Circulating cell-free DNA levels in haemodialysis patients and its association with inflammation, iron metabolism and rhEPO doses

Michaela Kohlova[1,2], Sandra Ribeiro[1,3], Maria do Sameiro-Faria[4,5], Petronila Rocha-Pereira[3,6], João Fernandes[2,3], Flávio Reis[2], Vasco Miranda[5], Alexandre Quintanilha[3,4], Luís Belo[1,3], Elsa Bronze-Rocha[1,3], Elísio Costa[1,3], Alice Santos-Silva[1,3]

1- Departamento de Ciências Biológicas, Laboratório de Bioquímica, Faculdade de Farmácia, Universidade do Porto, Porto; 2- IBILI, Faculdade de Medicina, Universidade de Coimbra, Coimbra; 3-Instituto de Biologia Molecular e Celular (IBMC), Universidade do Porto, Porto; 4- Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto; 5- Fresenius Medical Center, Dinefro – Diálises e Nefrologia, SA; Porto; 6- Centro Investigação Ciências Saúde, Universidade Beira Interior, Covilhã

INTRODUCTION/AIMS

Inflammation is a common feature in end-stage renal disease (ESRD) patients under haemodialysis (HD), which is enhanced in patients who develop resistance to recombinant human erythropoietin (rhEPO) therapy. The mechanisms/factors triggering the inflammatory process are still poorly clarified. It has been demonstrated, recently, a release of cell-free DNA (cfDNA) during dialysis procedure, which has been related with cellular necrosis and apoptosis, similarly to that found in other clinical situations.

The aim of this work was to evaluate the circulating cfDNA levels in ESRD patients and to search for relationships among haematological and inflammatory disturbances, as well as with iron metabolism, nutritional status and dialysis adequacy.

METHODS

This transversal study included 153 ESRD patients under HD (89 males and 64 females, mean [± SD] age: 65.7 [14.4] years). Patients were under therapeutic HD three times per week for the duration of 3-5 hours, for a median time of 2.13 (0.82-5.24) years. For the HD procedure, high-flux polysulfone FX-class dialyzer of Fresenius (Bad Hamburg, Germany) was used. The main causes of renal failure in these patients were as follows: diabetic nephropathy (n=55), hypertensive nephrosclerosis (n=20), nephritic syndrome (n=13), other diseases (n=19) and of uncertain aetiology (n=46). Patients with autoimmune diseases, malignancy, and acute or chronic infection, were excluded. All participants gave their written informed consent to participate in this study that was previously approved by the Ethics Committee from the clinic of dialysis. A group of healthy volunteers (n=20) with normal haematological and biochemical values, with no history of renal or inflammatory disease, and, as far as possible, age and gender matched with our patients, was also included.

Blood was collected immediately before the second dialysis session of the week. Hematological data were accessed by using an automatic blood cell counter (Sysmex K1000; Sysmex, Germany). Differential leukocyte and reticulocyte counts were performed by microscopy. Serum iron concentration was determined using a colorimetric method (Iron, Randox Laboratories Ltd., North Ireland, UK), whereas serum ferritin and transferrin were measured by immunoturbidimetry (Ferritin, Laboratories Ltd., North Ireland, UK; Transferrin, Laboratories Ltd., North Ireland, UK). Enzyme-linked immunosorbent assays were used to measure soluble transferrin receptor (sTfR; human sTfR immunoassay, R&D Systems, Minneapolis, USA). Plasma levels of hepcidin-25 were quantified using a peptide enzyme immunoassay (Bachem Group, Peninsula Laboratories, LLC, California). Serum C-reactive protein (CRP) was determined by nephelometry [CRP (latex) High-Sensitivity, Roche Diagnostics]; serum interleukin (IL)-6 was evaluated by enzyme immunoassays (Human IL-6 High Sensitivity ELISA, eBioscience, Austria). The circulating cfDNA levels were assessed directly in serum samples, as recently described [6]. Briefly, SYBR® Gold Nucleic Acid Gel Stain (Invitrogen, Paisley, UK) was diluted in dimethyl sulfoxide and phosphate buffer; 10 µL of the serum sample were mixed 40 µL of SYBR® Gold solution. Fluorescence was measured with a 96-well fluorometer, at emission wavelength of 535 nm and excitation wavelength of 485 nm.

Clinical and haematological data, iron metabolism, inflammatory and nutritional markers for ESRD patients and controls, as well as circulating cfDNA serum levels, are shown in Table 1.

Table 1. Clinical and hematological data, iron metabolism, inflammatory and nutritional markers, and circulating cfDNA serum levels for ESRD patients and controls.

	Controls (n=20)	HD patients (n=153)
Clinical data		
Diabetic patients, n (%)	-	52 (34)
Hypertensive patients, n (%)	-	93 (60.8)
Previous time on dialysis, years	-	2.1 (0.8-5.1)
Darbopoietin alpha, µg/Kg/week	-	0.4 (0.2-0.7)
Dialysis adequacy		
Kt/Ve	-	1.5±0.2
URR, %	-	75.8±6.4
Creatinine, mg/dL	-	8.4±2.9
Haematological data		
Hemoglobin, g/dL	14.8±0.9	11.7±1.5*
Hematocrit, %	43.9±2.8	36.2±4.7*
Erythrocytes, x1012 /L	4.8±0.4	3.8±0.5*
MCV, fl	88.6±13.1	95.8±6.1*
MCH, pg	31.0±1.4	31.1±2.4
MCHC, g/dL	33.9±0.4	32.3±1.2*
RDW, %	15.1±0.6	14.9±1.7
Reticulocytes, x109/L	55.8 (32.8-77.7)	49.3 (27.4-72.8)
RPI	1.2 (0.6-1.6)	0.9 (0.5-1.4)
Platelets, x109/L	261.8±59.1	178.5±50.6*
White blood cells, x109/L	6.8±1.5	6.4±2.0
Neutrophils, x109/L	4.3±1.3	3.9±1.5
Lymphocytes, x109/L	1.9±0.4	1.7±0.7*
Neutrophil/Lymphocyte ratio	2.2 (1.8-2.9)	2.3 (1.8-3.3)
Iron metabolism		
Iron, µg/dL	56.0 (43.3-70.8)	39.0 (30.0-54.0) *
Transferrin, mg/dL	307.5 (285.3-333.0)	181.0 (160.5-204.0) *
Transferin saturation, %	12.4 (8.4-15.8)	15.1 (11.7-20.3) *
sTfR, nmol/L	12.8 (11.1-15.3)	20.2 (14.7-27.0) *
Ferritin, ng/mL	58.9 (47.6-151.9)	439.2 (286.0-521.7) *
Hepcidin-25, ng/mL	178.4 (113.2-318.6)	1573.6 (804.6-2454.4) *
Inflammatory markers		
CRP, mg/dL	0.78 (0.43-1.47)	4.9 (2.3-13.0) *
IL-6, pg/mL	0.40 (0.27-0.59)	2.3 (1.3-4.3) *
Nutritional markers		
Albumin, g/dL	-	3.9±0.4
BMI, Kg/m2	21.7 ± 2.2	25.9±4.6*
Circulating cell-free DNA		
Circulating cell-free DNA, ng/mL	763.2 (612.0-935.6)	1178.2 (932.2-1350.4) *

For statistical analysis was used Statistical Package for Social Science version 17.0. Parameters are expressed as mean ± SD or as median values (inter quartile range) when appropriate.

*To compare data from patients on HD and after change to ON-HDF was used Wilcoxon test. Significance was accepted at $P < 0.05$. MCV: mean cell volume; MCH: mean cell hemoglobin; MCHC: mean cell hemoglobin concentration; RDW: red cell distribution width; RPI: reticulocyte production index; sTfR: soluble transferrin receptors; CRP: C-reactive protein, rhEPO: recombinant human erythropoietin; URR: urea reduction ratio

RESULTS

We found that HD patients presented, when compared with the control group, a significant decrease in haemoglobin concentration, haematocrit, erythrocyte and lymphocyte counts, and in mean cell haemoglobin concentration, and an increase in mean cell volume; significant changes in iron metabolism (a decrease in iron and transferrin, and an increase in transferrin saturation, sTfR, ferritin and hepcidin serum levels) and in inflammatory markers (increase in CRP and IL-6). Moreover, patients showed an increase in the circulating cfDNA levels, when compared with controls. Significant correlations were found between circulating cfDNA levels and IL-6 (r=0.333; p<0.001), CRP (r=0.418; p<0.001), iron (r=-0.237; p=0.002), transferrin (r=-0.545; p<0.001), sTfR (r=0.357; p<0.001) and rhEPO doses (r=0.284; p<0.001). Moreover, multiple regression analysis identified the serum levels of CRP (β=0.372; p<0.0001) as an independent variable associated with cfDNA serum levels.

CONCLUSIONS

Increased levels of cfDNA have been documented in blood under several healthy and pathological conditions, namely, age-associated inflammation and frailty, exhaustive exercise, cancer, lupus erythematous and in patients under HD [4,7,8]. Our data confirm that HD patients present high circulating cfDNA levels, which seems to be correlated with the inflammatory grade. It has been reported that cfDNA is able to selectively induce IL-6 production by monocytes [9], which could justify, at least in part, the enhanced inflammatory process found in HD patients and the subsequent disturbances in iron metabolism, and in the response to rhEPO therapy. It has been reported that there is an increase in cellular necrosis and apoptosis during HD procedure, which could be related with the leaking of DNA fragments from the nuclei of the leukocytes . Further studies are, therefore, required to understand, if the leucocytes or the body cells present the source of cfDNA.However, it is clear that cfDNA by contributing to enhance the inflammatory process could be an important player in the pathogenesis of ESRD.

REFERENCES

Costa E,et al. *Am J Nephrol* 2008;28:677-683
Costa E, et al. *J Clin Immunol* 2008;28:268-275
Jofre R, et al. *J Am Soc Nephrol* 2006;17(3):274-280
Junk K, et al *Clin Chim Acta* 2010;411:1611-1624
Tovbin D, et al. *Nephrol Dial Transplant* 2012;27:3929-3935
Goldshtein H, et al. *Ann Clin Biochem* 2009;46:488-94
Atamaniuk J, et al. *Clin Chem* 2004;50:1668-70
Jyhava J, et al. *Aging Cell* 2013, *in press*
Atamaniuk J, et al.. *Nephol Dial Transplant* 2012;27:902-905
Atamaniuk J, et al. *Clin Chem* 2006;52:523-26