OXYGEN TRANSPORTERS – PRODUCTION METHODS OF NATURAL AND ARTIFICIAL BLOOD

TRANSPORTERY TLENU – METODY POZYSKIWANIA NATURALNEJ I SZTUCZNEJ KRWI

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Summary: Oxygen carriers could be divided into three groups: natural, "in vitro obtained" and artificial. Last two types have to be as similar to the natural ones as possible to be considered effective. In the process of in vitro red blood cells production, proliferation, and differentiation, haematopoietic stem cells seem to be the most promising. Although the progress in this area is very dynamic, there are still some challenges that must be resolved like a low yield of red blood cell production and high costs of in vitro cell cultures. Another attempt is connected with the use of induced pluripotent stem cells. However, results of research in this field are still not satisfactory and do not allow to use this strategy in clinical applications. Artificial blood synthesis focuses mainly on the production of a carrier, that would transport oxygen in a similar way to haemoglobin. In the treatment of severe clinical cases, a different approach based on pharmacotherapy and hormonotherapy is currently used. It seems to be relatively safe and improves the production of blood cells directly in patients.
This work reviews current research on the production of oxygen carriers. Moreover, it shows the molecular basis of haemopoietic system development and erythropoiesis, which are essential to understand the process of blood cell formation.

Keywords: oxygen carriers, artificial blood, blood production, haematopoiesis

Streszczenie: Transportery tlenu można podzielić na trzy grupy: naturalne, „otrzymane *in vitro*" i sztuczne. Ostatnie dwa rodzaje muszą być podobne do naturalnej krwi, żeby można uznać je za efektywne. W procesie pozaustrojowej produkcji krwi, najbardziej obiecujące wydaje się być namnażanie

i różnicowanie krwiotwórczych komórek macierzystych. Chociaż obserwuje się dynamiczny postęp w tym zakresie, nadal wiele wyzwań, takich jak mała ilość otrzymywanych erytrocytów i wysoka cena hodowli komórkowych *in vitro,* czeka na rozwiązanie. Inne próby wiążą się z użyciem indukowanych komórek macierzystych. Jednakże wyniki badań nie są nadal satysfakcjonujące i nie pozwalają na ich zastosowanie w terapiach klinicznych. Synteza sztucznej krwi skupia się głównie na produkcji nośników, które będą w jak największym stopniu podobne do hemoglobiny. W ciężkich przypadkach klinicznych stosuje się inne podejście oparte na farmakoterapii i terapii hormonalnej. Uważa się je za stosunkowo bezpieczne i pozwalają one na poprawę produkcji komórek krwi bezpośrednio w organizmie pacjenta.
Ta praca stanowi przegląd najnowszych badań na temat produkcji sztucznych nośników tlenu. Ponadto, przedstawia podstawy molekularne rozwoju układu krwiotwórczego i erytropoezy, co jest istotne dla zrozumienia procesów tworzenia komórek krwi.

Słowa kluczowe: przenośniki tlenu, sztuczna krew, produkcja krwi, hematopoeza

INTRODUCTION

During each second, 2 millions of red blood cells are being replaced by the new ones in the human body [66]. Therefore, the disorders in haematopoiesis implicit severe clinical consequences. Some of them are associated with disturbances of the erythropoietic pathway. The others are connected with biochemical disorders, such as the lack of erythropoietin receptor.

Medical care in the modern world concentrates not only on the survival of haematological patients but also on the improvement of their life quality. Finding a reliable method of in vitro production of red blood cells or synthetic oxygen carriers generation would increase the access to blood transfusion. Because of that, more than any time before, researchers are trying to find an effective method of oxygen transport to the tissues.

The essential signal for erythropoiesis is erythropoietin (EPO). Steroid hormones can stimulate the above-mentioned process as well, but only in cooperation with EPO. Steroids impact also on the increased production of haemoglobin in further stages of erythropoiesis [85].

A complex insight into erythroblasts biology is crucial to understand haematopoietic system disorders better. On the one hand, the diversity of plasma membrane receptors on the erythroid progenitors and their ligands give a high chance of erythropoiesis modulation in severe clinical cases. On the other hand, understanding the biochemical bases of carrying oxygen gives an excellent chance for synthetic oxygen carriers production.

In this paper, we have gathered the current research about the in vitro attempts of red blood cells production and about artificial oxygen carriers.

ERYTHROID CELLS AND THEIR MARKERS

Erythropoiesis is a part of haematopoiesis process, which involves the development of red blood cells (Fig 1.). It begins with the differentiation of multipotential hematopoietic stem cells (HSC) into progenitor erythroid cells: early-stage burst-forming unit – erythroid (BFU-E) and late-stage colony – forming unit-erythroid (CFU--E). There can be distinguished two stages of stem cells before the differentiation occurs: haematopoietic stem cells (HSCs) and multipotential progenitors (MPPs).

HSCs proliferate to increase the population of stem cells. Moreover, they can differentiate into any type of cell due to the activity of the pluripotent cells. Their antigen properties can be described by the presence of CD150 (glycoprotein expressed on the surface of T, B, natural killer, and dendritic cells), CD201 (endothelial protein C receptor), CD110 (thrombopoietin receptor), specific for human CD90 (glycosylphosphatidylinositol (GPI)-linked glycoprotein receptor), CD49f (integrin α6) and low expression of Rho (GTPase), CD34 (transmembrane phosphoglycoprotein) [84, 4, 14, 10, 54, 92, 80]. The antigens absent onto HSCs are CD48 (B-lymphocyte activation marker [52]), CD135 (fms like tyrosine kinase 3 [40]), and, CD45RA (naive/memory common lymphoid marker [88]), Furthermore, specific antigens are expressed by all lines up to multipotential progenitors (MPPs) like CD34, CD117hi (transmembrane tyrosine kinase receptor), and Sca1hi (stem cells antigen-1), while Lin (mature cell lineage marker) and CD38 (cyclic ADP ribose hydrolase) are not detected [64].

MPPs have the ability to differentiate only into cells of the specific germ layer. There could be distinguished three types of MPPs: intermediate-term repopulating IT-MPP (CD150+, CD48-, CD34lo, CD135-, CD49b+), short-term repopulating ST-MPP (lacks CD150 and exhibits higher levels of CD34 in comparison to IT--MPP) and proper MPP line. Propper MPPs differ from ST-MPPs by the occurrence of CD48 antigen [64]. In contrast to LT-HSCs, all types of MPPs are CD49b+ [61].

MPPs further differentiate into common myeloid progenitors (CMP). CMPs are the first stage of cells, which do not possess an ability to differentiate into lymphoid progenitors, but rather only to megakaryocyte/erythrocyte or granulocyte/macrophage progenitors [41]. CMPs could be characterized by the presence of GATA-1 (Erythroid transcription factor, GATA binding protein 1), GATA-2 (GATA Binding Protein 2), NF-E2 (complex essential for regulation of erythroid maturation), TAL1 (bHLH transcription factor crucial for erythroid differentiation) proteins, but the lack of GATA-3 (GATA Binding Protein 3). These proteins are in an opposite state in common lymphoid progenitors (CLP) [1].

A burst-forming-unit-erythroid (BFU-E) is the first cell type that arises from CMP. Its characteristics include low frequency of dividing and the need to be sti-

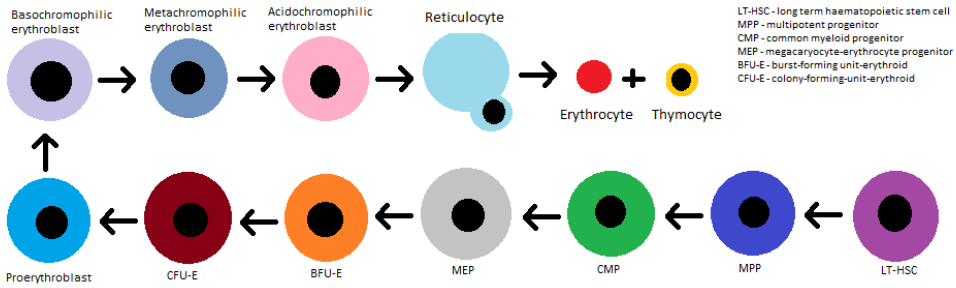

FIGURE 1. The process of haemathopoiesis: LT-HSC – long term heamatopoietic stem cell, MPP – multipotent progenitor, CMP – common myeloid progenitor, MEP – megakaryocyte–erythroid progenitor cell, BFU-E – Burst-forming unit-erythroid, CFU-E – colony-forming unit-erythroid
RYCINA 1. Proces hematopoezy: LT-HSC – *ang.* long term heamatopoietic stem cell, MPP – *ang.* multipotent progenitor, CMP – *ang.* common myeloid progenitor, MEP – *ang.* megakaryocyte–erythroid progenitor cell, BFU-E – *ang.* Burst-forming unit-erythroid, CFU-E – *ang.* colony-forming unit-erythroid

mulated by many growth factors, such as granulocyte-macrophage-colony-stimulating factor (GM-CSF), interleukin-3 (IL-3), EPO [91] as well as interleukin-9 (IL-9) [19].

BFU-E differentiates into colony-forming-unit-erythroid (CFU-E) – the next stage of cell in the erythropoiesis, which in opposition to BFU-E possess a great ability to proliferation and is more sensitive to EPO [19]. The name of the line comes from the ability to form colonies, after stimulation by IGF-1, EPO, but unlike in BFU-E, not IL-3 [19].

The earliest erythroid precursors are proerythroblasts, which differentiate into basochromophilic erythroblasts [82]. Both of these cellular precursors are rich in CD71 (transferrin receptor), CD36 (scavenger receptor) and CD235a (glycophorin A) antigens [90]. The CD34, which is considered as a marker of the stem and progenitor cells, disappears from erythroid precursor cells, although some investigations confirm its slight presence on proerythroblasts [90]. The expression of CD117 is detectable only in proerythroblasts, while CD38, CD45 (leukocyte common antigen) and HLA-DR (Human Leukocyte Antigen – DR isotype) levels decline along with the maturation of the cells. Even though proerythroblasts are mostly connected with the erythroid cell line, they also possess the ability to differentiate into other than basophilic erythroblasts cell lines in response to different signaling molecules [34].

Next transitions into metachromatophilic and ortochromatophilic erythroblasts, as well as reticulocytes, are accompanied by a further decrease of CD36. Only the expression of CD71 and CD235a remains in all-stage erythroblasts, although mature erythrocytes are CD71 negative. Metachromatophilic erythroblasts arise from basophilic erythroblasts and are the last stage, that possesses the ability to proliferate.

The final step of differentiation leads by reticulocytes into mature red blood cells, which remain the high expression of CD235a but lose completely CD36, CD117, HLA-DR antigens [90].

MORPHOLOGICAL CHANGES OF ERYTHROID CELLS

The erythroblast maturation is accompanied by dramatic changes in the morphology of the cells. The cell size decreases along with the erythropoiesis transformation. Proerythroblast radius ranges from 7.5 to 10 μmeters, what is about two times longer than in case of the other erythroid cells apart from basophilic erythroblasts [19]. Almost all intercellular space is occupied by a big, rounded nucleus, with visible many small nuclei. The distinct decondensation of chromatin is caused by intense gene transcription [62]. The cytoplasm of proerythroblast is moderately acidic, due to the extensive protein synthesis. The microscopic study considers the presence of Golgi apparatus at this stage of erythroid differentiation in contrast to basophilic erythroblasts. On the one hand, the haemoglobin synthesis is initiated, which leads to an increase in pH.

On the other hand, the cytoplasm is still strong basophilic because of an abundant number of ribosomes. The nuclear chromatin is heterochromatic [47]. The next steps of erythroid maturation lead to intensive nuclear size reduction and changes in ribosome amounts. The synthesis of alkaline haemoglobin is ended after the ribosome degradation in mature red blood cells [47]. Erythroblasts indicate specific for each stage pattern of haemoglobin (Hb) content. In comparison to the previous stages, metachromatophilic and ortochromatophilic erythroblasts posses the exceeded lifetime (up to 24 and 30 hours respectively) [67].

Directly after nuclei excretion, the cells turn into reticulocytes [30]. The process of nuclei excretion is the time-limiting point in red blood cells production attempts in mammals. Ennucleation includes, actin filaments polymerisation followed by the shift of the nucleus to random side of the cell. Some researchers suggest the participation of protein sorting and vesicle trafficking in erythroblast enucleation [26, 38, 78].

HAEMATOPOIETIC SYSTEM DEVELOPMENT

Haematopoiesis is initiated in the third week of human development in a yolk sac. Furthermore, in the 27th day, hematopoietic stem cells arise from the endothelial lining of the aorta and vitelline artery [74]. Therefore, primitive haematopoietic cells have the same mesodermal origin as endothelial cells [17]. The stem cells termed hemangioblasts form islets, which are responsible for maintaining the proper environment for the production of red blood cells [74].

After the end of the first month of development, the foetal liver begins to produce red blood cells and slowly replaces the yolk sack-derivated cells. The process of cell exchanging is completed by the end of the 3rd month [65]. In this organ, erythropoiesis takes place in star-shaped structures formed by the macrophages, which are surrounded by erythroblasts in different stages of maturation. More primitive cells like proerythroblasts are located close to the macrophage, while mature cells lose the connection with it [17]. Crucial for liver haematopoiesis seem to be Kruppel like factor 1 (KLF1), that induces beta-globin synthesis. As a transcription factor, which binds directly to the CACCC box (sequence CCACACCCT) in the beta-hemoglobin promoter, KLF1 enables the change of epsilon chains into mature beta chains of haemoglobin [54]. Another crucial factor is GATA binding protein 3 (GATA3), which is considered essential for proper foetal liver haematopoiesis and nervous system development [57].

After the second month of development, haematopoiesis centres are also formed in the spleen, which shares the same haematopoietic structure with the liver [16]. Some data suggest, that most splenic haematopoiesis centres represent further stages in the development of erythroid lineage, rather than multipotential or self-maintaining. Moreover, the cells are destined to disappear from the spleen within 72 h [39]. Further studies have shown, that haematopoietic cells in the spleen can be divided into two groups: pluripotent haematopoietic stem cells and more committed progenitors [27].

Splenic haematopoiesis is completely suppressed before the 8th month in contrast to process in the liver which is active almost to the birth. Since the 5th month of development, the bone marrow starts the production of red blood cells [89]. At that place, the HSCs settle the microenvironment called “niche” wherein more mature erythroblasts are localized in the central part in contrast to more primitive forms, which are pushed aside, next to the inner periosteum [87]. Protooncogene C-MYB, downregulated by miR-150, is crucial for splenic and liver embryonic haematopoiesis [29]. Other genes, which mutations in early stages of development result in death, are retinoblastoma (RB1), core-binding factor subunit alpha-2 (CBFA2) and previously mentioned EKLF [33, 70, 48]. Rb1 protein modulates cell-cycle progression via elongation factor 2 (E2F) regulation [49]. CBFA2 encodes a transcription factor, which is believed to be required for the transition from endothelial to haematopoietic cells [37].

In the postnatal human life, the red bone marrow level decreases over time and is replaced by the yellow bone marrow. In each bone, the level of marrow declines with different speed. In the vast majority of long bones, this amount falls to zero in the diaphysis, and only small quantities remain in the epiphysis. However, in some bones, the level of bone marrow stabilizes and remains constant throughout the whole life. The highest level of bone marrow could be found about 80% of the initial amount in vertebras and pelvis, as well as 50% and 25% in sternum and ribs respectively [65].

MOLECULAR REGULATION OF ERYTHROPOIESIS

During erythropoiesis, several intracellular changes occur. Alternations could be divided into two groups: biochemical and structural.

Biochemical changes are connected with haemoglobin occurrence, as well as with the expression of cell lineage-specific proteins, which mainly belong to hormone receptors and cell adhesive proteins. Mostly connected with EPO stimulation are proerythroblasts, which possess heterogeneity in EPO sensitivity among individual cells [31]. However, neither EPOR expression nor its affinity to EPO explains their varied respond for EPO stimulation. One of the potential explanation of this phenomena could be connected with different glycosylation forms of EPOR. The 62kDa isoform does not contain glycans whereas the highly glycosylated 78kDa protein is considered as an active receptor [29, 63]. The haemoglobin synthesis in EPO -stimulated proerythroblasts as well as its proliferation increases through stimulation of iron ions intake and DNA synthesis.

Similarly, steroid hormones induce haemoglobin synthesis, although the proliferation remains intact [71]. For instance, basophilic erythroblasts indicate an increased haemoglobin synthesis in response to androgens stimulation [85]. However, the proliferation of proerythroblasts (from primitive cell culture) is inhibited by incubation with glucocorticoids. Moreover, in the experiment, glucocorticoids have diminished effects of EPO on proliferation [85]. Erythroid maturation is thought to be inhibited by glucocorticoids, which act through both transcription and EPO receptor pathway regulation. Glucocorticoids do not exhibit any cellular specificity [35], whereas androgenic steroids have a stronger effect on more mature erythroid precursors. Extreme proliferation stimulation has been obtained by incubation with EPO and testosterone, and dihydrotestosterone. What is more, the presence of the mentioned hormones results in the decrease up to 10% of the initial level in minimal EPO concentration, required to support the culture [85]. The binding between the FAS and FAS ligand is mainly active in progenitors and acts in opposition to the EPO-induced pathway [15].

To direct proerythroblasts into red blood cells line GATA-1 is required [56]. IL-3 stimulation leads to mast cells differentiation [33], CCAAT-enhancer-binding proteins (C/EBP) and EPO to neutrophils, while GM-CSF treatment and GM-CSF differentiates proerythroblasts into macrophages [34]. At this step, differentiation into lymphocytes and megakaryocytes is impossible. That has been proved experimentally by stimulation with thrombopoietin (TPO), genetically modified proerythroblasts, which overexpressed the thrombopoietin receptor, [34].

In vitro studies on FVA cell line (immature murine erythroblasts infected with the anemia-inducing strain of Friend virus) shows, that EPO stimulates phosphorylation of T-cell acute lymphocytic leukemia protein 1 (TAL1) [31]. The EPO receptor, in response to stimulation, activates protooncogene serine/threonine-

-protein kinase RAF1. RAF1 binds to Src homology 2 (SH2) domain of growth factor receptor-bound protein 2 (GRB2). As a result, RAF1 changes its phosphorylation state and initiates a mitogen-activated protein kinases (MAPK) cascade [31]. Besides, there is an alternative pathway of MEK activation by phosphoinositide 3-kinase (PI-3K), which is also activated by EPOR [31]. TAL1 protein can be phosphorylated by serine/threonine kinase ERK1 or MAPK in response to endothelial growth factor (EGF) stimulation. The phosphorylation of serine 122 and probably 172 occurs with the presence of p300 coactivator. Phosphorylated TAL1 is an excellent indicator of EPO-induced PI-3K-MAPK signaling pathway [73]. Proerythroblasts are the first cells that have an ability to perform globin synthesis. Bioaccumulation of haemoglobin (Hb) varies in time in the cell cycle. It is postulated that in G1 and early S phase, Hb is accumulated in contrast to late S and G2 phase, when Hb concentration decreases, due to the dilution by other, newly synthesized proteins [93]. Heamoglobin synthesis can be stimulated by iron-containing porphyrins. The study on hemin shows that haemoglobin synthesis increases up to 4-5 times in rabbit reticulocytes, while in hepatocyte carcinoma (FLC cell line) it does not exceed 20% [44]. The effect decreases in time, and after long times of incubation, the level of haemoglobin has no significant difference with the sum of haemoglobin obtained from reticulocytes and FLC alone [44]. Long non-coding RNA (lncRNA) may also impact on haemoglobin synthesis regulation. Lack of urothelial cancer associated 1 lncRNA (UCA1) impairs heme biosynthesis and therefore stops erythroid differentiation [38].

Structural changes include chromatin remodeling, enucleation, cytoskeleton reorganization and changes in plasma membrane liquidity. More mature HSC stages of haematopoietic stem cells are more likely to exhibit an euchromatic genome state, due to the remodeling by SWItch/Sucrose Non-Fermentable (SWI/SNF) complexes. Curiously, the levels of histone deacetylases HDAC-1, HDAC-5, HDAC-6 and DNA methyltransferase DNMT3b are decreased, which correspond to downregulation of SMARCA1 and SMARCA4 genes [24].

The most spectacular cytoskeleton changes occur during enucleation. Researchers emphasize the role of Rac-1 GTPase, which in cooperation with ARF6 (ADP Ribosylation Factor 6) reorganizes the cytoskeleton [13]. p38 MAP – stress-induced protein kinase also plays a role in cytoskeleton reorganization [77]. Research performed on mouse erythroleukemia cell line SKT6 suggest, that EPO activates MAP and JNK kinases, but does not affect ERK [50]. Reticulocytes are the first erythropoietic cells that could be found in the peripheral blood. The occurrence of basophilic granulated endoplasmic reticulum is the attitude, which differentiates them from erythrocytes.

An interesting factor of proerythroblasts differentiation is the change in cell membrane liquidity. The liquidity decreases in response to differentiation signals but can be restored by an increase of the temperature [58].

CLINICAL SIGNIFICANCE

Pathological changes in erythroid progenitors result in severe clinical conditions because all further haematopoietic cells stages are affected. There could be distinguished three types of disturbances: primitive, acquired and quantitative: neoplasms and anaemias [35].

Acquired disorders can occur in response to physical damage, like irradiation of the haematopoietic organ [86]. The effect is highly dependent on the dose of radiation, time of exposure and life stage of developing human. Improper diet and lifestyle can develop into anaemia [18].

Primitive disorders, affecting crucial for erythropoiesis proteins, are much more complicated. Biochemical disturbances in erythropoiesis are mainly connected with pathological down or upregulation of cytokine receptors. Genes, which mutations in early stages of development result in death, are retinoblastoma gene, core-binding factor subunit alpha-2 (CBFA2), EKLF and C-MYB. In most cases, their absence does not affect yolk sac haematopoiesis, which indicates that yolk sac stem cells and foetal-liver stem cells differ in sensitivity to mutations [26, 33, 35, 51]. This could happen due to the different globin chains synthesis and its regulation in each organ.

During development, defective haematopoiesis in foetal liver can be caused by the inactivation of KLF1, that leads to the lack of beta-globin synthesis. The disruption of GATA-3 protein results in growth retardation, internal bleeding, deformities of brain and spinal cord [57].

CBFA2 is a transcription factor essential for early stages of haematopoietic system development [55]. Interestingly, the disruption of even one copy of the CBFA2 gene reduces the number of progenitors for erythroid cells [33].

Embryos with C-MYB knockout die at stage E15 due to the insufficiency of blood production. As a proliferation accelerator, it is present in a wide variety of neoplasms, for instance, acute myeloid leukemia. Neoplasms could be regulated by pharmacological targeting of C-MYB using mebendazole [83] and by downregulation of C-MYB translation by miR-150 [29].

Thalassemias are described as blood disorders characterized by anomalies in the biosynthesis of haemoglobin chains [20]. In most cases, improper haemoglobin chain is a result of a change in single amino acid in the polypeptide chain. For instance, abnormal human haemoglobins Iwate and Hyde Park form when the proximal histidine is replaced by tyrosine. Replacement of distal histidine in the beta chain of haemoglobin results in Boston and Saskatoon forms. The oxygen transport failure, caused by the mutations, is a result of stabilization of Fe (+III) in heme in haemoglobin structure [59]. Patients with beta-thalassemia exhibit increased the concentration of EPO in the blood. In this condition, ERK1/2 phosphorylation is enabled, therefore stimulating the differentiation into erythrocytes.

Interestingly, current research shows a significant difference in response to serum cytokines by normal and affected cells. The ERK1/2 phosphorylation is noticed only in abnormal erythroblasts, although other kinases like MEK, c-RAF and b-RAF phosphorylation remains at the same level. Further investigations revealed the increased cellular concentration of calcium ions and cAMP, which respectively activates MEK via PKC and EKR1/2 via PKA. Clinically significant could be the fact, that there is a high correlation between cAMP level in polychromatic and acidophilic erythroblasts and foetal haemoglobin (HBF) level in blood [85].

Anaemia is a complex condition characterized by the low concentration of erythrocytes or haemoglobin. Classification of anaemia is based on RBCs amount and properties, like mean corpuscular volume or heterogeneity [6]. The disease could be the result of impaired production or excessive destruction of RBC. Also, fluids equilibrium are essential in maintaining proper RBCs concentration. Improper diet and lifestyle can develop into anaemia as well. General symptoms include pale skin, fatigue, and rapid heartbeat.

Several factors, like disrupted haemoglobin synthesis (thalassemia) or iron deficiency lead to impaired RBCs production, sensitivity to EPO or deficiency of vitamin B12 results in anaemia as well [63]. Erythropoiesis can be interrupted at all previously mentioned stages, which results in decreased RBCs amount. Interestingly, case reports show condition in which proerythroblasts proliferate and differentiate properly, but the anaemia occurs [94]. Due to the cytotoxic patients' plasma, erythroblasts are being damaged. Application of immunosuppressive drugs as well as splenectomy could be beneficial in that condition.

Increased destruction of erythrocytes is often a result of inappropriate cell structure. In some cases, like hereditary spherocytosis or elliptocytosis, plasma membrane proteins, like spectrin or ankyrin are affected. In other cases, like sickle cell anaemia, the unoxygenated haemoglobin aggregates, which leads to shape deformation. Defective cells are captured and degraded by spleen [60]. Interestingly, enzymes defects, like glucose-6-phosphate dehydrogenase insufficiency, leads to accumulation of reactive oxygen species in erythrocytes, resulting in methaemoglobin accumulation and cell destruction [68].

Neoplastic cases contain proliferative disorders in blood composition. Erythroleukemias are neoplasms associated with erythropoiesis. The concerns only cell stages, that posses an ability to proliferate, for instance, proerythroblasts [43]. In this disorder, immature forms are predominated by myeloid blasts [36]. This phenomenon is most often caused by mutations within the P53 suppressor gene, which promote cancer progression [46]. The extensive proliferation of low differentiated bone marrow cells, including myeloid precursors, representing up to 80% of all cells [43] is characteristic in marrow-derived neoplastic diseases. The most severe and complex leukaemia, characterized by complex karyotypes, is pure erythroid leukaemia. Maturation arrest with hyperproliferation is responsible for a very aggressive clinical course [46].

HORMONAL THERAPIES THAT TRIGGER RED BLOOD CELLS MATURATION

When the structure and function of erythrocytes are proper, but the amount of cells is too low, the clinical focus on stimulation of the erythroid progenitors' maturation. Anaemias are diagnosed in 20-60% of patients with thyroid disorder [3]. Clinical studies carried out by Carmel et al. have revealed that among pernicious anaemias, 24,1% of cases suffered from clinical thyroid disease and 11,7% from hypothyroidism. Moreover, abnormal thyroid stimulating hormone (TSH) occurred in 48,3% of patients [9]. An observation that patients with thyroid insufficiency often suffer from anaemia has led to the conclusion, that thyroid hormones could be easily used as a maturation accelerator [21]. It has been established, that triiodothyronine (T3) acts on a transcriptional regulator Trip-1. Then, Trip-1 connects with tyrosine kinase Lyn, which is also able to interact with EPOR. Strikingly, T3 is responsible for cell divisions, but in contrast to EPO, it inhibits erythroid maturation [26].

Others, like glucocorticoids, stimulate cell proliferation during stress erythropoiesis. These hormones induce proliferation of erythroid progenitors and its maturation in cooperation with EPO [81]. In vivo, glucocorticoids are responsible for the physiological response to hypoxia via accelerating the expansion of erythroid progenitors in bone marrow [5].

IN VITRO PRODUCTION OF RED BLOOD CELLS

To provide great volumes of red blood cells (RBC) for transfusion, the development of in vitro production of erythrocytes is very desirable. The process should aim to differentiate HSC only into erythrocytes to reach maximum efficiency. Additionally, the fact of the presence of antigens, which are specific to donor, on progenitor and precursor cells should be considered in designed protocols. To avoid this problem, studies are concentrated mainly on the use of stem cells.

First attempts of RBC ex vivo production had a serious drawback because the feeder cells had to be present in the culture additionally [23]. That approach made the culture harder and increased its costs. On the other hand, feeder cells are crucial in coculture, because they stimulate HSC to express a high level of EPOR [7]. Further attempts have concentrated on the elimination of feeder cells from the culture. Miharada et al. have established the first protocol for production of enucleated red blood cells from umbilical cord HSCs without using feeder cells. The protocol includes four passages and stimulation with cytokines, i.e. EPO, SCF, IL-3, VEGF, IGF-II as well as mifepristone treatment, supplemented with D-mannitol, adenine, and disodium hydrogen phosphate dodecahydrate. The whole pro-

cess lasts twenty days and leads to obtain basochromophilic proerythroblasts and then, further self-extrusion of the nucleus in precursor cells [45].

In most cases, produced RBCs differ slightly in morphology and immunology in comparison to RBCs, obtained under physiological conditions. In general, stem cell-derived individuals have greater volume and up to 50% higher concentration of haemoglobin [69]. Usually, the haemoglobin contained mainly foetal chains, which possess higher affinity to oxygen, which could be problematic in clinical applications [44, 11, 2].

To optimize the yield of erythrocyte production, Giani et al. have incorporated genome editing in their studies. SH2B3 gene (lymphocyte adapter protein) product acts as a negative regulator of cytokine signaling, therefore inhibiting the HSC response to proliferation and maturation activators. Scientists targeted SH2B3 with CRISPR/Cas9 genome editing system to silence the gene expression. Such manipulations resulted in enhancement of the number of differentiated RBCs [22].

In order to obtained a cellular model for further investigation, Hiroyama et al. isolated mouse HSC and differentiated them into MEDEP progenitor cell line. Furthermore, they showed that immortalization of erythroid progenitor cells could be performed at various stages of differentiation [25].

Even though there are methods of ex vivo RBCs production, the potentially enormous costs of developing the technology prevent its implementation in clinical practice [51].

An alternative source of erythrocyte precursors can be induced pluripotent stem cells (iPSC), which are progenitor cells, obtained by dedifferentiation of mature cell lines. In order to be suitable for clinical applications, iPSC-derived erythroid cells have to be similar to normal adult erythroid cells. Scientists confirmed that iPSC--derived erythroblasts resemble control erythroblasts in terms of genes expression and general biological function [79]. It is noteworthy that significant differences in cytoskeleton were revealed by proteome comparing of iPSC-derived erythroid cells with normal human erythroid stem cells. That results in a low level of enucleation in iPSC-derived cells. Only 10-15% iPSC –derived erythroid cells turn into reticulocytes in comparison to normal erythroid cells [76]. Presented results indicate that iPSCs have potential use in ex vivo production of RBCs, but further research to make iPSCs more similar to natural ones, should be done in this field [72].

ARTIFICIAL BLOOD

Along with the rapid development of surgical medicine, there are problems with the collection and use of natural blood due to the insufficient number of donors, and the short shelf life of blood. Moreover, there are some limitations, caused by different blood types and the possibility of contamination with blood-borne

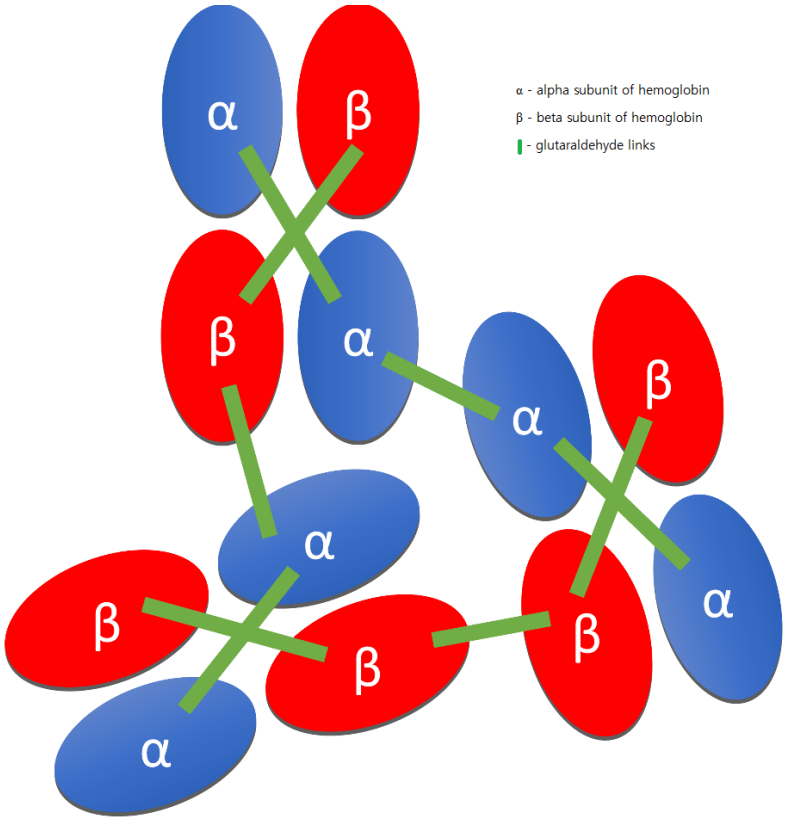

FIGURE 2. Schematic depiction of Hemopure[R], the green bars indicate glutaraldehyde links between hemoglobin proteins
RYCINA 2. Schematyczne przedstawienie Hemopure[R], zielone linie przedstawiają glutaraldehyd łączący hemoglobinę

viruses like HIV or Hepatitis [32]. It is estimated, that each year about 2 million people die of blood loss, which is about 12% of total deaths [9, 67]. To solve these problems, several alternative substitutes were developed and tested instead of human's blood like animal's blood, milk, urine or even beer. However, all these attempts ended without any success [11]. Finally, the idea of producing a partially or entirely artificial blood (AB1) roses.

The primary investigation has been focused on the development of materials with high affinity to oxygen. It turned out that hemoglobin-based oxygen carriers (HBOCs) are less toxic and more efficient in oxygen transport than other substitutes like a perfluorocarbon-based ABl [12]. The primary current source of HBOCs is expired human blood bags, but there are also other sources like blood from the meat industry and recombinant haemoglobin produced by transgenic bacteria or yeasts [48, 72].

The HBCOs can be divided into encapsulated and non-encapsulated. The first one are encapsulated with the lipid bilayer, monolayer or polymer and the second one are mostly cross-linked haemoglobin tetramers. Out of non-encapsulated HBCOs, the most promising is HemopureR, developed by OPK Biotech, which reached phase III clinical trial (clinical trial identifier: NCT01881503) to this point [8]. The HemopureR is bovine hemoglobin cross-linked with glutaraldehyde to form stable tetramers and further agglomerates (Fig. 2). Another one is Polyheme, which consists of human pyridoxylated human haemoglobin [8]. The ABl is similar to the Hemopure, and the difference is the use of pyridoxal to obtain more physiologic partial pressure of saturation (P50).

Nevertheless, the use of Polyheme was rejected by the US Food and Drug Administration due to hypertension problems after application. Most of non-encapsulated HBCOs cause severe pulmonary and systemic vasoconstriction due to nitric oxide (NO) scavenging property of haemoglobin [42]. However, these problems can be solved by the NO inhalation during the use of HBCOs.

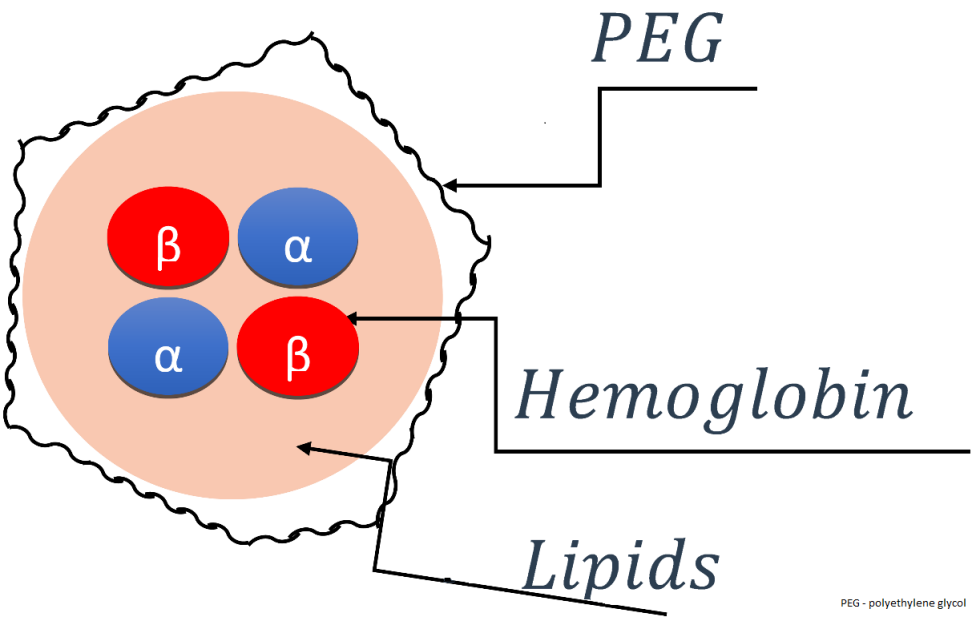

FIGURE 3. Example of NRCs, alfa and beta are subunits of hemoglobin tetramer; pink circle indicates lipids encapsulating the hemoglobin and black rope is PEG coating the lipid vesicle
RYCINA 3. Przykład NRC, α i β to podjednostki tetrameru hemoglobiny; różowe koło oznacza lipidy enkapsulujące hemoglobinę; czarna linia to PEG otaczający lipidowy pęcherzyk

Encapsulated HBCOs do not have the vasoconstriction problem in comparison to the non-encapsulated. The most known example are the Neo Red Cells (NRCs) developed by Terumo Corp in Japan, which contain hemoglobin from outdated blood cells. The NRCS consists of encapsulated inositol hexaphosphate coenzyme and substrates (to reduce methemoglobin back to haemoglobin) in liposomes. Lipid particles are coated with polyethylene glycol (PEG) (Fig. 3) to prolong half-life in the circulatory system and further modified with phosphatidylethanolamine to prevent aggregation [78]. The NRCs were tested on some animal species, like monkeys, rats, dogs and exhibited very prominent results of being safe and efficient. Further, they can be stored for about 320 days in room temperature which makes them cheap to store.

Another example is Hemoglobin-Vesicles (HbVs) developed at Waseda University. HvVs are encapsulated hemoglobin proteins and further coated with PEG. The HbVs toxicity was tested on pregnant mother rats showing minimal changes of lipids, bilirubin, and ferric iron (Fe3+). Furthermore, no accumulation in the fetus was observed [28]. Nevertheless, its storage life is lower than in the case of NRC (120h). Similarly, its half-life is also lower and depends on the health condition of animals [75].

CONCLUSIONS

Past efforts have mainly focused on the stimulation of erythroid precursors proliferation and maturation. The potential benefit of the strategy is that the standalone organism deals with the problem of hypoxia. Although efficient, pharmacological treatment has introduced physiological balance disorders. As the reason of that, future efforts mainly focus on artificial and ex vivo blood production. The strategy would mimic physiological body state better and would not dysregulate the natural balance. There are still problems, which have to be solved, like high costs or low effectiveness of erythrocytes production. However, the future seems to be very promising in the area of oxygen carriers development.

ACKNOWLEDGEMENT

The publication was supported by the project of High Ministry of Education NzN 3.0 (POWER.03.03.00-00-P011/18), by the Student Scientific Group “Biology of cancer cells” (SKN No. K 148) and Statutory Funds of Department of Molecular and Cellular Biology.

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Editor – Maciej Zabel

Received: 09.05.2019
Accepted: 30.06.2019
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