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RESISTANCE TO RECOMBINANT HUMAN ERYTHROPOIETIN THERAPY IN HAEMODIALYSIS PATIENTS – FOCUS ON INFLAMMATORY CYTOKINES, LEUKOCYTE ACTIVATION, IRON STATUS AND ERYTHROCYTE DAMAGE

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Abstract

Anaemia is a common complication in haemodialysis patients. This condition is associated to a decreased bone marrow production of erythrocytes, mainly due to the inability of the failing kidneys to secrete erythropoietin (EPO). The introduction of recombinant human EPO (rhEPO) therapy led to a significant reduction in anaemia and improved patients' quality of life. However, there is a marked variability in the sensitivity to rhEPO, with up to 10-fold variability in dose requirements to achieve correction of anaemia. Approximately 5-10% of the patients show a marked resistance to rhEPO therapy. rhEPO resistance is associated to an increased morbidity and mortality of haemodialysis patients.
In this paper a revision of the mechanisms underlying resistance to rhEPO therapy will be performed, with particular emphasis on inflammatory cytokines, leukocyte activation, iron status, and erythrocyte damage.

1. Introduction

Anaemia is a common complication that contributes to the burden of haemodialysis (HD) patients. It has also a negative impact on cardiovascular

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system, cognitive function, exercise capacity and quality of life, resulting in a significant morbidity and mortality in these patients. The introduction of rhEPO therapy in the early 1990s for treatment of anaemia of HD patients has led to a significant reduction in anaemia and to an improvement in patients' quality of life (1-4). There is, however, a marked variability in the sensitivity to rhEPO, with up to 10-fold variability in dose requirements to achieve correction of the anaemia. Furthermore, around 5-10% of the patients show a marked resistance to rhEPO therapy (2,4-6). The European Best Practice Guidelines define "resistance to rhEPO therapy" as a failure to achieve target haemoglobin levels (between 11 and 12 g/dL) with maintained doses of rhEPO higher than 300 IU/Kg/week of epoetin or of higher doses than 1.5 μg/Kg/week of darbopoietin-alfa (7).

The reasons for this variability in rhEPO response are unclear (4, 8-12). There are a lot of conditions reported as associated with rhEPO resistance, namely, inflammation, oxidative stress and iron deficiency, as major causes (4, 8-14), and blood loss, hyperparathyroidism, aluminium toxicity and vitamin B12 or folate deficiency, as minor causes. However, exclusion of these factors does not eliminate the marked variability in sensitivity to rhEPO (15).

In this paper a revision of the mechanisms of resistance to rhEPO therapy will be performed, with particular emphasis on inflammatory cytokines, leukocyte activation, iron status, and erythrocyte damage.

2. Inflammatory cytokines

Inflammation is the physiological response to a variety of noxious stimuli such as tissue injury caused by infection or physical damage. It is a complex process that involves the participation of several cells and molecules, and may present different intensities and durations.

Inflammation usually refers to a localised process. However, if the noxious stimulus is severe enough, distant systemic changes may also occur, and these changes are referred as "acute phase response", which is accompanied by signs and symptoms such as fever, anorexia, and somnolence. This acute phase response may include neuroendocrine, metabolic and haematopoietic changes, as well as changes in non-protein plasma constituents (16). The haematopoietic response includes leukocytosis and leukocyte activation, thrombocytosis, and anaemia secondary to erythrocyte damage and/or decreased erythropoiesis (17).

Inflammatory stimuli induces the release of cytokines, including tumour necrosis factor (TNF)-α, interleukin (IL) -1, IL-6, and interferon (IFN)-γ, which may be produced by several cells, including leukocytes, fibroblasts and

endothelial cells (18). This release of cytokines causes many systemic changes, including increased synthesis and release of positive acute-phase proteins, such as C-reactive protein (CRP) and fibrinogen, as well as the suppression of negative acute-phase proteins, such as albumin and transferrin (4,11,19).

The causes for the inflammatory response in HD patients are not well clarified. There are several potential sources, including bacterial contamination of the dialyser, incompatibility with the dialyser membrane and infection of the vascular access. However, the dialysis procedure may only be partially responsible for the inflammatory response, because even patients with renal insufficiency who are not yet on dialysis present raised inflammatory markers, which rise further after starting regular HD treatment, suggesting that the disease per se triggers an inflammatory response (6,13,15).

Along an inflammatory response, the iron from the erythropoiesis traffic is mobilised to storage sites within the reticuloendothelial system, inhibiting erythroid progenitor proliferation and differentiation, and blunting, therefore, the response to erythropoietin (EPO) (endogenous and/or exogenous). An erythropoiesis-suppressing effect has been also attributed to increased activity of pro-inflammatory cytokines reported in this inflammatory condition, and this relationship has been proposed as a potential factor associated to rhEPO therapy resistance (6,13,15,20). Actually, it was reported that pro-inflammatory cytokines, such as IL-1, IL-2, IL-4, IL-6, TNF-α and INF-γ diminish BFU-E and CFU-E cells, resulting in suppression of erythropoiesis (5,21,22). Moreover, it was reported (5) that serum derived from haemodialysed patients suppresses erythroid colony-forming response to EPO, in a manner that can be inhibited by antibodies against TNF-α and INF-γ. These data suggest a key role in the rhEPO response for these inflammatory mediators (8,11,23,24).

Recently, our group demonstrated an activation of T-cells and an enhanced ability of this cells to produce Th1 related cytokines (IL-2, INF-γ and TNF-α) after short term in vitro stimulation, although these cytokines were undetectable in serum. This increased capacity to produce Th1 cytokines could justify, at least in part, the anaemia found in HD patients. However, this enhanced capacity to produce cytokines was not associated to the refractoriness to rhEPO therapy, as previously described in non-responders HD patients (11). We also demonstrated that non-responders patients presented higher C-reactive protein (CRP) and lower albumin serum levels as compared to responders (25), suggesting a relationship between resistance to rhEPO therapy and the inflammatory response (Table I).

Table 10- Inflammatory markers among HD patients (responders and non-responders to rhEPO therapy).

	rhEPO responders	**rhEPO non-responders**
White cell counts	N	N
Neutrophils counts	↑	↑
Albumin serum levels	N	↓
CRP serum levels	↑	↑↑
IL-6 serum levels	↑	↑↑
Soluble IL-2 receptor serum levels	↑	↑
Elastase plasma levels	↑	↑↑

N: normal values when compared with healthy controls; ↑: increase values when compared with healthy controls; ↓: decrease values when compared with healthy controls; ↑↑ increase values when compared with responders patients. Adapted from references 25 and 40.

3. Leukocyte activation

Leukocytosis and recruitment of circulating leukocytes into the affected areas are hallmarks of inflammation. Leukocytes are chimio-attracted to inflammatory regions and their transmigration from blood to the injured tissue is primarily mediated by the expression of cell-adhesion molecules in the endothelium, which interact with surface receptors on leukocytes (26,27). This leukocyte-endothelial interaction is regulated by a cascade of molecular steps that correspond to the morphological changes that accompany adhesion. At the inflammatory site, leukocytes release their granulation products and may exert them phagocytic capacities.

In acute inflammation, the leukocyte infiltration is predominantly of neutrophils, whereas in chronic inflammation a mononuclear cell infiltration (predominantly macrophages and lymphocytes) is observed. Although leukocyte-endothelial cell interaction is important for leukocyte extravasation and trafficking in physiological situations, there is increasing evidence that altered leukocyte-endothelial interactions are implicated in the pathogenesis of diseases associated with inflammation, possibly by damaging the endothelium or altering endothelial function (28,29).

Leukocytosis is essential as the primary host defence, and neutrophils, the major leukocyte population of blood in adults, play a primordial role. It is well known that neutrophils have mechanisms that are used to destroy invading microorganisms. These cells use an extraordinary array of oxygen-dependent and oxygen-independent microbicidal weapons to destroy and remove infectious agents (30). Oxygen-dependent mechanisms involve the production of reactive

oxygen species (ROS), which can be microbicidal (31), and lead to the development of oxidative stress. Oxygen-independent mechanisms include chemotaxis, phagocytosis and degranulation. The generation of microbicidal oxidants by neutrophils results from the activation of a multiprotein enzyme complex known as the reduced nicotinamide adenine dinucleotide phosphate oxidase, which catalyzes the formation of superoxide anion ($O2\cdot-$). Activated neutrophils also undergo degranulation, with the release of several components, namely, proteases (such as elastase) and cationic proteins (such as lactoferrin).

Elastase is a member of the chymotrypsin superfamily of serine proteinases, expressed in monocytes and mast cells, but mainly expressed by neutrophils, where it is compartmentalized in the primary azurophil granules. The intracellular function of this enzyme is the degradation of foreign microorganisms that are phagocytosed by the neutrophil (32). Elastase can also degrade local extracellular matrix proteins (such as elastin), remodel damaged tissue, and facilitate neutrophil migration into or through tissues. Moreover, elastase also modulates cytokine expression at epithelial and endothelial surfaces, up-regulating the production of cytokines, such as IL-6, IL-8, transforming growth factor β (TGF-β) and granulocyte-macrophage colony-stimulating factor (GM-CSF); it also promotes the degradation of cytokines, such as IL-1, TNF-α and IL-2. There is evidence in literature that high levels of elastase are one of the major pathological factors in the development of several chronic inflammatory lung conditions (33).

Plasma lactoferrin is predominantly neutrophil derived and its presence in the specific granules is often used to identify these types of granules. Lactoferrin is also found in other granules, in the tertiary granules, though in lower concentrations (34-37). Lactoferrin is a multifunctional iron glycoprotein, which is known to exert a broad-spectrum primary defence activity against bacteria, fungi, protozoa and viruses. It can bind to large amounts of free iron. The iron bound to lactoferrin is taken up by activated macrophages, which express specific lactoferrin receptors. During inflammation, this causes iron deprivation of the erythroid precursors, which fail to express lactoferrin receptors (38). Other mechanisms in which lactoferrin is implicated include a growth regulatory function in normal cells, coagulation, and perhaps cellular adhesion modulation (39).

There are no considerable data concerning to the correlation between leukocyte activation and resistance to rhEPO therapy, particularly to the neutrophil activation. We recently found that patients under HD, particularly non-responders patients, present higher elastase plasma levels, compared to

healthy controls (40). Moreover, we demonstrated neutrophil activation, triggered by HD procedure. However, the HD procedure does not explain the higher elastase levels found in non-responders patients. Moreover, we found statistically significant positive correlations between elastase levels and CRP, suggesting that the rise in elastase levels is part of the inflammatory process found in HD patients, being particularly enhanced in non-responders. The statistically significant correlation that we also found between elastase levels and weekly rhEPO doses also corroborate this hypothesis; in fact, non-responders to rhEPO therapy patients requiring higher weekly rhEPO doses to achieve target hemoglobin levels, are also associated with an increased inflammatory process.

The increase plasma levels of elastase found in HD patients can be associated to alterations in erythrocyte membrane proteins, which could decrease erythrocyte half life in HD patients, particularly in non-responders, and consequently increase the degree of anaemia in these patients.

Table II - Iron status markers among HD patients (responders and non-responders to rhEPO therapy).

	rhEPO responders	**rhEPO non-responders**
Haemoglobin concentration	↑	↓↓
Mean cell haemoglobin	N	↓
Mean cell haemoglobin concentration	N	↓
RDW (red cell distribution width)	↑	↑↑
Transferrin saturation	N	N
Ferritin serum levels	↑	↑
Transferrin serum levels	↓	↓
s-TfR serum levels	N	↑
Prohepcidin serum levels	↑	↑

N: normal values when compared with healthy controls; ↑: increase values when compared with healthy controls; ↓: decrease values when compared with healthy controls; ↑↑ increase values when compared with responders patients; ↓↓ decrease values when compared with responders patients. Adapted from reference 61.

4. Iron status

Iron is an essential trace element that is required for growth and development of living organisms, but excess of free iron is toxic for the cell (41-43). Mammals lack a regulatory pathway for iron excretion, and iron balance is maintained by the tight regulation of iron absorption from the intestine (42,44). The intestinal

iron absorption is regulated by the level of body iron stores and by the amount of iron needed for erythropoiesis (41,42,44,45).

In HD patients, iron absorption is similar to that found in healthy individuals; however, when under rhEPO therapy, the absorption of iron increases as much as 5 times (46). This increased iron absorption is not sufficient to compensate for the iron lost during the HD procedure, and with the frequent blood draws performed on these patients. For this reason, intravenous iron administration has become a standard therapy for most patients receiving rhEPO. To avoid iron overload, with potentially harmful consequences, there is a need to monitor iron therapy by performing regular blood tests reflecting body's iron stores.

Recently, a complex regulatory network that governs iron traffic emerged, and points to hepcidin as a major evolutionary conserved regulator of iron distribution (47-49). This small hormone produced by the mammalian liver has been proposed as a central mediator of dietary iron absorption. High levels of hepcidin were found to be associated with a decrease in both iron uptake from the small intestine and in iron release from macrophages; a decreased placental iron transport was also observed (50).

The synthesis of hepcidin is stimulated by inflammation and iron overload. The in vitro stimulation of fresh human hepatocytes by pro-inflammatory IL-6 showed strong induction of hepcidin mRNA, indicating that this cytokine may be an important mediator of hepcidin induction in inflammation (51-57). It is synthesized as preprohepcidin, a protein with 84 amino acids. This peptide is cleaved leading to prohepcidin with 60 aminoacids, which is further processed giving rise to the 25 aminoacids protein, hepcidin (51,52).

Hepcidin was reported to bind to the transmembrane iron exporter ferroportin, which is present on macrophages, on the basolateral site of enterocytes, and also on hepatocytes. It has been demonstrated in vitro that hepcidin induces the internalization and degradation of ferroportin, a crucial protein for cellular iron export (53). By diminishing the effective number of iron exporters on the membrane of enterocytes and of macrophages, hepcidin suppresses iron uptake and release, respectively. This is the phenotype of ferroportin disease, where iron accumulation is observed mainly in macrophages and often combined with anaemia (54).

This increased hepcidin expression during inflammation explains sequestration of iron in the macrophages and inhibition of intestinal iron absorption, the two hallmarks of the anaemia of inflammation, which is normocytic or microcytic iron-refractory (47,50,52). This decreased availability in iron may be a host defence mechanism against invading microorganisms.

The biological importance of the precursor molecule of hepcidin, named prohepcidin, in regulating iron metabolism, is still undetermined. However, there is some evidence that prohepcidin levels are a reliable indicator of hepcidin levels and activity (50,52,58,59).

Hepcidin synthesis is regulated by inflammation, a common finding in HD patients. Thus, increased levels of prohepcidin have been reported in HD patients (50,52,58-60).

We recently demonstrated that HD patients non-responders to rhEPO therapy present a mild to moderate anaemia, even with the administration of higher rhEPO doses (61). This anaemia is hypochromic [decreased mean cell haemoglobin (MCH) and mean cell haemoglobin concentration (MCHC)], and is associated with a more accentuated anisocytosis than in responder patients. Considering that all patients, in our study, were under iron and folate prophylactic therapies, and, that iron status and vitamin B12 serum levels were normal, these changes could not be attributable to a deficiency in these erythropoietic nutrients. Actually, the HD patients non-responders to rhEPO therapy seem to present a "functional" iron deficiency, characterized by the presence of adequate iron stores as defined by conventional criteria, but apparently with an inability to sufficiently mobilize iron to adequately support erythropoiesis. In fact, no statistically significant differences were found in serum iron status markers between the groups of patients (responders and non-responders), except for the soluble transferrin receptor (s-TfR), which was statistically significant higher among non-responders (Table II). This "functional" iron deficiency is reflected by a decrease in MCH and in MCHC, and by a more accentuated anisocytosis (high RDW) in non-responders patients. Furthermore, the inverse correlation that we have found between CRP and mean cell volume, MCH, serum iron and transferrin saturation, are in agreement with the observations that the chronic inflammation enhanced in non-responders, may lead to trapping of iron within the macrophages and to a reduction in serum iron levels. Additionally, HD patients non-responders to rhEPO therapy presented lower prohepcidin serum levels when compared with responders. As previously referred, hepcidin is up-regulated by inflammation; however, on the other hand EPO downregulates liver hepcidin expression (62). As non-responders patients were treated with much higher doses of rhEPO compared with responders, the lower prohepcidin levels found in those patients could be explained by this mechanism.

5. Erythrocyte damage

The erythrocyte, presenting a limited biosynthesis capacity, suffers and accumulates physical and/or chemical changes, which become more pronounced with cell aging, and whenever an unusual physical or chemical stress develops (63).

The erythrocyte membrane is a complex structure comprising a lipidic bilayer, integral proteins and the skeleton. Spectrin is the major protein of the cytoskeleton, and, therefore, the major responsible for erythrocyte shape, integrity and deformability. It links the cytoskeleton to the lipid bilayer, by vertical protein interactions with the transmembrane proteins, band 3 and glicophorin A (64). In the vertical protein interaction of spectrin with band 3 are also involved ankyrin (also known as band 2.1) and protein 4.2. A normal linkage of spectrin with the other proteins of the cytoskeleton assures normal horizontal protein interactions.

Erythrocytes are physically stressed during the HD process, and metabolically stressed by the unfavourable plasmatic environment, due to metabolite accumulation, and by the high rate of haemoglobin autoxidation, due to the increase in haemoglobin turnover, a physiologic compensation mechanism triggered in case of anaemia (64,65). The erythrocytes are, therefore, continuously challenged to sustain haemoglobin in its reduced functional form, as well as to maintain the integrity and deformability of the membrane.

When haemoglobin is denatured, it links to the cytoplasmic pole of band 3, triggering its aggregation and leading to the formation of strictly lipidic portions of the membrane, poorly linked to the cytoskeleton. These cells are probably more prone to undergo vesiculation (loss of poorly linked membrane portions) whenever they have to circulate through the haemodialysis membranes or the microvasculature. Vesiculation may, therefore, lead to modifications in the erythrocyte membrane of HD patients (66,67).

Erythrocytes that develop intracellular defects earlier during their life span are removed prematurely from circulation (68,69). The removal of senescent or damaged erythrocytes seems to involve the development of a senescent neoantigen on the plasma membrane surface, marking the cell for death. This neoantigen is immunologically related to band 3 (70). The deterioration of the erythrocyte metabolism and/or of its antioxidant defences may lead to the development of oxidative stress within the cell, allowing oxidation and linkage of haemoglobin to the cytoplasmatic domain of band 3, promoting its aggregation,

the binding of natural antiband 3 autoantibodies and complement activation, marking the erythrocyte for death.

The band 3 profile [high molecular weight aggregates (HMWAg), band 3 monomer and proteolytic fragments (Pfrag)], differs among younger, damaged and/or senescent erythrocytes. Older and damaged erythrocytes present with higher HMWAg and lower Pfrag. Younger erythrocytes show reduced HMWAg and higher Pfrag (69). Moreover, several diseases, known as inflammatory conditions, presented abnormal band 3 profiles, suggestive of oxidative stress development (68,69,71).

HD patients present a reduction in erythrocyte membrane spectrin, though without statistically significance, was found in non-responders patients. This spectrin deficiency was accompanied by the production of hypocromic and anisocytic erythrocytes, and this anisocytosis could reflect an even more enhanced change in membrane protein interactions in non-responders (73,74). Non-responders patients also showed a decrease in Pfrag and in Pfrag/band 3 monomer ratio (Fig. 1) and a trend to higher values of membrane bond haemoglobin (75), suggesting that they present a higher erythrocyte damage that may result from an even more adverse plasmatic microenvironment. When studying the differences in membrane protein composition before and after the haemodialysis procedure, we found that this spectrin reduction was enhanced in the non-responder group (76); moreover, we observed that this could be associated to an enhanced inflammation process and/or to the interaction with proteases released by neutrophils as we also found significant rise in elastase, product of leukocyte activation.

6. Conclusions

Although the etiology of resistance to rhEPO therapy is still unknown, inflammation seems to have an important role in its pathophysiology. Resistance to rhEPO therapy is also associated with “functional” iron deficiency, higher elastase plasma levels, and alterations in erythrocyte membrane protein structure and in band 3 profile.

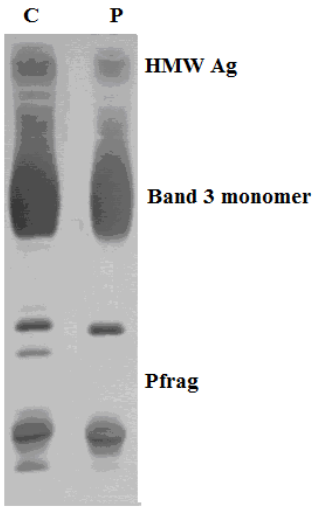

Figure 6 - Illustration of two band 3 profiles. C: control; P: HD patient; HMW Ag: high molecular weight aggregates; Pfrag: proteolytic fragments.

The exact origins of the inflammatory process remain unclear. We speculate that the release of elastase during the HD procedure could have an important role in amplifying the inflammatory process in HD patients, particularly in non-responders (Fig 2). Furthermore, this elastase release could also have an important role in the alterations found in erythrocyte membrane protein structure and in band 3 profile, further contributing to worsening of anaemia (Fig 2). The inflammatory process, the rhEPO doses administrated and the lactoferrin release during haemodialysis could have an important role in iron uptake from the small intestine, in the release of iron from macrophages and, finally, in the availability of iron for erythropoiesis..

Further studies are needed to understand the rise in inflammation with the associated need in higher doses of rhEPO and reduced iron availability. It will be also important to clarify the mechanism of lymphocyte loss, by studying the role of IL-7 levels and/or the peripheral lymphocyte apoptosis; the effect of higher levels of elastase in the inflammatory process, and in the alterations in erythrocyte membrane protein composition and in band 3 profile. Of great importance would be also the development of animal studies, by using a rat

model of chronic renal failure, as some histological and molecular studies, cannot be performed in humans, such as the erythropoietic and renal studies.

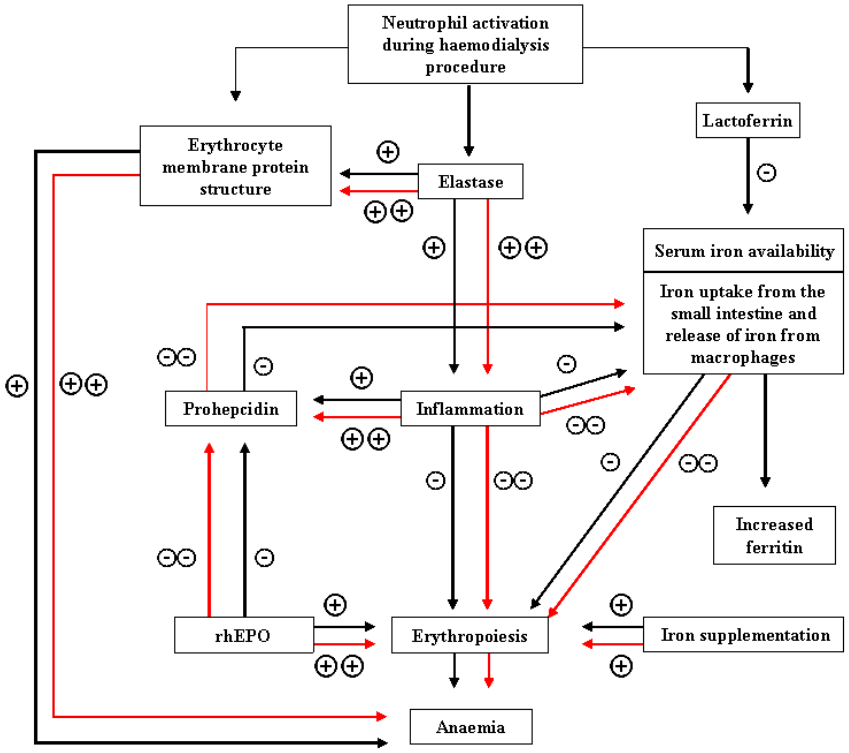

Figure 7 - Potential mechanisms involved in the pathophysiology of resistance to rhEPO therapy. Black lines represents responders patients and red lines non-responders patients; (+) represents activation and (-) inhibition.

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