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VASCULAR WALL AND ENDOTHELIUM

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LECTURES

POSTERS

ALTERED ERYTHROCYTE MEMBRANE BAND 3 PROFILE IN CHRONIC RENAL FAILURE PATIENTS UNDER HAEMODIALYSIS

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ABSTRACT

Our aim was to study changes in RBC membrane band 3 profile, as a cumulative marker of RBC changes, in chronic renal failure (CRF) patients under haemodialysis and recombinant human erythropoietin (rhEPO) therapy and its linkage with resistance to this therapy.

We studied 63 CRF patients, 32 responders and 31 non-responders to rhEPO therapy, and 26 healthy individuals matched for age and gender. We evaluated the band 3 profile and membrane-bound haemoglobin (MBH). Total serum bilirrubin, glutathione peroxidase (GPx) and superoxide dismutase activities, RBC count, haematocrit, haemoglobin concentration, haematimetric indices and reticulocyte were also evaluated. CRF patients presented anaemia, slightly regenerative, as showed by the decreased RBC count, Hb and haematocrit, alongside with an increased reticulocyte count, RPI and RDW values. CRF patients showed a statistically significant decrease in high molecular weight aggregates and proteolytic fragments (Pfrag), and a rise in Band 3 monomer. A rise in GPx and a trend to lower values of MBH were also found in CRF patients. A positive correlation was found between Pfrag and, Hb and haematocrit. When comparing the haematological data between the two groups of CRF patients,

we found that non-responders patients were more anaemic, and presented a statistically significant decrease in Pfrag, and a trend for a rise in MBH, suggesting a higher RBC damage.

Our data suggest that band 3 profile seems to be a good marker of erythrocyte changes in CRF patients. These changes seem to be associated with a younger RBC population, but also with a rise in RBC damage, which is enhanced in non-responders CRF patients. Band 3 profile could be used as a marker of RBC changes in these patients and in the understanding of the mechanism of resistance to rhEPO therapy.

Key-Words: Chronic renal failure, Band 3, rhEPO, Erythropoietin.

INTRODUCTION

Band 3 protein is the major integral protein of the red blood cell (RBC) membrane. It is known as the senescent neoantigen, as modifications in band 3 protein, by proteolytic cleavage, clustering or exposure of unusual epitopes, trigger the binding of specific anti-band 3 autoantibodies and complement activation, marking RBC for death. An abnormal band 3 profile [an increase in high molecular weight aggregates (HMWAg) and a decrease in band 3 monomer and proteolytic fragments (Pfrag)], has been associated with RBC damage/aging in inflammatory conditions associated with oxidative stress, namely in cardiovascular disease, pregnancy and acute physical exercise[1-3].

Chronic renal failure (CRF) has also been associated with both inflammation and oxidative stress. A deficient renal erythropoietin secretion underlies the development of an anaemia, which is usually corrected by therapy with recombinant human erythropoietin (rhEPO). However, about 25% of the patients do not respond to this therapy[4].

Our aim was to study the erythrocyte membrane band 3 profile, as a cumulative marker of RBC changes, in CRF patients under haemodialysis and rhEPO therapy.

MATERIALS AND METHODS

SUBJECTS AND SAMPLES

We studied 89 individuals including 63 CRF patients, 32 responders and 31 non-responders to rhEPO therapy, and 26 healthy controls. The rhEPO maintenance dose for responder's patients was 8.03 ± 5.97 U/Kg/week/Hb and for non-responders was 56.70 ± 22.40 U/Kg/week/Hb. The two groups of patients were matched for age, gender, weight, body mass index, mean time under haemodialysis, urea reduction ratio, urea Ktv and parathyroid hormone serum levels. No laboratory indicators of iron deficiency and/or vitamin B12 and folate deficiencies were found in CRF patients

Peripheral blood samples were collected into EDTA containing tubes.

The causes of renal failure in patient's population were as follows: diabetic nephropathy (n=19), chronic glomerulonephritis (n=10), polycystic kidney disease (n=3), hypertensive nephrosclerosis (n=3), obstructive nephropathy (n=3), pyelonephritis associated with neurogenic bladder (n=1), nephrolithiasis (n=1), chronic interstitial nephritis (n=1), Alport syndrome (n=1), renal vascular disease due to polyarteritis (n=1) and chronic renal failure of uncertain aetiology (n=17).

Patients with autoimmune disease, malignancy, haematological disorders, and acute or chronic infection were excluded. All patients gave their informed consent to participate in this study. Classification of CRF patients in responders or non-responders, was performed in accordance with the European Best Practice Guidelines (5), that defines resistance to rhEPO as a failure to achieve target haemoglobin levels with doses of epoetin more than 300 IU/Kg/week or 1,5 mg/Kg/week of darbopoietin-alfa.

Age and gender-matched individuals, with normal haematological and biochemical values, without any history of renal or inflammatory disease, were used as controls.

In all individuals (patients and controls), we evaluated RBC count, haematocrit, haemoglobin concentration (Hb), haematimetric indices, red cell distribution width (RDW) (by using a blood cell counter); reticulocyte count (brilliant cresyl blue staining), reticulocyte production index (RPI); membrane bound haemoglobin (MBH) (by spectrophotometry)[1], total serum bilirubin levels, glutathione peroxidase (GPx) (RANSEL, Randox, UK) and superoxide dismutase (SOD) activities (RANSOD, Randox, UK); band 3 profile [% of band 3 monomer, high molecular weight aggregates (HMWAg) and proteolytic fragments (Pfrag)].

Band 3 profile

RBC membranes were treated with an equal volume of a solubilisation buffer containing 0.125M Tris HCl pH 6.8, 4% sodium dodecil sulfate (SDS), 20% glycerol, and 10% 2-mercaptoethanol, heat-denatured and submitted to polyacrylamide gel electrophoresis (SDS--PAGE), using the discontinuous Laemmli system. Membrane proteins were electrophoretically transferred from SDS gels to a nitrocellulose sheet. Additional reactive sites on the nitrocellulose were blocked by incubation in 5% low fat dry milk and 0.1% Triton-X 100 in PBS (phosphate buffered saline) pH 7.0, for overnight at 4ºC and under gentle rotation. Band 3 immunoblot was performed; monoclonal antibodies anti-human band 3, produced in mouse, recognising an epitope located in the cytoplasmic pole of the band 3 molecule (Sigma), were added (dilution 1:3000) and incubated for 4 h; the washing of the nitrocellulose was followed by the addition and incubation with antimouse Ig peroxidase linked (Sigma) for 1 h (dilution 1 : 4000). The incubations were carried out at room temperature; the dilutions of the antibodies were prepared with PBS pH 7.0 containing 0.1% Triton-X 100 and 0.5% low fat dry milk (9,10). In the washes, the same buffer without low fat dry milk was used. Hydrogen peroxide and α-cloronaphtol were used to develop the immunoblot. The band 3 immunoblots were scanned (DarkroomCN UV/wl, BiocaptMW version 99, Vilbert Lourmat) and quantified by densitometry (Bio 1D++version 99, Vilbert Lourmat).

Data analysis

Statistical analyses were carried out using the *SPSS* package. Multiple comparisons between groups were performed by one-way ANOVA supplemented with Tukey´s honestly significant difference (HSD) *post hoc* test. For data not normally distributed, differences between the three groups were evaluated by the Kruskal-Wallis test; for single comparisons (two groups), the Mann--Whitney *U* test was used. Significance was accepted at p less than 0.05.

RESULTS

CRF patients presented anaemia, slightly regenerative, as showed by the decreased RBC count, Hb and haematocrit, alongside with an increased reticulocyte count, RPI and RDW values. A rise in GPx and a trend to lower values of MBH were also found in CRF patients (Table I). CRF patients showed a statistically significant decrease in HMWAg and Pfrag, and a rise in Band 3 monomer (Table I; Fig. 1). A positive correlation was found between Pfrag and Hb and haematocrit (Fig. 2). When comparing the haematological data between the two groups of CRF patients, we found that non-responders patients were more anaemic, and presented a statistically significant decrease in Pfrag, and a trend for a rise in MBH, suggesting a higher RBC damage.

Table I – Haematological and biochemical data for controls and CRF patients – responders and non-responders to rhEPO therapy.

	Controls (n=26)	All patients (n=63)	Responders (n=32)	Non-responders (n=31)
Hb (g/dL)	13.90 (13.2-15.00)	10.90 (10.30-12.30) b)	11.70 (10.83-12.68) b)	10.4 (9.00-11.30) b) c)
Haematocrit (%)	43.10 (40.10-46.70)	34.20 (30.60-37.10) b)	35.15 (32.25-38.35) b)	31.10 (27.70-35.20) b) c)
RBC ($x10^{12}$/L)	4.72 ± 0.59	3.68 ± 0.54 b)	3.76 ± 0.42 b)	3.58 ± 0.64 b)
MCV (fL)	92.00 (90.00-94.00)	93.80 (90.00-98.20) a)	95.80 (92.48-98.08) a)	92.30 (85.40-100.30)
MCH (pg)	29.83 ± 1.39	30.15 ± 3.04	31.29 ± 1.53 b)	28.97 ± 3.73 c)
MCHC (g/dL)	32.48 ± 0.58	32.03 ± 2.37	33.16 ± 1.77	30.85 ± 2.35 a)c)
RDW (%)	12.79 ± 0.52	15.92 ± 2.56 b)	14.56 ± 1.23 b)	17.32 ± 2.83 b)c)
RBC production / damage / removal				
Reticulocytes ($x10^9$ /L)	33.57 ± 22.78	61.03 ± 31.36 b)	55.12 ± 30.98 a)	67.14 ± 31.06 b)
RPI	0.42 (0.19-0.66)	0.98 (0.58-1.40) b)	1.08 (0.72-1.51) b)	0.92 (0.52-1.24) a)
Total Bilirubin (mg/dL)	0.62 ± 0.25	0.61 ± 0.24	0.61 ± 0.23	0.62 ± 0.24
MBH ($x10^{-4}$ %)	53.00 (37.75-89.75)	50.00 (28.00-82.00)	45.50 (25.25-80.75)	58.50 (30.50-100.75)
SOD (IU/g Hb)	1039.8 (737.4-1331.6)	898.6 (679.4-1454.2)	858.97 (662.4-1256.5)	1074.76 (581.6-2638.7)
GPx (IU/g Hb)	35.62 ± 8.83	45.82 ± 13.69 a)	48.73±13.46 a)	43.11±13.87
Band 3 profile				
HMWAg (%)		15.23 (13.38-19.40) a)	14.86 (11.30-20.19) a)	15.92 (14.28-18.68) a)
Band 3 monomer (%)		61.84 (56.87-64.41) b)	61.26 (56.08-65.06) a)	62.17 (58.01-64.29) b)
Pfrag (%)		22.70 ± 6.01 a)	24.01 ± 6.03	21.34 ± 5.78 a) c)

a) $p<0.05$, *vs* controls; b) $p<0.001$, *vs* controls; c) $p<0.05$ *vs* responders.

DISCUSSION

Our data suggest that band 3 profile seems to be a good marker of erythrocyte changes in CRF patients. These changes seem to be associated with a younger RBC population, but also with a rise in RBC damage, which is enhanced in non--responders CRF patients. Band 3 profile could be used as a marker of RBC changes in these patients and in the understanding of the mechanism of resistance to rhEPO therapy.

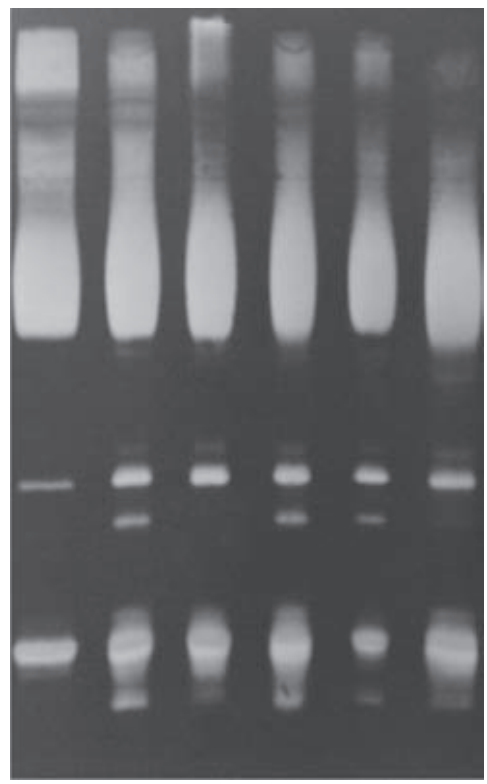

Fig. 1 – Illustration of Band 3 profiles. C1, C2 – Controls; P1 and P2 – responders CRF patients; P3, P4 – non-responders CRF patients.

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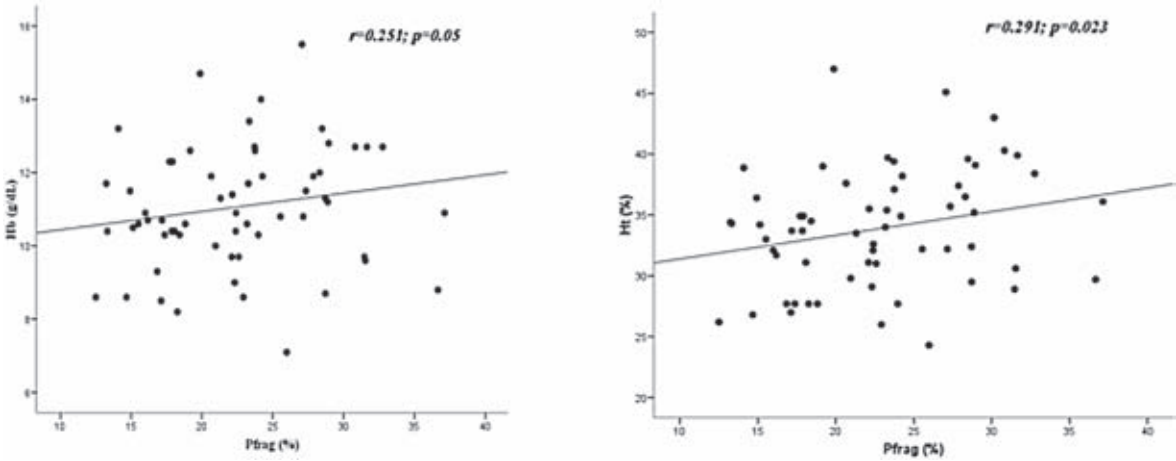

Fig. 2 – Correlation of Pfrag with Hb and haematocrit (Ht) in CRF patients.

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