Vascular Wall and Endothelium

Editores
J. Martins e Silva
Carlota Saldanha

2008
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Lisboa – Portugal

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Actas de Bioquímica
Apartado 4098
1500-001 Lisboa – Portugal

Subscription Information
Subscription price is $25.00 (twenty five US dollars) or €25,00 (twenty five euros) per volume. An additional charge of €5,00 (five US dollars) per volume is requested for post delivery outside Portugal. Payment should accompany all orders. Correspondence concerning subscription should be addressed to the mailing address above.

ISBN: 972-590-076-6
VASCULAR WALL AND ENDOTHELIUM

LISBON (PORTUGAL),
SEPTEMBER 14, 2007

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Fundação Merck Sharp e Dohme
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VASCULAR WALL AND ENDOTHELium

Proceedings of the Symposium on Vascular Wall and Endothelium
(7th Advanced Course on Applied Biochemistry)
Held in Lisbon, September 14, 2007.
Organized by the Institute of Biopatologia Química, Faculdade de Medicina, and Unidade de Biopatologia Vascular do Instituto de Medicina Molecular, Universidade de Lisboa

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Published by
Instituto de Biopatologia Química
Faculdade de Medicina
Universidade de Lisboa
Agradecimentos à Fundação Merck Sharp e Dhome pelo apoio financeiro concedido à realização e publicação dos textos do 7.º Curso Avançado de Bioquímica Aplicada.

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Printed in Portugal
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ALTED ERYTHROCYTE MEMBRANE BAND 3 PROFILE IN CHRONIC RENAL FAILURE PATIENTS UNDER HAEMODIALYSIS

Elísio Costa¹²³, Susana Rocha¹², Petronila Rocha-Pereira¹, Elisabeth Castro¹², Flávio Reis⁵, Frederico Teixeira⁴, Vasco Miranda⁶, Maria do Sameiro Faria⁶, Alfredo Loureiro⁷, Alexandre Quintanilha²⁸, Luis Belo¹², Alice Santos-Silva¹²

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⁸ Instituto Ciências Biomédicas Abel Salazar, Universidade do Porto.

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 bilirubin, 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 Pf rag, 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 comple-
ment activation, marking RBC for death. An ab-
normal band 3 profile [an increase in high mo-
lecular 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 exercise1-3.

Chronic renal failure (CRF) has also been as-
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 hu-
man erythropoietin (rhEPO). However, about 25%
of the patients do not respond to this therapy4.

Our aim was to study the erythrocyte mem-
brane band 3 profile, as a cumulative marker of
RBC changes, in CRF patients under haemodi-
alysis 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 rhE-
PO 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 hor-
mone serum levels. No laboratory indicators of
iron deficiency and/or vitamin B12 and folate de-
ficiencies were found in CRF patients
Peripheral blood samples were collected into
EDTA containing tubes.

The causes of renal failure in patient’s popula-
tion were as follows: diabetic nephropathy (n=19),
chronic glomerulonephritis (n=10), polycystic
kidney disease (n=3), hypertensive nephrosclero-
sis (n=3), obstructive nephropathy (n=3), pyelo-
nephritis associated with neurogenic bladder
(n=1), nephrolithiasis (n=1), chronic interstitial
nephritis (n=1), Alport syndrome (n=1), renal vas-
cular 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. Class-
ification 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
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), 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-chlornaphtol were used to develop the immunoblot. The band 3 immunoblots were scanned (DarkroomCN UV/wl, Bio captMW 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.
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.

ACKNOWLEDGEMENTS

We are very grateful to FMC, Dinefro – Diálises e Nefrologia, SA and Uninefro – Sociedade Prestadora de Cuidados Médicos e de Diálise, SA, and to their nurses for the technical support. This study was supported by a PhD grant (SFRH/BD/27688/2006) attributed to E. Costa by FCT and FSE.

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

<table>
<thead>
<tr>
<th></th>
<th>Controls (n=26)</th>
<th>All patients (n=63)</th>
<th>Responders (n=32)</th>
<th>Non-responders (n=31)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb (g/dL)</td>
<td>13.90 (13.2-15.00)</td>
<td>10.90 (10.30-12.30) b)</td>
<td>11.70 (10.83-12.68) b)</td>
<td>10.4 (9.00-11.30) b) c)</td>
</tr>
<tr>
<td>Haematocrit (%)</td>
<td>43.10 (40.10-46.70)</td>
<td>34.20 (30.60-37.10) b)</td>
<td>35.15 (32.25-38.35) b)</td>
<td>31.10 (27.70-35.20) b) c)</td>
</tr>
<tr>
<td>RBC (10^12/L)</td>
<td>4.72 ± 0.59</td>
<td>3.68 ± 0.54 b)</td>
<td>3.76 ± 0.42 b)</td>
<td>3.58 ± 0.64 b)</td>
</tr>
<tr>
<td>MCV (fL)</td>
<td>92.00 (90.00-94.00)</td>
<td>93.80 (90.00-98.20) a)</td>
<td>95.80 (92.48-98.08) a)</td>
<td>92.30 (85.40-100.30)</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>29.83 ± 1.39</td>
<td>30.15 ± 3.04</td>
<td>31.29 ± 1.53 b)</td>
<td>28.97 ± 3.73 c)</td>
</tr>
<tr>
<td>MCHC (g/dL)</td>
<td>32.48 ± 0.58</td>
<td>32.03 ± 2.37</td>
<td>33.16 ± 1.77</td>
<td>30.85 ± 2.35 a)c)</td>
</tr>
<tr>
<td>RDW (%)</td>
<td>12.79 ± 0.52</td>
<td>15.92 ± 2.56 b)</td>
<td>14.56 ± 1.23 b)</td>
<td>17.32 ± 2.83 b)c)</td>
</tr>
</tbody>
</table>

**RBC production / damage / removal**

<table>
<thead>
<tr>
<th></th>
<th>Controls (n=26)</th>
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<th>Responders (n=32)</th>
<th>Non-responders (n=31)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reticulocytes (10^6/L)</td>
<td>33.57 ± 22.78</td>
<td>61.03 ± 31.36 b)</td>
<td>55.12 ± 30.98 a)</td>
<td>67.14 ± 31.06 b)</td>
</tr>
<tr>
<td>RPI</td>
<td>0.42 (0.19-0.66)</td>
<td>0.98 (0.58-1.40) b)</td>
<td>1.08 (0.72-1.51) b)</td>
<td>0.92 (0.52-1.24) a)</td>
</tr>
<tr>
<td>Total Bilirubin (mg/dL)</td>
<td>0.62 ± 0.25</td>
<td>0.61 ± 0.24</td>
<td>0.61 ± 0.23</td>
<td>0.62 ± 0.24</td>
</tr>
<tr>
<td>MBH (x10^4 %)</td>
<td>53.00 (37.75-89.75)</td>
<td>50.00 (28.00-82.00)</td>
<td>45.50 (25.25-80.75)</td>
<td>58.50 (30.50-100.75)</td>
</tr>
<tr>
<td>SOD (IU/g Hb)</td>
<td>1039.8 (737.4-1331.6)</td>
<td>898.6 (679.4-1454.2)</td>
<td>858.97 (662.4-1256.5)</td>
<td>1074.76 (581.6-2638.7)</td>
</tr>
<tr>
<td>GPx (IU/g Hb)</td>
<td>35.62 ± 8.83</td>
<td>45.82 ± 13.69 a)</td>
<td>48.73±13.46 a)</td>
<td>43.11±13.87</td>
</tr>
</tbody>
</table>

**Band 3 profile**

<table>
<thead>
<tr>
<th></th>
<th>Controls (n=26)</th>
<th>All patients (n=63)</th>
<th>Responders (n=32)</th>
<th>Non-responders (n=31)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HMWAg (%)</td>
<td>15.23 (13.38-19.40) a)</td>
<td>14.86 (11.30-20.19) a)</td>
<td>15.92 (14.28-18.68) a)</td>
<td>15.92 (14.28-18.68) a)</td>
</tr>
<tr>
<td>Band 3 monomer (%)</td>
<td>61.84 (56.87-64.41) b)</td>
<td>61.26 (56.08-65.06) a)</td>
<td>62.17 (58.01-64.29) b)</td>
<td>21.34 ± 5.78 a)c)</td>
</tr>
<tr>
<td>Pfrag (%)</td>
<td>22.70 ± 6.01 a)</td>
<td>24.01 ± 6.03</td>
<td>24.01 ± 6.03</td>
<td>24.01 ± 6.03</td>
</tr>
</tbody>
</table>

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

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


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