of lymphocytes, CD4+ or CD8+ T cells (Figure 3B). Typically, 10,000 events were acquired in the gated region. The threshold level for annexin V-positive cells was set for each sample at the intersection of the histogram curves obtained from unstained cells and the specific monoclonal antibody.

**Staining and analysis of surface Fas, TNFRI, TNFRII and CD28 receptors on CD4+ and CD8+ T lymphocytes**

The samples of venous blood (100 µl per tube) and cultures (1 × 10^7 cells per tube) were vortexed and were divided in aliquots into 12 × 75 mm plastic tubes. The appropriate samples were stained with a single sample of an mAb and with the following four-colour combinations to visualize expression of the examined receptors: CD3/CD8/CD4/Fas, CD3/CD8/CD4/TNFRI, CD3/CD8/CD4/TNFRII and CD28/CD8/CD4/CD3 (20 µg of each mAb per tube). In addition, an appropriate set of isotype control samples was used. After incubation (30 min in the dark at 4°C), the samples were subsequently lysed and fixed using Immuno-prep reagents (Immunotech, USA) with the Q-prep Immunology Workstation (Coulter, USA). Listmodes were acquired on the Epics XL flow cytometer (Coulter, USA) and analysed using the WinList software, version 5.0 (Verity, USA). The percentages of CD4+ and CD8+ cells with surface expression of Fas, TNFRI, TNFRII and CD28 receptors were obtained from anti-CD4 or anti-CD8 vs the SSC dot plot window (Figure 4). As in the case of annexin V assay analysis, the statistical results obtained using the CD4 or anti-CD8 vs the SSC dot plot approach were more meaningful than the FSC vs SSC dot plot approach. Thus, they were also the ones chosen and presented below. Typically, 10,000 events were acquired in the gating region. The threshold level for positive cells was set for each sample at the intersection of the histogram curves obtained for cells stained with the isotype control and the specific mAb.

**Statistics**

We analysed the resultant data using the computer program Statistica, version 5.5 (Statsoft, Poland), and by applying descriptive statistics, ANOVA/MANOVA tests for repeated measures and the least significant difference test as a post hoc test. Only significant differences are presented in the full form. The level of significance in all was \( P \leq 0.05 \).

**Results**

**CD4+ and CD8+ T lymphocytes in peripheral blood**

The amounts of CD8+ T lymphocytes in peripheral blood decreased, while that of the CD4+ T cells remained constant during rHuEpo therapy. A significant decline in the CD8+ T cells as compared with initial values was noted only after 6 months of follow-up (Figure 1).

**Apoptosis in PBMC cultures (sub-G1 peak)**

Flow cytometry analysis of apoptosis, visualized as the sub-G1 peak (Figure 2A), revealed significantly higher percentages of cells in apoptosis in the PBMC cultures stimulated with PHA and camptothecin than in the non-stimulated cultures (Figure 2B).

![Fig. 1. Comparison of the amounts of CD4+ and CD8+ T lymphocytes in the peripheral blood of HD patients treated with rHuEpo and in healthy subjects, for the duration of the study. The figure presents the mean ± SD [CD8+ ANOVA, \( F = 4.2 \) \( P = 0.003 \), significant LSD before/6 months \( P = 0.004 \) LSD before/1 year \( P = 0.003 \); CD4+ ANOVA, differences insignificant].](image1)

![Fig. 2. The intensity of apoptosis in PBMCs, measured as the sub-G1 peaks immediately ex vivo and in the cultures of PBMCs stimulated with different stimulants. (A) The image shows examples of analyses performed using flow cytometry, their results confirmed by using DNA gel electrophoresis (characteristic apoptotic DNA ladder from the PHA + camptothecin well). (B) Detailed results obtained during follow-up in the PBMC cultures from HD patients treated with rHuEpo and from healthy controls. The figures present the mean ± SD [MANOVA non-stimulated/PHA + camptothecin, \( R^2 = 7.2 \) \( P = 0.007 \), significant LSD before/6 months \( P = 0.01 \) LSD before/1 year \( P = 0.009 \), other differences insignificant; MANOVA healthy/HD patients, \( R^2 = 5.1 \) \( P = 0.02 \)].](image2)
The effect was present as early as after 6 months of rHuEpo treatment in HD patients. Nevertheless, the amplitude of the increase in the percentage of apoptotic cells following such a stimulation was higher in samples from the healthy controls than from the HD patients. In all examined groups, samples stained ex vivo or stimulated with different concentrations of rHuEpo were not different from unstimulated samples.

The characteristic pattern of the DNA ladder resulting from the products of DNA cleavage in late apoptosis, the so-called ‘low molecular weight DNA’, was noted, confirming that the sub-G₁ peaks exactly reflected apoptosis (Figure 2A).

**Apoptosis in the populations of peripheral blood CD4⁺ and CD8⁺ T lymphocytes**

Two approaches were applied in the analysis. The first was based on the lymphocyte gate generated by using an FSC/SSC dot plot (Figure 3A), while the second included only the CD4⁺ or CD8⁺ T lymphocytes, which was achieved by gating them electronically using the fluorescence of anti-CD4 or anti-CD8 antibodies combined with the SSC (Figure 3B). The phenotyping of CD4⁺ or CD8⁺ cells with annexin V revealed the gradually increasing ability of the CD8⁺ T cells to undergo apoptosis in HD patients treated with rHuEpo. This effect was visible as early as 6 months after the initiation of rHuEpo therapy. However, the maximal in vitro apoptotic response in cultures stimulated with PHA and camptothecin was stronger in healthy controls than in HD patients treated with rHuEpo throughout the follow-up. Interestingly, throughout the study, the increased binding of annexin V to the CD8⁺ T cells derived from HD patients treated with rHuEpo was similar to that in the stimulated cells.

Throughout the study, the intensity of apoptosis in the CD4⁺ T cells from HD patients treated with rHuEpo stimulated with PHA and camptothecin was lower than in those from the controls. However, the intensity of apoptosis, measured as the control/stimulated amplitude, was significant in PBMC cultures derived from HD patients treated with rHuEpo as early as 1 year after the start of the study.

Different concentrations of rHuEpo did not affect either the CD8⁺ or CD4⁺ population of lymphocytes in terms of apoptosis in vitro.

**Surface expression of Fas receptor on peripheral blood CD4⁺ and CD8⁺ T lymphocytes**

The Fas receptor was expressed on the surfaces of the majority of CD8⁺ T cells from HD patients at a significantly higher level than in cells from healthy controls, and this expression did not change throughout the follow-up. The ex vivo levels of Fas-positive CD8⁺ T cells from HD patients were equal to those from the PHA-stimulated cultures in vitro and significantly higher than in the non-stimulated cultures (Figure 4).

The expression of the Fas receptor on the surface of CD4⁺ T cells from HD patients was constant throughout the follow-up and comparable between HD patients and the control subjects. The ex vivo levels of CD4⁺Fas⁺ T cells from HD patients were equal to those in the non-stimulated cultures (Figure 4).

For both CD8⁺ and CD4⁺ T lymphocytes, the only efficient stimulant of in vitro Fas expression was PHA, while different concentrations of rHuEpo did not affect it.

**Surface expression of TNF receptors on peripheral blood CD4⁺ and CD8⁺ T lymphocytes**

At baseline of the study, the surface expression of TNFRI on the CD8⁺ T cells from HD patients was significantly higher than in the healthy controls. During follow-up, this expression decreased gradually to the level noted in the healthy control subjects. As in the CD8⁺Fas⁺ T cells, the ex vivo percentage of CD8⁺TNFRI⁺ T cells from HD patients was equal to that in the in vitro PHA-stimulated cultures and significantly higher than in the unstimulated cultures (Figure 4).

There were no differences between HD patients and healthy controls in terms of TNFRII expression on CD8⁺ T cells. This expression was constant throughout the follow-up, and none of the stimulants used affected it significantly.

As in the CD8⁺Fas⁺ T cells, the expression of TNFRI receptor on the CD4⁺ T cells from HD patients was constant throughout the follow-up and comparable between HD patients and the healthy controls. Nevertheless, the ex vivo levels of CD8⁺TNFRI⁺ T cells noted in HD patients were equal to those in the PHA-stimulated cultures and significantly higher than in the non-stimulated cultures (Figure 4).

The level of TNFRII expression was constant throughout the follow-up. However, the levels of CD4⁺TNFRII⁺ cells were significantly higher in the non-stimulated cultures than in the stimulated cultures or ex vivo samples. As for the expression of the Fas molecule, the only efficient stimulant of TNFRII expression in vitro on both CD8⁺ and CD4⁺ T lymphocytes was PHA, while different concentrations of rHuEpo did not affect it. None of the stimuli used were able to increase the percentage of TNFRII receptor. In the case of the CD4⁺TNFRII⁺ T cells, some of the stimulants decreased it, as compared with control unstimulated cultures.

**Surface expression of CD28 receptor on peripheral blood CD4⁺ and CD8⁺ T lymphocytes**

The percentages of CD4⁺ and CD8⁺ T lymphocytes expressing the CD28 receptor on their surface increased significantly 1 month after the beginning of rHuEpo.
treatment. This increase had two important features: first, although the increase concerned both CD4⁺ and CD8⁺ T cells, only the percentage of CD8⁺ T cells increased to the levels observed in the healthy controls. Secondly, despite the decrease in the absolute number of CD8⁺ T cells throughout the study, the \( \text{CD8}^+ \text{CD28}^+ / \text{CD8}^+ \text{CD28}^- \) ratio remained constant from the initial increase up to the end of follow-up (Figure 5).

The percentage of CD28⁺ T lymphocytes increased in both populations of lymphocytes in HD patients treated with rHuEpo, but more visibly in the CD8⁺ T cells.

**Changes in HD patients not treated with rHuEpo**

In the HD patients who were not being treated with rHuEpo, all studied parameters throughout the

![Fig. 3. The intensity of apoptosis in CD4⁺ or CD8⁺ T lymphocytes measured as the percentage of annexin V–FITC-positive cells. Two approaches were applied in the analysis: the first was based on the lymphocyte gate containing all lymphocytes and generated using an FSC/SSC dot plot (A, both graphs below the dot plot were generated using the R1 region). The second included only the CD4⁺ or CD8⁺ T lymphocytes; this was achieved by gating them electronically using the dot plots of anti-CD4 or anti-CD8 vs SSC (B, both graphs below the dot plot were generated using the R2 region). The percentages of CD4⁺ annexin V⁺ or CD8⁺ annexin V⁺ T cells were measured immediately \textit{ex vivo} and in the cultures of PBMCs stimulated with PHA, camptothecin, their mixture or with different concentrations of rHuEpo. The figures present the mean ± SD [CD8⁺; MANOVA\(_{\text{HD patients - course of the treatment}}\) \( R_{\text{rao}} = 2.4 \) \( P = 0.01 \), significant LSD\(_{\text{before/6 months}}\) \( P = 0.004 \) LSD\(_{\text{before/1 year}}\) \( P = 0.001 \); other differences insignificant; MANOVA\(_{\text{healthy/HD patients}}\) \( R_{\text{rao}} = 13.4 \) \( P = 0.004 \); CD4⁺; MANOVA\(_{\text{HD patients - course of the treatment}}\) all differences insignificant; LSD\(_{\text{non-stimulated/PHA + camptothecin}}\) 6 months \( P = 0.11 \), 1 year \( P = 0.03 \), MANOVA\(_{\text{healthy/HD patients}}\) \( R_{\text{rao}} = 2.13 \) \( P = 1.3 \times 10^{-4} \)).
Fig. 4. The percentage of CD4\(^+\) or CD8\(^+\) T cells expressing Fas receptor, TNFRI and TNFRII. The approach applied in the analysis was to include only the CD4\(^+\) or CD8\(^+\) T lymphocytes (upper dot plot). The percentages of CD4\(^+\) or CD8\(^+\) T cells expressing Fas receptor, TNFRI and TNFRII were measured immediately \textit{ex vivo} and in the cultures of PBMCs stimulated with PHA or with different concentrations of rHuEpo. The figures present the mean±SD [CD8\(^+\) Fas\(^+\): MANOVA healthy/HD patients, \(R_{\text{rao}} = 15.1\ P = 0.002\); MANOVA non-stimulated/PHA, \(R_{\text{rao}} = 5.3\ P = 0.01\), other differences insignificant; CD4\(^+\) Fas\(^+\): MANOVA healthy/HD patients, insignificant; MANOVA non-stimulated/PHA, \(R_{\text{rao}} = 3.5\ P = 0.02\), other differences insignificant; CD8\(^+\) TNFRI\(^+\): MANOVA healthy/HD patients before therapy, \(R_{\text{rao}} = 9.3\ P = 0.006\), MANOVA healthy/HD patients 1 year of the therapy, \(R_{\text{rao}} = 0.33\ P = 0.47\); MANOVA non-stimulated/PHA, \(R_{\text{rao}} = 6.3\ P = 0.002\), MANOVA non-stimulated ex vivo, \(R_{\text{rao}} = 4.8\ P = 0.009\), other differences insignificant; CD4\(^+\) TNFRII\(^+\): MANOVA healthy/HD patients, insignificant; MANOVA non-stimulated/PHA, \(R_{\text{rao}} = 7.8\ P = 0.001\), MANOVA non-stimulated ex vivo, \(R_{\text{rao}} = 5.5\ P = 0.004\), other differences insignificant; CD8\(^+\) TNFRII\(^+\): all differences insignificant; CD4\(^+\) TNFRII\(^+\): MANOVA non-stimulated/PHA, \(R_{\text{rao}} = 10.3\ P = 0.003\), MANOVA non-stimulated ex vivo, \(R_{\text{rao}} = 7.2\ P = 0.008\), other differences insignificant].
follow-up were equal to those noted at baseline in HD patients treated with rHuEpo; therefore, we decided not to present those results in the figures.

**Discussion**

In this study, we found that the long-term treatment of HD patients with rHuEpo correlated with an increase in the intensity of apoptosis in CD8\(^+\) T lymphocytes, but it exerted hardly any visible effect on CD4\(^+\) T lymphocytes. The apoptotic activity of these kinds of cells seemed to be related to changes in the surface expression of TNFRs. In addition, both examined populations exhibited rapid increases in the surface expression of TNFRs. In addition, both examined populations exhibited rapid increases in the proportion of CD28\(^+\) T cells.

Our current results are in strict accord with those described in the report of our previous study [3], in which we found that long-term treatment with rHuEpo decreased the percentage of CD8\(^+\) T lymphocytes, mainly CD8\(^+\)CD152\(^+\) T cells, exerting no effect on the percentage of CD4\(^+\) T cells. It is known that the surface expression of the CD152 receptor is correlated with the death of activated CD152\(^+\) cells [4]; so, a decrease in the percentage of CD8\(^+\)CD152\(^+\) T cells during rHuEpo treatment was concordant with the increasing ability that we found in our current study of CD8\(^+\) T cells to undergo apoptosis during rHuEpo therapy.

The disturbed capacity of CD8\(^+\) T lymphocytes to undergo apoptosis might be connected with the HD-induced chronic proinflammatory activity state in HD patients; high levels of some proinflammatory cytokines, an increased activity of monocytes and the high level of expression of proinflammatory receptors [2,3,9]. In the current study, the higher level of markers of such activation, such as Fas and TNFRs, on T lymphocytes from peripheral blood (i.e., cells bathed in serum), compared with T lymphocytes from non-stimulated cultures (cells bathed in culture media), confirmed the stimulatory conditions existing *in vivo* in HD patients. The increased expression of the Fas receptor on lymphocytes during the immune response is a commonly known phenomenon; the Fas-dependent process is a major pathway of clearing lymphocytes after they have successfully finished eradication of the pathogen. However, in the case of HD patients, repetitive HD induces an ‘artificial’ proinflammatory state due to the contact of leukocytes with dialysers membranes and exposure to the components of dialysis fluids [13]. This status mimics some of the changes present during an actual chronic infection, thus elevating, also chronically, Fas expression on lymphocytes.

According to the current view, a long, moderate stimulation seems to be unprofitable for acquired immunity, causing a so-called ‘immune attrition’. Briefly, if the amount of proinflammatory stimuli exceeds a given threshold, immune responsiveness is greatly reduced [14]. Although Fas-mediated apoptosis is a major pathway of apoptosis in activated lymphocytes, our finding of the continuously high expression of the Fas molecule did not elucidate the mechanism behind the increased sensitivity of CD8\(^+\) T cells to apoptosis during rHuEpo therapy. The other important process by which activated lymphocytes may undergo apoptosis is regulated via TNFRs, mainly TNFRI [15]. As in the CD8\(^+\)Fas\(^+\) T cells, high levels of CD8\(^+\)TNFRI\(^+\) T cells may be interpreted to indicate the increased activity of CD8\(^+\) T cells in HD patients as well as the upregulation of the TNFRI receptor, related to a low ability of CD8\(^+\) T cells to undergo apoptosis. In light of these facts, the administration of rHuEpo may be considered to have been a trigger, which allowed CD8\(^+\)TNFRI\(^+\) lymphocytes to be gradually eliminated via apoptosis, which was visible *in vivo* throughout the follow-up.

On the other hand, the number of CD4\(^+\) T cells remained stable during rHuEpo treatment. *In vitro* studies shed more light on this ‘unresponsiveness’ of CD4\(^+\) T cells. We found that the levels of CD4\(^+\)TNFRII\(^+\) cells were higher in non-stimulated cultures compared with the stimulated ones. Bearing in mind that TNFRII is a ‘decoy’ that traps TNF-\(\alpha\) [15], a decline in the percentage of CD4\(^+\)TNFRII\(^+\) T cells after the stimulation might have been related to their enhanced cross-linking with TNF-\(\alpha\). The CD4\(^+\) T cells might have bound preferentially to TNF-\(\alpha\) via TNFRII, avoiding cross-linking of TNF-\(\alpha\) with TNFRI, thus protecting themselves from apoptosis.

The annexin V assay allows the assessment of apoptosis present as a control/stimulated amplitude and the mobility of phospholipids in the plasma.
Apoptosis of CD8+ T cells in HD patients treated with rHuEpo

membrane. All samples from all individuals were prepared in the same way; therefore, it may be stated that spinning, staining or fixation affected all samples in the same ways. The results indicate that the plasticity of the plasma membranes of lymphocytes from HD patients was lower than of those from healthy subjects. Such a change in the structure of plasma membranes in HD patients, thought to be caused by membrane lipid peroxidation, has already been described in the case of erythrocytes, and accounts for their impaired function [16]; however, it may be of more consequence in the case of lymphocytes, which live longer than erythrocytes or other leukocytes. Accelerated senescence, resistance to apoptosis and, as a result, impaired immunity may be among the most important consequences of changes in the structure of plasma membranes.

Taking into account that the lymphocytes from HD patients may share some features with senescent lymphocytes [17], the increasing intensity of apoptosis in them seems to be a sign of the positive rearrangement of this cell compartment. In our opinion, resistance to apoptosis along with the increased expression of proinflammatory markers noted in HD patients, are the features of anergic CD8+ T clones, also active during human senescence and characterized by a low intensity of apoptosis [18]. In the elderly, such clones are generated as a result of chronic proinflammatory states, and, as was already mentioned, a similar state is present in HD patients. In addition, unspecific stimulation with proinflammatory cytokines, whose levels are increased in HD patients [3], keeps the activity of such clones persistently elevated [19]. Elimination of those residual clones, which are believed to be a source of immune disturbances, is the prerequisite to replacing them with new, specific and fully active effector CD8+ T cells that maintain a proper cellular response [18,19]. The claim that the immune system in HD patients is affected by immunosenescence seems to be confirmed by the low percentage of CD28+ T cells found in HD patients before the administration of rHuEpo. This state is a commonly recognized feature of aged anergic immune cells [18,19]. It is of note that the administration of rHuEpo increased the proportion of CD28+ T cells in peripheral blood. It is also important that the increasing percentage of CD28+ T cells was noted mainly among CD8+ T cells, in which it preceded the decrease of their absolute numbers. Such a positive rearrangement of the CD8+ T compartment is proof of the rejuvenating influence of rHuEpo on lymphocytes, as CD8+CD28+ T cells are thought to be ‘young’, mainly naive cells that can appear in the periphery only when expanded anergic CD8+CD28− T clones are eliminated. This rearrangement is also in agreement with the findings of our previous study, in which we reported an improvement in cellular immunity during rHuEpo treatment [3]. On the other hand, the barely perceptible increase in the percentage of CD4+CD28+ T cells might be insufficient to bring about a similar healthy rearrangement in the CD4+ T compartment.

Nevertheless, the question of how rHuEpo supplementation may induce the above-described effects is still unanswered, because in vitro cultures with rHuEpo revealed no or only a few direct effects. Being a multipotent agent, rHuEpo may affect the above changes indirectly, through other tissues, for example [10–12]. The improvement in the general health of HD patients that is dependent on the recovery from anaemia (treated with rHuEpo) is also known to be one of the factors contributing to a better immune response [20].

In summary, we demonstrated that the depletion of CD8+ T lymphocytes during long-term treatment with rHuEpo occurred in an apoptotic manner. The mechanisms involved in this process might have been mediated through the TNFRI; they were independent of the Fas molecule. That phenomenon might be related to the rHuEpo-dependent increase in the proportion of CD8+CD28− T cells in the periphery, which preceded changes in their susceptibility to apoptosis. Further studies are required to explain the role, which seems to be indirect, of rHuEpo in these phenomena.

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