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New alternatives for erythropoietin therapy in chronic renal failure

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Abstract: Erythropoietin (EPO) is one of the main cytokines involved in the regulation of erythropoiesis. The main site of EPO production are the kidneys. An altered EPO production leads to pathological conditions such as anemia and polycythaemia. Due to the progressive loss of renal peritubular cells, patients with chronic kidney disease (CKD) have low EPO plasma levels. This decreases erythron stimulation with the direct consequence of developing anemia. Before the introduction in the clinical practice of rHuEpo, in the late 1980s, the only solution for treating this type of anemia were blood transfusions and anabolic steroids. Even rHuEpo has proven to be safe and effective for treatment of anemias, there are some concerns about its cost, the need for frequent parenteral administration, and development of anti-EPO antibodies. These inconveniences prompted the search for novel erythropoiesis stimulating agents. Different strategies lead to isolation or chemical synthesis of such agents as darbepoetin alfa and EPO mimetics. In this review, we present some general aspects of EPO biology, with emphasis on chronic renal failure, and expose some of the alternatives to EPO used for anemia correction.

Keywords: Erythropoietin, darbepoetin, hematide, synthetic erythropoiesis protein, chronic kidney disease, hepcidin

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1 Introduction

Erythropoietin is a cytokine that regulates red blood cell production (erythropoiesis) by direct interaction with a specific receptor expressed by erythroid progenitor cells. EPO is distinct among hematopoietic growth factors because it is produced primarily in the kidneys rather than the bone marrow.

The first hypothesis sustaining the idea of a humoral factor that controls erythropoiesis was proposed in 1906, and at that time it was coined the term "haemopoietin" [1]. Bondsdorff and Jalvisto who linked EPO solely with red blood cell production introduced the term "erythropoietin" in 1948 [2]. In 1950s Jacobson and colleagues established that the kidney is the main site of EPO production [3]. Later the liver was identified as another source of EPO production [4]. Purification in 1977 of EPO isolated from urine collected from patients suffering of aplastic anemia [5], enabled sequencing of human EPO gene in 1985 [6]. Later EPO gene was cloned and was developed a transfected CHO cell line to provide recombinant human EPO (rHuEpo) for use in treatment of different anemias [7]. The first clinical trial with rHuEpo began in 1989 in uremic patients [8]. Currently rHuEpo is approved for use in the treatment of anemias of different etiology: (i) anemia of chronic renal failure; (ii) anemia associated with HIV patients treated with AZT; (iii) anemia associated with cancer chemotherapy. In the later years, a number of compounds with EPO mimetic properties have been developed.

2 Erythropoietin and erythropoietin receptor

2.1 Erythropoietin structure and EPO gene regulation

EPO is a 30.4 kDa glycoprotein belonging to the cytokine family of proteins. The human EPO gene is located on chromosome 7q11-22, and consists of five exons and four introns. In the 3' enhancer region of EPO gene are located specific DNA sequences, 5'-RCGTG-3', termed hypoxia response elements (HRE) [9]. They are recognized by hypoxia-inducible transcription factors (HIF), which are involved in regulation of gene expression under hypoxic conditions.

HIF is a dimer of alpha and beta subunits, for each of which there are three isoforms numbered 1 to 3. The most studied to date is HIF-1, which is composed by a α subunit (HIF-1 , 120 kDa) and a β subunit (HIF-1β, 91-94 kDa) [10]. All three HIF-α isoforms, HIF-1α, HIF-2α and HIF-3α are continuously translated, but not detectable under normoxic conditions, and have specific expression patterns [11]. By contrast, HIF-β is constitutively synthesized and its stability is not influenced by O_2 level [12].

Oxygen regulates both HIF activity and stability through hydroxylation on specific Pro and Asn residues. These reactions are catalyzed by specific prolyl-4-hydroxylases (HIF-PHDs), and an HIF-α-specific asparaginyl hydroxylase, termed factor inhibiting HIF-1 (FIH) [10]. There are known 3 HIF-PHDs isoforms that regulate HIF-α in a non-redundant manner. All of the 3 HIF-PHDs require 2-oxoglutarate as a co-substrate, and

Fe(II) and ascorbate as co-factors.

In normoxia, HIF-1 is hydroxylated on Pro^{564} and Pro^{402} residues belonging to an O_2-dependent degradation domain (ODDD) [13]. Hydroxylated HIF-1α will interact with von Hippel-Lindau tumor suppressor protein (pVHL) forming a complex that is polyubiquitinilated by an E_3 ligase with subsequent proteasomal degradation [14–16]. By contrast, in hypoxia, HIF-PHDs became less active leading to HIF-1α stabilization. HIF-1α translocates into nucleus where it heterodimerizes with HIF-β forming the active HIF-1, which will bind to DNA HRE motifs [17]. HIF-1 induces transcription of genes that will ameliorate the effects of hypoxia (transferrin and transferrin receptor gene, glycolytic enzymes, glucose transporters like GLUT-1 and GLUT-3 etc).

Hydroxylation of HIF-1α by FIH on Asn^{803} blocks its transcription activity by inhibiting interaction with co-activator CBP/p300. In hypoxia, FIH also became inactive enabling hydroxylation and subsequent interaction between HIF-1α and CBP/p300 [18].

It was found that HIF-2 is the primary transcription factor responsible for induction of EPO and EPO-R genes expression under hypoxic condition [19].

The final product of the EPO gene is a single polypeptide chain containing 193 amino acids, which undergoes post-translational modifications: (i) glycosylation, (ii) disulphide bond generation, (iii) removal of a 27 amino acid hydrophobic secretory sequence, and (iv) cleavage of C-terminal arginine (*Arg166*) [20, 21]. The carbohydrate moiety contains three *N*-linked oligosaccharides (*Asp24, Asp38* and *Asp83*) and one *O*-linked oligosaccharide (Ser126). Human EPO has two disulphide bonds (*Cys7-Cys161* and *Cys29-Cys33*). Glycosylation and disulphide bond patterns maintain the correct conformation needed for *in vivo* biological activity and interaction with EPO receptor [22, 23]. Deglycosylation retains full biological activity of EPO but leads to a very rapid plasma clearance by the liver [24].

2.2 Erythropoietin receptor

EPO-R is a 72-78 kDa glycosylated transmembrane protein belonging to the superfamily of cytokine receptors. Interaction of EPO with EPO-R leads to receptor homodimerisation, followed by its phosphorylation and activation of Janus kinase 2 (Jak-2) [25]. Jak-2 is a tyrosine kinase constitutively associated with the cytosolic domain of EPO-R. Activated Jak-2 promotes activation of a number of signaling pathways, including signal transducer and activator of transcription 5 (STAT-5), mitogen-activated protein kinase (MAPK), and the phosphoinositol 3 kinase (PI3K)/Akt with anti-apoptotic and mitogenetic general effects [26–29]. The PI3K pathway through Akt is widely viewed to be essential to the EPO's antiapoptotic action.

The carbohydrate moiety of EPO is thought to prevent EPO-R binding through electrostatic interactions, and as a consequence, the affinity for receptor decreases with EPO glycosylation level [30].

EPO-receptor is expressed in various degrees by erythroid cell progenitors. The number of EPO-R per cell gradually decreases along erythroid cell line differentiation, reticulo-

cytes and mature RBCs lacking EPO-R [31, 32]. EPO-R is expressed primarily between CFU-E and pronormoblast stage of erythroid cell development [31, 32]. A very small number of EPO-R is also expressed by BFU-E [31]. The overall action of EPO on erythroid progenitors is to rescue them from apoptosis, sustaining their proliferation and differentiation [33].

3 Renal failure and rHuEpo

3.1 Renal failure

Renal failure can be either acute (sudden and rapid onset) or chronic (gradual onset). Without treatment, both forms lead to end-stage renal failure, requiring dialysis or transplantation. Therapy of renal failure aims to ameliorate one or more risk factors or to compensate the decline in renal function.

Anemia is a common consequence of chronic renal failure. It can significantly affect morbidity, mortality and quality of life of chronic kidney disease patients. The World Health Organization defines anemia as a hemoglobin concentration lower than 13 g/dL in men and post-menopausal women, and lower than 12 g/dL in other women.

Different factors are reported to contribute to the anemia in CKD patients among which blood loss during hemodialysis session, shortened RBCs life span, vitamin deficiencies, uraemia *per se*, EPO and iron deficiencies, and inflammation are most prominent [34]. However, little is known by which extent these different factors contribute to the disease etiology.

Renal anemia has been associated with cardiovascular complications, including left ventricular hypertrophy, congestive heart failure, reduced cognitive and mental function, impaired quality of life, and the need for regular transfusions [35–39].

There are different studies suggesting that EPO can also reduce the renal dysfunction and injury caused by oxidative stress, hypoxia, and haemorrhagic shock, generally by reducing caspase activation and apoptotic cell death [40–44]. Identification of EPO-R on renal tubule cells suggests that EPO could have non-haemopoietic roles in kidney [45]. By promoting mitogenesis, EPO could have renoprotective effects.

Before the availability of rHuEpo, the only treatment for patients with anemia of chronic renal failure was blood transfusion, a solution with many side effects like iron overload and immune response. The first clinical trials on humans showed that rHuEpo restored packed cell volume, abrogated the need for regular blood transfusions in patients requiring dialysis, and improved the overall wellbeing [46–48]. As a consequence of these results in 1988 rHuEpo was granted with a license as a therapeutic agent for patient with anemia of chronic renal failure.

3.2 Forms of rHuEpo

There are currently four different rHuEpo forms: alpha, beta, delta, and omega. However, until recently, only EPO-alfa and EPO-beta were commercially available. As mentioned above glycosylation pattern is very important for maintaining and influencing biological activity. There are minimal differences in respect to glycosylation pattern between natural EPO, EPO-alfa and EPO-beta depending upon the cell type used for their production [49]. These differences are reflected by differences in both pharmacokinetic and pharmacodynamic profiles between the natural and the recombinant forms, and among the recombinant forms. Both EPO-alfa and EPO-beta have similar turnover times in the plasma with a half-life of about 7 to 8 hours.

In CKD patients receiving hemodialysis rHuEpo could be administered by intravenous and subcutaneous route [50]. Subcutaneous route has some advantages versus intravenous: (i) it does not require any venous access; (ii) significantly prolongs the increase of serum EPO, thus sustaining the stimulation of erythropoiesis. One major side effect was pure red cell aplasia (PRCA), an immunological complication encountered in some patients receiving recombinant erythropoiesis-stimulating agents (see later in the text). In these situations it is recommend changing the route of rHuEpo administration from subcutaneous to intravenous [51]. The frequency of administration depends upon clinical status of the patient: from one to three times daily, and from once to twice weekly.

One strategy to delay drug clearance was to increase the glycosylation degree. The potential benefit of such modification is a less frequent administration of the drug by prolonging the half-life of EPO in circulation. By site mutagenesis were introduced in the polypeptide backbone of EPO two additionally N-glycosylation sites. The glycoprotein generated in this manner was called darbepoetin alfa. Darbepoetin alfa is considered to be a second-generation erythropoietic stimulating agent. It has five sites for N-glycosylation and a 3-fold increased half-life in plasma [52]. In July 2002 the US Food and Drug Administration approved the use of darbepoetin alfa for treatment of chemotherapy-associated anemia in patients with nonmyeloid malignancies. Based on clinical trials it is recommended the administration of darbepoetin alfa once weekly or once every two weeks [53]. In a recent trial it was showed that darbepoetin alfa, administrated once monthly, is able to maintain hemoglobin level in most dialysis patients stabilized previously on once every two weeks dosing [54].

3.3 Hyporesponsiveness to erythropoietin

Although introduction in clinical use of rHuEpo and darbepoetin alfa was a major breakthrough in the management of anemia in CKD patients, it was found that approximately 5-10% of patients show a suboptimal response. US guidelines define hyporesponsiveness as a failure, in the presence of adequate iron stores, to achieve and maintain the target hemoglobin level at a rHuEpo dose of 450 IU/kg/week when administered intravenously or 300 IU/kg/week when administered subcutaneously [55].

There are many potential causes of EPO hyporesponsiveness including iron deficiency, persistent infections, inflammation, chronic blood loss, aluminium overload, vitamin deficiencies (folic acid, vitamin B_{12}, and vitamin C) etc, some of these causes being interconnected [56, 57]. Among all of these factors, we will briefly present two of them.

Patients with CKD have a chronic inflammatory response characterized by sustained chronic release of pro-inflammatory cytokines IL-1β, IFN-γ, TNF-α, IL-6 and IL-12. These pro-inflammatory cytokines inhibit the development on erythroid lineage at different stages leading to an overall suppressive effect upon erythropoiesis [58–60]. It was also found that pro-inflammatory cytokines could act *in vitro* as direct inhibitors of EPO secretion [61].

Direct inhibition of EPO synthesis and counteracting EPO effect on erythrocyte progenitors are causes of anemia in CKD patients.

Another major cause of EPO hyporesponsiveness in CKD patients is iron deficiency. Transferrin saturation (TSAT) and ferritin are the most valuable serum tests used to evaluate iron stores. Iron deficiency could be absolute (TSAT $< 20\%$ and ferritin serum concentration is less than 100 ng/mL) and functional (when TSAT $< 20-30\%$ and serum ferritin concentration is greater than 100 ng/mL) [62].

Chronic inflammation and iron deficiency are linked through hepcidin. Hepcidin, a small antimicrobial peptide expressed by the liver, has a central position in iron metabolism and homeostasis regulation [63]. Hepcidin synthesis is induced in response to inflammation. In hepatocytes IL-6 promotes activation of STAT-3 which in turn will enhance transcription of hepcidin gene [64–66]. Hepcidin interact with ferroprotin, the main iron export protein in mammal cells, down-regulating iron efflux from enterocytes and macrophages [67]. As a consequence erythron compartment will not have sufficient iron for erythropoiesis and EPO treatment will fail to raise hemoglobin level.

4 Novel erythropoietin mimetics

In recent years, the development of different erythropoietin stimulating agents (ESA) was an attempt to overcome the limitations of rHuEpo use: efficiency, duration of activity, route of administration, and the deal with concomitant iron deficiency and inflammation. These agents belong to the third generation erythropoietin stimulating agents, and include continuous erythropoiesis receptor activator and erythropoietin mimetic peptides. Some of these ESA are polyethylene glycol conjugates of EPO (PEGylated EPO).

4.1 Continuous erythropoies receptor activator

Continuous erythropoiesis receptor activator (CERA) was created by insertion in the EPO molecule of a methoxy-polyethylene glycol polymer (PEGylated EPO) [68]. The polymer could be linked to the N-terminal amino group, to the ε-amino of Lys^{52} or Lys^{45}. This modification reduces the clearance of the product, leading to a half-life of $70-122$ hours after intravenous administration and $102-147$ hours after subcutaneous administration.

These variations in the half-life of CERA depend upon the dose. Administration of a single dose of CERA on healthy human volunteers results in a dose-dependent rise in reticulocytes with a maximal response after approximately 7 days. The clinical trials available to date showed that CERA has a few if significant adverse effects in healthy individuals or patients. These studies demonstrate the absence of an immune response in humans, and subsequent absence of CERA antibodies. CERA has passed all phases of clinical trials and now is waiting for US Food and Drug Administration approbation.

4.2 Erythropoietin mimetic peptides

Erythropoietin mimetic peptides are a group of peptides, discovered by phage display technology, which are able to mimic erythropoietin activity. For the prototype of these peptides the proposed trade name is Hematide. Hematide is a synthetic peptide without any sequence homology to rHuEpo [69]. It is highly PEGylated to increase stability and extend the plasma half-life. Hematide has both in vitro and in vivo EPO-like activity (binding to EPO-R, induction of cell proliferation and differentiation). Administration in CKD patients of a single dose of 0.05 mg/kg of Hematide once monthly, lead to an increase of hemoglobin level similar with the increase observed in healthy volunteers after a 0.1 mg/kg dose of Hematide. It was observed that antibodies directed to EPO, isolated from patients with pure red cell aplasia (PRCA), did not cross react with Hematide. This observation raised the possibility of treating anemia in CKD patients with PRCA that fall to respond to rHuEpo or analogues [70]. Hematide has passed the phase I clinical trials and now are ongoing phase II studies in CKD and oncology patients.

4.3 Polymer modified erythropoiesis protein

Synthetic erythropoiesis protein (SEP) is a 51 kDa chemically synthesized protein consisting of a 166 amino acids polypeptide backbone [71]. Two non-coded amino acids [Lys^{24} (N -levulinyl) and Lys^{126} (N -levulinyl)] are attachment sites for polymer chains with a controlled length and a total of eight negative charges. Due to the absence of glycosylation, SEP is not recognized by asialoglycoprotein receptor, which is predominantly responsible for renal EPO clearance [72, 73]. As a consequence SEP has plasma half-time two to three times longer than EPO [71]. In the same study, it was showed that SEP has a superior hematopoietic activity than EPO.

4.4 Inhibitors of HIF prolyl hydroxylases

HIF-PHDs inhibitors represent a novel class of molecules with high potential clinical applications in the treatment of congenital and acquired anemias (i.e., anemia of CKD). A series of small molecular mass HIF-PHDs have been developed. These compounds inhibit HIF-PHDs preventing HIF-α subunit degradation.

One such compound is FG-2216. A recent study indicated FG-2216 as a potent and reversible inducer of endogenous Epo in non-human primates [74]. It was also found that induction of Epo was followed by a robust increased of hemoglobin level. This observation could be explained by the fact that some of the genes coding for proteins involved in iron metabolism and EPO gene are regulated in a coordinated manner by HIF transcription factorv [75]. HIF down-regulates hepcidin gene and up-regulates ferroportin and EPO genes enabling iron transfer to the erythron with subsequent hemoglobin synthesis stimulation.

Recently was identified a compound that increase viability and exercise performances under hypoxic conditions in mice [76]. Ethyl-3,4-dihydroxybenzoate (EDHB) treatment of mice was followed by elevation of HIF-1α in liver and EPO concentration in serum.

5 EPO gene therapy

Gene therapy is an attractive alternative to the use of therapeutic serum proteins. Although rHuEpo and darbepoetin alfa considerably improved the management of anemia in CKD patients, there are some undesirable aspects, which can be overcome using gene therapy. There are descriptions of different approaches in this subject.

An approach was the generation of engineered cells that could release EPO depending on the pO_2 [77]. This was achieved using a vector that contains human EPO cDNA placed under the control of the hypoxia-responsive phosphoglycerate kinase promoter.

Recently it was designed an adenovector which contains EPO gene placed under the control of the cytomegalovirus promoter [78]. In this study, a tissue protein factory based on dermal cores (Biopump) was developed. The dermal cores, harvested from patients with CKD, were transduced ex vivo with the adenovector and then implanted in an autologous manner. EPO serum concentration increased significantly to therapeutic levels from day 1 after implantation and it reached a peak during the first week of follow-up. Despite the absence of significant drug-related side effects, gradually EPO expression decreased, and this decrease was correlated with an accumulation of CD8 cytotoxic T cells in derma.

The use of simple recombined vectors for delivering EPO gene for anemic subjects has some risks. In a study, adeno-associated virus vectors containing EPO gene were intramuscularly delivered to macaques. Some animals developed a severe autoimmune anemia [79].

Another proposed solution was to combine stem cell therapy with gene therapy [80]. In this study bone marrow stromal cells harvested from mice with chronic renal failure anemia were genetically engineered ex vivo to secrete EPO at a rate of approximately 3-4 EPO units/10^6 cells/24 hours. These cells were embedded in a collagen-based matrix and then implanted subcoutaneously in donor mice. Noted was an increase of hematocrit value. A major advantage of this therapy is avoidance of autoimmune complications that usually are associated with immunogenic vector systems.

6 Erythropoiesis - stimulating agents (ESA) and anemia correction - some observations

Despite the fact that rHuEpo was introduced in clinical practice in the late 1980s, there are still controversies in regards to the hemoglobin level at which ESA therapy should be initiated and the targeted hemoglobin level. Current evidence suggest that a hemoglobin level greater than 11 g/dL is associated with increase in physical performance, with reduced risk for hospitalization and with a significant regression of left ventricular hypertrophy of uremic patients with anemia. One important question is to what extent anemia in CKD patients could be corrected without negative effects upon these patients.

The recent recommendations National Kidney Foundation Kidney Disease Outcomes Quality Initiative (K/DOQI) suggest that ESA therapy should be initiated when hemoglobin level falls bellow 9.0 g/dL [81]. These recommendations also suggest that the target hemoglobin level should be 11.0 g/dL or greater, with cautions when hemoglobin level is intentionally maintained at 13.0 g/dL.

Paoletti et al. analyzing results of different randomized studies showed that normalization of hemoglobin level is associated with an improvement of the quality of life but not with reduced mortality and hospitalization rate in uremic patients [82].

A recent Canadian study indicated that a level of hemoglobin between 12 and 14 g/dL had no effect upon left ventricular hypertrophy [83].

Trying to completely correct anemia with ESA therapy could have a negative effect due to increase of the hematocrit level, which will lead to increase blood pressure, hypertension and risk of thrombosis.

Two recent studies tried to address the issue of relation between anemia correction and cardiovascular outcome and risks: (i) the Cardiovascular Risk Reduction by Early Anemia Treatment with Epoetin Beta (CREATE) [84] trial and (ii) the Correction of Hemoglobin and Outcomes in Renal Insufficiency (CHOIR) [85]. The CHOIR trial reported that using target hemoglobin level of 13.5 g/dL increases the risk of death caused by cardiovascular events among patients with CKD anemia. On the other hand, the CREATE study reported that between two groups of CKD patients (stage 3 or 4) were no differences in regard to the risk of cardiovascular events when using a target hemoglobin levels of 13.0 to 15.0 g/dL, and respectively a lower target range of 10.5 to 11.5 g/dL. Also, in the CREATE study it was found that high target hemoglobin levels did not ameliorate left ventricular hypertrophy.

Both CREATE and CHOIR studies proved that a high target hemoglobin level is not beneficial for CKD patients as they increase the risk of cardiovascular events and mortality. As a consequence, both studies recommend a partial correction of anemia in uremic patients.

6.1 Side effects generated by administration of rHuEpo and its analogues

Initially it was believed that EPO-R expression is confined to the erythroid progenitors, and so it was appreciate that rHuEpo therapy will have a high degree of specificity with few if any nonerythropoietic effects. Further studies showed that EPO-R is expressed by a very large number of normal and tumoral cells, suggesting more pleiotropic functions for EPO [45, 86–88]. This observation raised the question of potential side effects of high doses of rHuEpo or analogues outside of the erythron. The fact that EPO induces, through interaction with EPO-R, angiogenesis [89, 90] and enhancement of cancer cells growth is a major concern about clinical use of rHuEpo and analogues [91]. Therefore, more studies are required to fully understand if human EPO (rHuEpo and analogues) is able to induce tumor growth, as present studies offer controversial results.

Although rHuEpo and its analogues have proved to be very effective in treating anemias of different etiologies, there are also some side effects such as hyperthension, thrombosis and allergic reactions. One of the most important side effects is a condition known as pure red cell aplasia (PRCA).

PRCA is defined by the absence of erythroblasts in the bone marrow. Antibodies generated against rHuEpo will neutralize not only the recombinant protein, but also the native erythropoietin, leading to the absence of red cell precursors in the bone marrow. In these conditions patients will develop an EPO-resistant anemia and will need blood transfusions. The immunological mechanism for developing antibody-mediated PRCA is unknown, but there are some factors incriminated for increasing immunogenicity of EPO and analogues and EPO-mimetics. Such factors could be the degree and nature of glycosylation, the manufacturing process, handling and storage, and components and properties of the product formulation.

The rates of antibody-mediated PRCA in CKD patients treated with epoetin alfa and epoetin beta were similar between 1989 and 1998 [92]. After 1999 the frequency of antibody-mediated PRCA increased significantly in the case of an epoetin-alfa formulation (EprexTM) [93]. It was suggested that the cause was the removal of human serum albumin (HSA) from one epoetin alfa formulation in 1998 [94]. Those formulations of EprexTM where HSA had not been removed lead to a very low incidence of antibody-mediated PRCA. In those formulations where HSA was removed it was replaced with polysorbat 80 and glycine. These compounds had lead to a reduction of protein stability and an increase of immunogenicity [95].

7 Conclusions

The full understanding of the EPO gene regulation, of the EPO - EPO-R interaction, and of how this interaction activates different signaling pathways will permit the rational design of new erythropoietic agents. These new agents could be used to correct the anemia of different etiologies and to improve the overall state of wellbeing of patients affected by anemia. An important aspect of future studies on erythropoietic agents is their selective

action upon erythron, without stimulation of other types of cells, in particular cancer cells.

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