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Impact of anemia treatment with methoxy polyethylene glycol-epoetin beta on polymorphonuclear cells apoptosis in predialysis patients with chronic kidney disease

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ABSTRACT

Background: Some data in literature indicate increased apoptosis of polymorphonuclear cells (PMNs) in chronic kidney disease (CKD), what seems to be connected with anemia. Erythropoiesis-stimulating agents, used in anemia treatment in CKD may affect cells apoptosis. Aim of this study was to investigate impact of anemia treatment with methoxy polyethylene glycol-epoetin beta (CERA) on PMNs apoptosis in predialysis patients with CKD.

Methods: Percentage of early and late apoptotic PMNs was measured by flow cytometry based on annexin V and propidium iodide binding. CD90 (Fas), CD95L (FasL), CD16 and CD11b expression on PMNs were evaluated by flow cytometry after incubation with respective monoclonal antibody.

Results: Percentage of PMNs in early and late apoptosis in CKD patients before CERA treatment was significantly higher to control group, which was accompanied by significantly higher Fas and Fas-L expression and significantly lower expression of CD16. CERA treatment downregulated significantly percentage of early, apoptotic PMNs but percentage of late apoptotic cells did not change and was still significantly higher to control group. In all investigated groups we observed a significant negative correlation between hemoglobin concentration and percentage of apoptotic PMNs, as well as Fas and FasL expression and significant positive correlation between Hb and CD16 expression.

Conclusions: Our results indicate that PMNs apoptosis is increased in predialysis patients with CKD and anemia treatment with CERA may diminish readiness of PMNs to undergo apoptosis. This antiapoptotic impact of anemia treatment with CERA seems to concern early apoptotic PMNs before they undergo to late, irreversible stage of apoptosis.

Introduction

Apoptosis, programed cell death, plays an important role in living organisms [1]. A review of available literature regarding chronic kidney disease (CKD) patients indicates increased apoptosis in various cells including immune cells [2,3]. Increased polymorphonuclear cells (PMNs) apoptosis, quantified by flow cytometry, is well documented in CKD patients with uremia under dialysis treatment [4]. Uremia is associated with immune system dysfunction, dysregulation of homeostasis, what may be directly related to abnormal apoptosis regulation [5]. Besides uremic toxins two main mechanisms seem to be responsible for increased apoptosis in these patients. The first one (extrinsic apoptosis pathway) could be activated Fas-Fas ligand (Fas-FasL) system in accordance with CKD progression [6]. The second mechanism (intrinsic apoptosis pathway) seems to be hypoxia [7] due to anemia, which is commonly present in patients with CKD and is caused by erythropoietin deficiency [8]. Anemia causes deterioration of quality of life and is strongly connected with cardiovascular complications in these patients with worse outcomes [9,10]. Erythropoiesis-stimulating agents (ESAs), commonly used in anemia treatment in CKD patients, besides improvement of patients'

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outcomes and their quality of life, seem to have nephroprotective effects and slow progression of CKD [11]. It may have clinical importance, because could delay the start of dialysis treatment, what is very important for predialysis patients with CKD [12]. Most data in literature about nephroprotective effects of anemia treatment concern short half-life ESAs and indicate on antiapoptotic, antioxidant, anti-inflammatory and antifibrotic effects [13]. Darbepoetin and especially methoxy polyethylene glycol-epoetin beta (continuous erythropoietin receptor activator – CERA), as a long half-life ESAs seem to be the best choice to achieve the long nephroprotective effect in predialysis patients with CKD, including antiapoptotic effect [14,15]. The lack of data in the available literature concerning PMNs apoptosis in predialysis patients with CKD and impact of anemia treatment with CERA on PMNs apoptosis and expression of some surface antigens was the reason for conducting this study.

Subjects and methods

Patient population

Thirty five predialysis patients (20 men and 15 women) with anemia and CKD in stage IV and V treated with methoxy polyethylene glycol-epoetin beta (CERA) (Mircera, Roche, Basel, Switzerland) were enrolled in the study. Relatively small sample size, depend on many excluding criteria, was satisfactory to give statistical power (70–100%). Causes of CKD were: primary hypertension with chronic kidney disease (40%), chronic tubule-interstitial nephritis (23%), chronic glomerulonephritis (20%) and polycystic kidney disease (17%). The control group included 20 volunteers (12 men and 8 women) without CKD (eGFR $>$ 60 ml/min, normal hemoglobin level, normal urine analysis and kidney ultrasound).

CKD patients with hemoglobin (Hb) level $<$10 g/dl and eGFR $<$30 ml/min, after excluding bleeding, iron deficiency, hemolysis, infection and severe secondary hyperparathyroidism, received a subcutaneous injection of Mircera in doses of 0.6 μg/kg once monthly. Treatment was continued until reaching the target Hb level 11–12 g/dl, achieved by 30 patients, who was enrolled in the second part of the study. Average treatment time was 227 days (from 108 to 428 days) average Mircera dose was 50 μg/monthly (from 30 to 75 μg). Patients with diabetes, blood transfusion in the past 3 months, acute infection, chronic infection (hepatitis B and C), autoimmune disease, immunosuppressive therapy, with increased C reactive protein (CRP) over 25 mg/l or history of malignancy were excluded from the study. All patients with CKD received ACE inhibitors and statins, they did not receive any vitamins. All investigated subjects have not been smoking at least 5 years. More data of patients who achieved target Hb and the control group are presented in Table 1.

The study was approved by the Ethics Committee of Research of the Medical University of Lodz – number RNN/97/09/KB. Only patients who signed informed consent were included in the study.

Table 1
Characteristics of Hb, creatinine and eGFR in examined subjects.

	Patients before treatment (n = 30) (Me; 25–75%)	Patients after treatment (n = 30) (Me; 25–75%)	Control group (n = 20) (Me; 25–75%)
Hb (g/dl)	9.6 (9.1–10.0)	11.6 (11.1–12.0)	14.4 (13.3–14.9)
Creatinine (μmol/l)	300 (249–395)	298 (239–396)	90 (77–108)
eGFR (MDRD) (ml/min/1.73 m^2)	16.4 (13.9–21.8)	16.6 (14.0–23.3)	65 (63–74)
CRP (mg/l)	18 (14.1–22.8)	16 (12.2–20.4)	4.5 (3.7–6.9)

PMN cells preparation

PMNs were isolated from heparinized peripheral blood drawn from CKD patients two times, before treatment with Mircera and after achieving target Hb. Hb level along with the other routine laboratory tests was evaluated monthly. From healthy volunteers peripheral blood was drawn once. PMNs were separated using density gradient centrifugation on Polymorphprep (Axis-Shield PoC As, Norway) at 450 × g at room temperature for 30 min. The lower phase, containing mainly PMNs and residual erythrocytes, was treated with hypotonic erythrocyte lysis solution. The PMNs were washed twice and resuspended in PBS. Cell purity and viability were determined by May–Grünwald–Giemsa staining and trypan blue exclusion test, respectively. Experiments were conducted on populations of PMNs whose purity and viability were not lower than 98%.

Measurement of PMNs apoptosis and expression of surface antigens by flow cytometry

The percentage of apoptotic PMNs was measured based on annexin V protein and propidium iodide (Sigma Chemical Co., St. Louis, MO, USA) according to the manufacturer's specifications (Annexin V/FITC Kit, Bender MedSystems Diagnostics GmbH, Vienna, Austria). A 5 μg aliquot of annexin V-FITC and 10 μg of propidium iodide (final concentration 1 μg/ml) were added to each cell suspension (10^6/200 μl); then they were incubated for 25 min in the dark at room temperature. Apoptotic PMNs were evaluated by flow cytometry (FACScan analyzer; Becton Dickinson; San Diego, CA, USA). Cells staining for annexin V-FITC positive were considered as early apoptotic ANX$^+$ and propidium iodide positive cells were recognized as late apoptotic cells PI$^+$ [16]. The expression of CD90 (Fas) (Ancell Corporation, Bayport, MN, USA), CD95L (Fas-L) (Ancell Corporation, Bayport, MN, USA), CD 16 and CD11b (BD Biosciences, Becton Dickinson, San Diego, CA, USA) was assessed after incubation of cells with respective mouse anti-human monoclonal antibody according to the manufacturer's specifications. Staining of the cells with monoclonal antibodies was performed in the dark. An appropriate isotype control was prepared in each experiment. Median fluorescence intensity (MFI) of the positively stained cells was detected by flow cytometry (FACScan flow cytometer, Becton Dickinson, San Diego, CA, USA).

Statistical analysis

Data are presented as the median and interquartile range (Me; 25–75%). Evaluation of the statistical significance was performed by Wilcoxon signed ranks test for paired data and by Mann–Whitney U test for unpaired data. Correlation between Hb and investigated apoptosis parameters was evaluated by calculation of Kendall's tau coefficient values. Statistical significance was assumed at a p value $<$0.05. It was used Statistica 9.0 (StatSoft, Poland) as statistical package.

Results

The results concerning patients with CKD before treatment and the control group are shown in Table 2.

The percentage of PMNs in early ANX$^+$ and late PI$^+$ stages of apoptosis was significantly higher in CKD patients. Fas and FasL expression were significantly higher in patients with CKD, but expression of CD16 was significantly lower in CKD patients in comparison to the control group.

The results concerning patients with CKD after CERA treatment and the control group are shown in Table 3.