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Erythropoietin as an Immunomodulating Agent

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ABSTRACT

Erythropoietin (EPO) is a glycoprotein produced by peritubular capillary epithelial kidney cells in response to hypoxia to control erythropoiesis. It stimulates growth and differentiation of red blood cells progenitors and protects them from apoptosis by binding to receptor (EPO-R) on CFU-E (erythroid colony-forming unit) and BFU-E (erythroid burst-forming unit) colonies. Although it seems that primary role of EPO is the regulation of red cells production, EPO-R has been found in/on other tissues and cells, including human polymorphonuclear leukocytes, monocytes and lymphocytes. Both EPO-R structure and its presence on these cells suggest that beyond erythropoietic function, EPO might possess some immunomodulatory properties. Progress of chronic kidney disease (CKD) gradually leads to kidney failure, uremia, hypertension and anemia resulting from decreased EPO production and presence of uremic toxins and proiflammatory cytokines. At the same time, CKD patients also show signs of the deficiency state in both cell mediated and humoral immunity, which is even deepened by dialysis procedure. High levels of proinflammatory cytokines produced by chronically activated monocytes and decreased IL-2 level reflecting weakened T lymphocytes function are observed. Deficient T lymphocytes responses lead to impaired humoral immunity presented by the decreased immunoglobulin levels in response to hepatitis B vaccination and increased frequency of different infections. Since late 80s recombinant human erythropoietin (rhEPO) is commonly used for treatment of anemia related to chronic kidney disease (CKD). Current review describes immunological aspects of rhEPO therapy in CKD patients. The aim of this work is to pay attention to the fact that observed improvement of the immunological responses described in last 15 years in CKD patients is not only the result of anemia correction during rhEPO treatment. Changes of crucial activation and co-stimulation antigens of T lymphocytes of rhEPO treated CKD patients along with EPO-R presence on human leukocytes indicate that EPO/rhEPO can directly influence immunological responses.

Implication for health policy/practice/research/medical education:
This review describes interesting immunological aspects of rhEPO therapy in CKD. RhEPO can directly influence lymphocytes and monocytes responses, so it is recommended reading this article to nephrologist and oncologist.

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1. Introduction

Erythropoietin (EPO), a 34 kDa glycoprotein produced by peritubular capillary epithelial kidney cells, is the key regulator of red blood cells production (1). It protects erythroid cells from apoptosis and promotes their proliferation and differentiation (1). EPO structure presents elements of cytokines composition and that is why it is considered that this hormone, apart from its influence on red blood cells system, can regulate immunological responses. Its receptor, EPO-R, together with IL-2, IL-4, IL-7, IL-13 or GM-CSF belongs to the superfamily of hematopoietic receptors (2). EPO binding

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to EPO-R results in receptor homodimerization followed by activation of JAK (*Janus kinase*) family members (3). Interactions between these kinases and EPO-R result in phosphorylation of several signaling proteins including signal transducer and activator of transcription 5 (STAT5) that plays important anti-apoptotic role in erythroid cells through direct Bcl-x_L activation (4) *(Figure 1)*. The very same STAT5 in T lymphocytes is triggered by IL-2, what increases expression of the other anti-apoptotic protein, Bcl-2 (5). Both IL-2 and EPO seem to activate similar pathways leading to promotion of cell growth, what is not very surprising, when we take into consideration the fact that their receptors belong to one family and share similar structure. EPO binding also induces other signaling molecules like rat sarcoma and MAP (*mitogen activated protein kinase*) kinases (6), phospholipase Cγ1 (PLCγ1) (7) or phosphatidylinositol 3-kinase (PI3K) (8), all promoting red blood cells progenitors survival *(Figure 1)*.

Although primary role of EPO is the regulation of red cells production, EPO-R has been found in other tissues and on other cells including central nervous system (9), endothelial cells (10) and some tumors cells (11). EPO-R was also detected on human polymorphonuclear leukocytes (12), monocytes (13) and lymphocytes (13). Novel quantitative flow cytometric method allowed in 1997 to analyze the cell surface expression of EPO-R on bone marrow red blood cells precursors (14). And so, the number of EPO-R is the highest in the most immature erythroid cells (approximately 1600 molecules per cell) and its expression decreases with erythroid maturation (14). In our previous article, we demonstrated that basic levels of EPO-R ex vivo on lymphocytes is quite low (less than 100 molecules/cell) and higher on monocytes (13). However, in vitro experiments revealed that on $CD4^+$ T lymphocytes stimulated with anti-CD3 EPO-R number increases after 48 hour of cell culture (13). The EPO-R number we observed on stimulated $CD4^+$ T lymphocytes reaches or even exceeds the one reported to be typical for the erythroid cells, which suggests that EPO/rhEPO could influence lymphocytes directly and somehow modulate their immunological responses.

2. Immunological Disorders In Chronic Kidney Disease

Chronic kidney disease (CKD) is a progressive loss in renal function over a period of years. As the kidney function decreases, patients develop uremia, hyperkalemia, hyperphosphatemia, hypertension and anemia (15). The goal of therapy is to slow down progression of disease but when patient reaches stage 5 of CKD (ESRD, end stage renal disease) renal replacement therapy is required (dialysis or kidney transplantation) (15). Anemia in CKD patients is a major consequence of kidney damage, although several other factors such as blood loss, iron deficiency or presence of proinflammatory cytokines are also included. However, in the absence of EPO, red blood cells precursors undergo apoptosis and anemia occurs (16). Recombinant human erythropoietin (rhEPO) is well known treatment used in clinical practice for CKD anemia (17). Another erythropoietic agent is darbepoetin, whose half-life is longer and biological activity higher in comparison to rhEPO (18). Both agents act by stimulating EPO-R directly, which results in cell proliferation and inhibits apoptosis of erythroid cells (17, 18). CKD patients also present improper immunological parameters of both humoral and cellular immunity that seem to be deepened by hemodialysis (HD) process. The most common findings include very high levels of proinflammatory cytokines TNF-α, IL-1 and IL-6 (19-21) produced by chronically activated monocytes during dialysis process *(Figure 2)*. Also, decreased levels of IL-2 and INF-γ (22) indicate deficient reactions of T cells to mitogen stimulation (22, 23) *(Figure 2)*. Additionally, lower number of $CD3^+$, $CD4^+$ and $CD8^+$ peripheral lymphocytes are observed in hemodialyzed (HD) patients (24). Poor T lymphocytes responses at last lead to impaired humoral immunity presented as the decreased immunoglobulins levels in response to hepatitis B vaccination and increased frequency of various infections due to both B and T lymphocytes impairments (25). Monocytes activation is usually explained by direct contact of peripheral blood mononuclear cells (PBMC) with dialytic membranes and possible back-transport of bacterial LPS (lipopolysaccharide) from the dialysate to the blood stream (26), while deficiency of T and B lymphocytes is less understandable. At this point we know that $CD4^+$ T lymphocytes (helper lymphocytes taking part in antigen presentation crucial for immune response) of HD patients are characterized by reduced expression of a major costimulatory CD28 molecule (23). Lower CD28 expression in CKD patients probably depends on high levels of TNF-α that directly influence CD28 gene transcription (27) and therefore could be one of the reasons of deficient T lymphocytes responses. More detailed analysis also reveals decreased percentage of proliferating $CD4^+$ T lymphocytes, which strongly

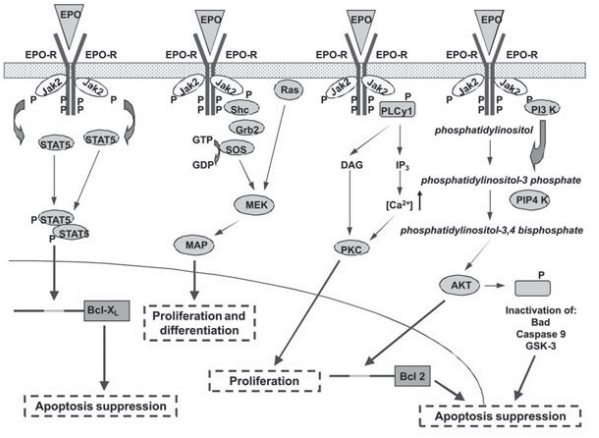

Figure 1. Signaling Pathways Activated by EPO.

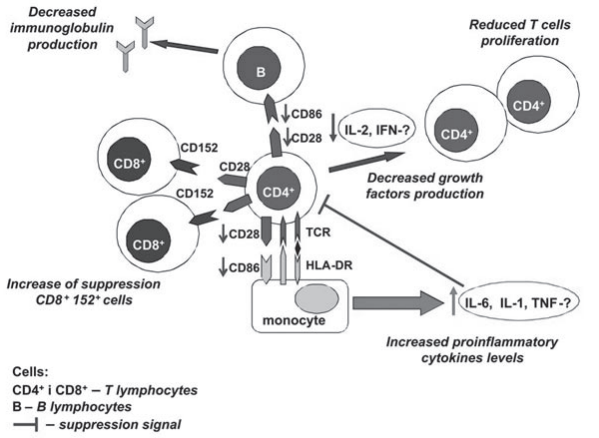

Figure 2. Immunological Disturbances in CKD Patients.

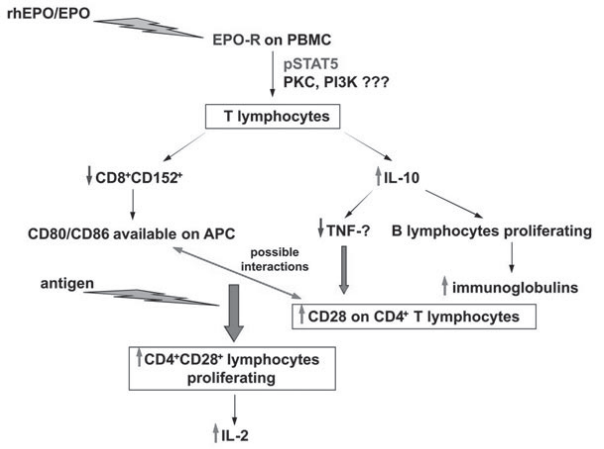

Figure 3. Immunological Effects of rhEPO Treatment.

depends on low expression of CD28 and also CD69 on T lymphocytes surface (23). Also, high percentage of $CD152^+$ cells are detectable ex vivo in HD patients (28). CD152 is the molecule that binds the CD80 molecule on antigen presenting cells (APCs) with 20 times as high affinity as that of the CD28 causing down-modulation of T-cell responses (29). Stimulation with phytohemagglutanin (PHA) additionally increases percentage of $CD152^+$ cells suggesting that the elevated ex vivo percentage of the $CD152^+$ lymphocytes in HD patients might result from a constant activation of blood cells (28).

3. Immunological Aspects of Rhepo Therapy in Chronic Kidney Disease

CKD patients receive rhEPO or darbepoetin for the correction of anemia. Although it seems that rhEPO treatment has no influence on renal function by improving red cells parameters (30), it has an impact on non-specific and specific immunity. Increase of IL-2 level was seen both after stimulation of whole blood cells cultures of patients with PHA as well as with rhEPO in concentration of 0.05 or 0.1 U/mL *(Figure 3)* (31). Experiments have shown that rhEPO could affect IL-2 production by stimulation of the beta chain of IL-2R and that the EPO-R belongs to the same superfamily of hematopoietic receptors (2). RhEPO therapy also influences levels of proinflammatory and antiinflammatory cytokines as decreased production of TNF-α and elevated secretion of IL-10 in whole blood cell cultures stimulated with PHA (32) are detected after few months of treatment *(Figure 3)*. However, these observations are not confirmed by all researchers. There are studies presenting higher levels of TNF-α in CDK patients receiving rhEPO (33), what could be explained by release of large amount of pro-inflammatory cytokines during dialysis in the situation when samples were obtained after dialysis process. Even though, in vitro experiments prove direct influence of rhEPO on blood cells. IL-10, on the other hand, inhibits production of macrophages derived proinflammatory cytokines (34) and influences B lymphocytes activity by switching the production of immunoglobulins in humans (35). In fact, in early 90s it was shown that rhEPO directly enhances B lymphocytes antibodies (IgG, IgM and IgA) production in serum-free medium (36). After few months of rhEPO treatment, number of $CD4^+CD45RA^+$ (naive) T lymphocytes gradually increases in HD patients with the elevation of hematological parameters, which suggests that rhEPO-induced immunomodulation is dependent on general improvement of patients' condition (24).

RhEPO itself does not influence T lymphocytes proliferation directly (24), but rhEPO treatment normalizes phenotype of $CD4^+$ T lymphocytes (23). In vitro experiments revealed that the expression level of costimulatory CD28 molecule and early activation marker – CD69 antigen on $CD4^+$ T lymphocytes of CKD patients treated with rhEPO for several months is similar to that of healthy people and significantly increased when compared with patients not receiving rhEPO (23). Additionally, proliferation parameters such as percentage of proliferating $CD4^+$ T lymphocytes or time between G0 and G1 phase are similar when compared between CKD patients receiving rhEPO and healthy controls; while in patients not receiving rhEPO, these parameters are significantly decreased (23). Long time between G0 and G1 phase (G0→G1 time) and decreased percentage of proliferating T lymphocytes correlate with decreased expression of CD28 and CD69 antigens in patients without rhEPO (23). More decreased CD28 and CD69 expression is, longer time G0→G1 T lymphocytes need to start to proliferate, which suggests strict connection between these antigens' function and time that lymphocytes need to respond to stimulation. This connection has been already described in healthy people, where the negative correlation was found between G0→G1 time and CD28 expression on $CD4^+$ T lymphocytes (37). Thus, decreased expression of CD28 and CD69 antigens can to some extent explain reduced T lymphocytes responses measured as increased incidence of infections observed in CKD patients. On the other hand, rhEPO treatment reduces percentage of $CD8^+CD152^+$ lymphocytes ex vivo as well as after stimulation with PHA or different concentrations

of rhEPO (28), which can be another mechanism of a better immunoglobulin profile and better resistance to infections seen with rhEPO therapy.

4. Conclusions

EPO-R presence on all populations of immune cells implies that EPO/rhEPO can influence lymphocytes, monocytes and granulocytes directly and somehow modulate their immunological responses. RhEPO promotes phosphorylation and activation of STAT5 in EPO-R-transfected lymphoid cells, what in the end leads to transcription of anti-apoptotic proteins (38). In T lymphocytes this signaling pathway is triggered by IL-2 (5) and that is why STAT5 was believed as most likely to be stimulated by EPO/rhEPO in lymphocytes. However, rhEPO itself does not induce STAT5 phosphorylation (39). Proportion of $CD4^+$ T lymphocytes expressing phosphorylated STAT5 is changed only in combination with anti-CD3 and in low concentrations after 6 hours (39). This is not surprising, because in vivo $CD4^+$ T lymphocytes respond only to antigen presentation through TCR/CD3 complex and EPO-R should rather act as a costimulatory molecule. Another effect of direct influence of rhEPO is increase of CD95 (Fas/APO-1, member of the death receptors family) antigen expression on $CD4^+$ T lymphocytes surface (39). This effect is analogous to that observed in red blood cells progenitors grown in presence of EPO (40). Almost certainly, CD95 gene promoter is activated by protein kinase C (PKC) (41) which has been already reported to be stimulated by EPO (42). CD95 enhances CD69 and CD25 antigens expression on $CD4^+$ and $CD8^+$ T lymphocytes and supports cytokine secretion (43), which can be seen in case of CKD patients treated with rhEPO (23, 31). EPO probably can modulate other signaling pathways in lymphocytes including those containing phospholipase Cγ1 (PLCγ1) or phosphatidylinositol 3-kinase (PI-3K) –same signaling pathways activated in red blood cells progenitors (7, 8). It is worth mentioning that studies carried out on mice demonstrated direct rhEPO influence on macrophages. Phosphorylation of STAT1, STAT3 and STAT5 and elevated levels of pro-inflammatory IL-12 were seen in bone marrow-derived cells treated with rhEPO (44). Furthermore, macrophages display activation of the PI-3K, MAPK and NF-κB signaling pathways (44). Presented studies describe the influence of rhEPO on activation parameters of T and B lymphocytes as well as macrophages function. At the present, clinical observations along with in vitro studies demonstrate that rhEPO can directly influence different cell populations of the immune system. Therefore, it looks like positive changes of immunological responses in CKD patients do not only depend on typical treatment, e.g. hemodialysis, but also on direct influence of rhEPO on patients' immune system. RhEPO definitely is an independent immunomodulatory agent and its receptor plays important costimulatory role on lymphocytes, monocytes and probably granulocytes.

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Conflict of interest

None declared.

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