2. Pathophysiology

2.1. Erythropoiesis

The 3 major factors controlling the rate of erythropoiesis and at the core of the pathogenesis of the anemia of chronic disease include:

1. the bone marrow with all its cell and growth factor machinery
2. erythropoietin (EPO)
3. iron

Iron metabolism will be addressed in Chapter 2.2. Other important co-factors, such as folate and vitamin B12, will not be discussed here, because they are not part in the pathogenesis of the anemia of chronic diseases (☞ Chapter 2.3.).

2.1.1. Erythropoiesis

Human erythrocytes have a mean lifespan of about 120 days. This implies that approximately 200 billion new erythrocytes, carrying collectively 6 g of hemoglobin, are produced per day, i.e. 2-3 million new red cells every second. The principal function of mature erythrocytes is to transport oxygen from the lungs to other body tissues. In situations of anemia, which decreases oxygen supply, red cell production can expand up to 20 times over baseline rates, underlying the very dynamic nature of erythropoiesis. One can distinguish 3 stages of erythropoiesis during development. The first stage corresponds to primitive embryonic erythropoiesis that takes place in blood islands within the yolk sac and is characterized by EPO-independency. The second stage corresponds to definitive erythropoiesis in the fetal liver. In the third stage, adult erythropoiesis results from the migration of hematopoietic stem cells to the bone marrow.

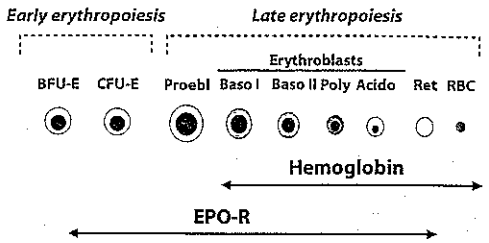

Figure 2.1: **Erythropoiesis.** Erythropoiesis is a process of continuous cell proliferation and differentiation that can be schematically separated into progenitors, precursor cells and mature red cells, most of which express EPO receptors (EPO-R). The earliest progenitor totally committed to the erythroid lineage is the burst-forming unit-erythroid (BFU-E) that evolves into colony-forming unit-erythroid (CFU-E). The earliest erythroid precursor is the pro-erythroblast, followed by basophilic erythroblast, polychromatophilic erythroblast and orthochromatic or acidophilic erythroblast. The changes in cytoplasm appearance reflect the progressive accumulation of hemoglobin. Sufficiently mature erythroblasts expulse their nucleus and become reticulocytes. Two days later the reticulocytes have lost their RNA and become fully mature red blood cells (RBC).

■ Erythroid cells

Erythropoiesis is a process of continuous cell proliferation and differentiation that can be schematically separated into 4 compartments, i.e. stem cells, progenitors, precursor cells and mature red cells (☞ Figure 2.1). Stem cells are rare, primitive cells that are both capable of self-renewal and of differentiation into all blood cell lineages, qualifying them as multipotential cells. Commitment to differentiation into one or more lineages generates progenitor cells. The progenitor cell compartment comprises colony-forming units of mixed lineage (CFU-mix), followed by a bipotential megakaryocytic-erythrocytic progenitor. The earliest progenitor totally committed to the erythroid lineage is the burst-forming unit-erythroid (BFU-E) that forms large bursts of erythroblast colonies within 2 weeks in semi-solid culture. BFU-E evolves into colony-forming unit-erythroid (CFU-E) that forms single colonies of up to 64 erythroblasts within a few days in semi-solid cultures. The erythroid precursor cell compartment comprises

bone marrow cells that are easily recognized under the microscope. The earliest erythroid precursor is the pro-erythroblast, followed by basophilic erythroblast, polychromatophilic erythroblast and orthochromatic erythroblast. The changes in cytoplasm appearance reflect the progressive accumulation of hemoglobin whose production is made possible through strong expression of transferrin receptors facilitating iron acquisition. Cell division ends at the polychromatophilic stage. Then sufficiently mature erythroblasts expulse their nucleus and become reticulocytes that are still actively producing hemoglobin in the circulation. Two days later the reticulocytes have lost their RNA and become fully mature red blood cells.

Growth factors

The whole process of erythropoiesis is strongly influenced by a number of cytokines and growth factors that affect the survival, proliferation and maturation of erythroid progenitors. Most of these growth factors are produced in the bone marrow micro-environment that comprises stromal cells (fibroblasts, endothelial cells), accessories cells (T-cells, macrophages, monocytes) and a protein-carbohydrate scaffold that forms the extracellular matrix. The major growth factors that regulate erythropoiesis include both positive regulators and negative regulators. The major positive regulators are stem cell factor (SCF), Flt-3 ligand (Flt-3L), thrombopoietin (TPO), interleukin-11 (IL-11), IL-3, granulocyte-monocyte colony-stimulating factor (GM-CSF) and erythropoietin (EPO). EPO is the major positive regulator and will be described in more details below. Indeed, EPO and EPO receptors appear to be required for definitive erythropoiesis and the terminal phases of erythropoietic differentiation. SCF and Flt-3L are early-acting growth factors that promote the survival and proliferation of early progenitors, but SCF is also the major growth factor for BFU-E. Besides its action on the megakaryocytic lineage, thrombopoietin also influences early progenitors up to the stage of BFU-E. IL-3 and GM-CSF act on more mature progenitors of all myeloid cell lineages, including BFU-E. Contrarily to EPO that is absolutely required for red cell differentiation, all these growth factors mostly impact on progenitor cell survival and proliferation and rather prevent cell differentiation. The process of erythropoietic differentiation involves a complex network of transcription factors (1). Among them, GATA-1 plays a major role by binding to GATA-binding sites present in the promoters and/or enhancers of all erythroid-specific genes (2), explaining its essential function both in erythroid survival and maturation. Other transcription factors, such as FOG-1, exert their activity through protein-protein interactions, with FOG-1 binding to GATA-1. Another important transcription factor is Tal-1 that forms transcriptional complexes with other transcription factors.

Negative regulators

Multiple apoptotic mechanisms are strongly involved in the negative regulation of erythropoiesis, explaining the small baseline level of ineffective erythropoiesis (1). EPO deprivation or stimulation of death receptors result in activation of caspases-3, -8 and -10, which then lead to cleavage of the transcription factors GATA-1 and TAL-1, thereby triggering apoptosis. However, caspase activation plays also an important role in the normal process of erythroid maturation. Cell death receptors and their ligands belong to the TNF receptor and TNF superfamilies, respectively. The Fas/Fas ligand (FasL) system is involved in a regulatory feedback mechanism exerted by mature erythroblasts against immature erythroblasts. Erythroid colony-forming cells express low levels of Fas. However, Fas is upregulated in early erythroblasts and this high expression is maintained through terminal differentiation, while expression of FasL is selectively induced in late differentiating erythroblasts. However, FasL-mediated cell death is only effective in less mature erythroblasts where it is antagonized by high doses of EPO. Indeed EPO mediates erythroid expansion during an erythropoietic stress by suppressing Fas/FasL coexpression. GATA-1 and Tal-1 cleavage appears to be responsible for Fas-mediated apoptosis (in the absence of EPO) and maturation blockage (in the presence of EPO). TNF-related apoptosis-inducing ligand (TRAIL) is also a negative regulator of normal erythropoiesis. TRAIL receptors are expressed more densely on immature compared to mature erythroblasts, while TRAIL is preferentially expressed by mature erythroblasts, a pattern very similar to that of the Fas/FasL system. TRAIL selectively decreases the number of glycophorin A intermediate erythroblasts without affecting immature or more mature erythroblasts. TWEAK, another member of the TNF superfamily, is also able

to inhibit erythroid cell growth and differentiation through caspase activation.

2.1.2. Erythropoietin

Regulation of EPO production

It has been known for a long time that EPO production is regulated by a feedback mechanism through which anemia-induced hypoxia stimulates EPO secretion in the circulation that in turn corrects the hypoxia by stimulating the generation of additional red cells (☞ Figure 2.2). Polycythemia induced by transfusion or prior exposure to hypoxia increases mouse sensitivity to EPO through reduction in endogenous EPO production. From its serum concentration of about 20 mU/ml and its serum half-life of about 7-8 hours, it can be estimated that baseline endogenous EPO production amounts to about 3 U/kg/day. In response to hypoxia, EPO levels increase after about 60-90 minutes, peak after about 20 hours and then decrease to an intermediate steady state level that is proportional to the severity of hypoxia (3). Metabolic factors also influence EPO levels, suggesting that increased oxygen demand also stimulates EPO production. Oxygen-sensing mechanisms are operative in anemic hypoxia (tissue hypoxia with normal arterial oxygen tension) as well as hypoxic hypoxia (tissue hypoxia with decreased arterial pO_2) (3). Thus it appears that regulation of EPO production is based on tissue oxygen tension. Cobalt also stimulates EPO secretion. Hence, one unit of erythropoietin was defined as the equivalent to the effect of 5 µMol $CoCl_2$.

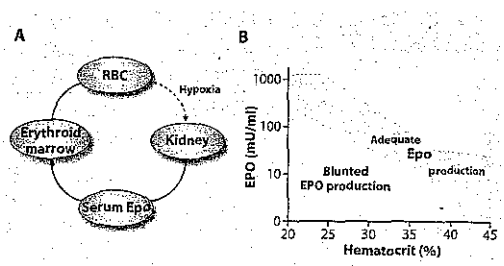

***Figure 2.2*: EPO physiology.** (**A**) EPO production is regulated by a feedback mechanism through which anemia-induced hypoxia stimulates EPO secretion that in turn corrects the hypoxia by stimulating the generation of additional red cells. (**B**) Therefore, serum EPO levels increase exponentially when an anemia develops.

Site of EPO production

Nephrectomy experiments identified the kidney as the major site of EPO production. The liver was later recognized as a secondary source of EPO, particularly during fetal development. EPO and EPO receptors are widely expressed in many non-erythroid tissues, including the brain, and appear to be involved in local tissue protection from ischemia and hypoxic damage. However this small amount of EPO production outside of the liver and kidney does not contribute to the endocrine function of EPO. The human EPO gene was cloned in 1985. This advance allowed the identification of EPO-producing cells as cortical interstitial cells adjacent to proximal tubules in the kidney. These cells were identified as fibroblasts. Only a minority of renal interstitial fibroblasts expresses EPO mRNA even during severe hypoxia. These peritubular fibroblasts generate EPO in a all-or-none fashion so that EPO production can only be increased through recruitment of additional cells. In the liver, EPO is produced by hepatocytes and interstitial cells that feature the same type of recruitment in response to hypoxia. Studies in human hepatoma cell lines showed that EPO-producing cells were the same as oxygen-sensing cells.

EPO and hypoxia

When the degree of anemia worsens, the area of EPO-producing cells spreads from the inner cortex to the entire cortex in the kidney and from the central venous area towards the portal area in the liver. The gradient of EPO production in the liver may be explained by an inverse gradient of oxygen tension that is minimal in the pericentral part of the hepatic lobe. The oxygen gradient in the kidney cortex results from high oxygen consumption together with restricted oxygen delivery because close contact allows oxygen to diffuse directly from arteries to veins before reaching the tubular capillaries. A major determinant of renal oxygen consumption is tubular sodium reabsorption that is proportional to the glomerular filtration rate, which in turn depends on renal blood flow. Therefore, renal blood flow does not impact considerably on EPO production, since it influences oxygen supply and oxygen consumption in parallel.

EPO gene regulation

The EPO gene is located on chromosome 7, encoding for a polypeptide with 193 amino-acids that is

cleaved during secretion to release an EPO protein with 165 amino-acids in the circulation. The regulation of the erythropoietin gene has been studied in details (4). EPO secretion mostly depends on EPO mRNA levels, which primarily result from increased EPO transcription, but also enhanced mRNA stability. Hypoxia response elements are found not only in the EPO enhancer sequence but also in the close vicinity of many other genes regulated by oxygen. This suggests the existence of a common oxygen-sensing mechanism both in EPO-producing cells in the kidney and liver, but also in many other cells where it regulates a variety of other processes (3). In response to hypoxia, EPO transcription is increased through the involvement of an enhancer element located 3" to the EPO gene. At the core of this process is the hypoxia-inducible transcription factor (HIF) (5). HIF consists of a heterodimer of an oxygen-regulated α subunit and a constitutive β subunit (6). HIF-1α forms a transcription factor complex binding to the EPO enhancer element. HIF-1α is rapidly induced by hypoxia and $CoCl_2$, but also deferoxamine, suggesting that oxygen sensing involves an iron-containing protein. Upon resolution of hypoxia, HIF-1α is rapidly ubiquitinated and degraded in proteasomes after binding to the von Hippel-Landau (VHL) protein. Binding of HIF-1α to the VHL protein depends on the hydroxylation of 2 specific prolines. HIF-1α proline hydroxylation is itself reliant on a non-heme iron-containing hydroxylase. Therefore, the following regulatory mechanism has been identified. In hypoxia, HIF-1α is not hydroxylated, does not bind to the VHL protein and thus persists to ensure continued EPO production. In the presence of oxygen, HIF-1α is rapidly hydroxylated, enabling it to bind to the VHL protein that targets it for ubiquitination and degradation in the proteasome. In addition, oxygen-dependent hydroxylation of HIF-1α on an asparagine by another hydroxylase (Factor-inhibiting HIF-1 or FIH) inhibits binding to another protein in the HIF-1α transcription complex, thereby preventing it from playing its enhancing role in EPO gene transcription. Other sequences that are located 5' to the coding sequence may be also involved in the regulation of EPO transcription. Negative regulators of EPO gene expression include GATA-2 and NF-κB. GATA-2 is a transcription factor that binds to the EPO promoter in the 5' flanking region of the EPO gene. GATA-2 DNA-binding is reduced under hypoxic condition. NF-κB also inhibits the EPO promoter. Gene suppression by NF-κB may be explained by competition between NF-κB and other transcription factors for the co-factors p300/CBP that are critically involved in hypoxia-induced EPO expression.

EPO receptor (EPO-R)

The EPO-R belongs to the cytokine class I receptor superfamily (5). EPO signalling involves numerous proteins, including JAK2 that chaperones EPO-R to the plasma membrane, kinases involved in the PI3K, AKT kinase and Ras pathways, and the signal transducer and activator of transcription Stat-5 that influences the expression of several genes controlling cell proliferation and differentiation and activates the expression of several anti-apoptotic genes in erythroid cells. EPO binding to EPO-sensitive cells results in increased DNA and RNA synthesis, glucose uptake, globin gene transcription, transferrin receptor expression and hemoglobin production. CFU-E is the most EPO-sensitive cell, while BFU-E is not. Rather than a proliferation factor, EPO appears to be mostly a survival factor that prevents apoptosis of CFU-E through basophilic erythroblasts.

Serum EPO levels

Serum EPO levels may vary considerably (7). Levels are usually between 10 and 20 mU/ml in normal subjects, may decrease somewhat in primary polycythemia, but increase exponentially when an anemia develops below an Hct of 30-35 % (☞ Figure 2.2). Therefore, a serum EPO value must always be evaluated in relation to the degree of anemia. From the relationship between Hct and EPO in normal subjects and patients with hemolytic anemia, it is possible to derive 95 % confidence limits (0.80-1.20) for the O/P ratios of observed/predicted log (EPO) (8). EPO levels inappropriately low for the degree of anemia (O/P <0.80) are encountered in renal failure, but also in a number of other conditions, including the anemia of chronic disease. Inappropriately high serum EPO levels (O/P >1.20) are seen in secondary polycythemia, a feature permitting its separation from primary polycythemia. In addition, one should take into account the inverse relationship between marrow erythropoietic activity and serum EPO levels: the higher the number of erythroid precursors, the

lower the serum EPO value. Hence serum EPO levels are the result of a balance between the rate of EPO production and its utilization by the erythroid marrow.

2.1.3. References

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2.2. Iron deficiency

Iron deficiency is the most important pathophysiological mechanism leading to anemia in IBD. It occurs in over one third of the patients suffering from Crohn's disease or ulcerative colitis (1). Iron metabolism is an active research field and recent advances have been made in understanding pathophysiological processes involved in the regulation of iron homeostasis.

2.2.1. Physiology of iron metabolism

Iron absorption

Iron is an essential element for most organisms from prokaryotes to mammals, being a part of numerous enzymes participating in redox reactions or oxygen delivery (2-4). The major source of iron is food, where it comes in the form of heme-bound or non-heme iron. Iron absorption occurs only in the duodenum and upper jejunum. Heme-bound iron has a considerably higher bioavailability than non-heme iron. Heme, released proteolytically from myoglobin and hemoglobin (mostly from meat), binds to the heme carrier protein HCP-1 and enters the mucosa epithelium unchanged via endocytosis, where iron is subsequently freed from the complex by the low pH; however, this process has not yet been studied in detail.

More is known about the absorption of non-heme iron. Most iron is available in the form of ferric (Fe^{3+}) ions, which do not readily pass the mucosal barrier; therefore a reduction to ferrous ions (Fe^{2+}) is needed to facilitate transport. Reducing agents are present in the food itself, the most common example being vitamin C. An additional mechanism utilizes the membrane-bound ferrireductase DcytB (duodenal cytochrome B). Ferrous iron then passes through the brush-border membrane via the divalent metal transporter DMT-1, which operates via a proton electrochemical gradient (2, 4-6).

Several factors influence iron uptake in the gut mucosa, among them the form of iron and its redox state in the food, the pH in the intestinal lumen, the presence of chelating agents in the food (e.g. phytic acid, oxalic acid) and the expression levels of DMT-1 in the epithelial cells. As a consequence, only a small percentage of the ingested iron (physiologically 1-2 mg per day) is absorbed (6).

Once iron has entered the cell, it is either used for metabolic purposes, incorporated into the iron storage protein ferritin, or is released into the circulation for the needs of the organism. The exact pathway by which iron crosses the gut epithelial cell from the apical brush border to the basolateral side has not been clarified sufficiently. Both transcytosis as well as a participation of iron chaperone proteins have been suggested to play a role in this process (7, 8). Once iron reaches the basolateral surface, it leaves the enterocyte via ferroportin and is packed into the iron transport protein transferrin to enter the circulation. Prior to binding to transferrin, the ceruloplasmin - related copper-containing membrane-bound ferroxidase hephaestin oxidises Fe^{2+} back to Fe^{3+} (6) (summarized in Figure 2.3).