ERYTHROPOIETIN AND THE NERVOUS SYSTEM

Novel Therapeutic Options for Neuroprotection

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Cover illustration: Dorsal root ganglion sensory neurons stained with anti-βIII tubulin antibody (green) over Schwann cells stained with anti-erythropoietin antibody (red).

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Dedicated to my parents, Gülsen and Safa Höke

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PREFACE

The idea for this book originated at the annual Society for Neuroscience meeting in the fall of 2003. As someone new to the field, I was pleasantly surprised to see many investigators from diverse disciplines working on erythropoietin. Even though erythropoietin was initially identified as the growth factor that induced red blood cell proliferation, it has multiple actions on a diverse population of cells and tissues, as is the case with many growth factors. What sets erythropoietin apart is the fact that it is one of the earliest recombinant proteins that has been in clinical use as a drug. This past experience with dosing and side effect profile makes it an ideal candidate for further development for neuroprotective therapies. In this book we have strived to bring a current state-of-the-art review of multiple aspects of erythropoietin research as it relates to the nervous system. Our hope is that this book will stimulate new research on erythropoietin and the nervous system and bring new investigators to the field.

First, I would like to thank all of the contributors to the book. Without their efforts and patience we could not have finished this book. I would also like to thank Marcia Kidston at Springer US and my assistant, Stephanie David for help and patience in getting the chapters formatted properly. Finally many thanks go to my wife, Nishi and my children, Maya and Erol, for their unwavering support and love.

Ahmet Höke MD, PhD
Baltimore, 2005

Chapter 1

HISTORY AND BIOLOGY OF ERYTHROPOIETIN IN HEMATOPOIETIC AND NON-NEURAL TISSUES

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Abstract: Erythropoietin (EPO) is a glycoprotein hormone, which is produced in kidney and liver, and is mainly involved in regulating proliferation and maturation of red blood cells. EPO gene expression is induced by hypoxia through the transcription factor hypoxia inducible factor-1, which has been found to be the main regulator of oxygen homeostasis in the body. Suppression of apoptosis is the principle mechanism of action of EPO in maintaining erythropoiesis. It has been recently recognized that EPO is a member of the cytokine type I superfamily and it has multiple effects in organs and tissues different from the hematopoietic system. Recent evidence of EPO as a protective factor in various injury models in the nervous system and heart has raised the possibility that EPO can exert protective effects in many organs in the body. However, whether the mechanism of protective action involves inhibition of apoptosis remains to be seen.

Key words: erythropoietin, history, erythropoiesis, hypoxia inducible factor-1, apoptosis, and tissue-protection

1. INTRODUCTION

The hormone EPO is an essential growth factor for the production of red blood cells. In healthy adult humans, one percent of all red blood cells are destroyed every day and replaced by reticulocytes. Blood oxygen availability is the principle regulator of erythropoiesis; hypoxia induces EPO gene expression in the kidneys and liver (Jelkmann et al., 2001). The plasma level of EPO may rise 1000-fold above the normal value and as a consequence the

basal rate of production of red cells (2-3 x 10^{11} per day) may increase 10-fold in hypoxic stress (Jelkmann, 1992).

In the recent years EPO and its receptor (EPOR) have been shown to be present in other tissues not involved in hematopoiesis. These include the brain, the reproductive tract, (Marti et al 1996, Kobayashi et al 2002, Masuda et al 2000), the lung, the spleen and the heart (Fandrey et al, 1993). These discoveries raised the possibility of EPO acting not only as an erythropoietic hormone but also as a possible protective factor in many organs. In particular, increased expression of EPO and EPOR in the brain following an ischemic injury (Siren et al, 2001), suggests a more likely paracrine rather than an endocrine mode of action of EPO at these sites. Moreover EPO in the brain and testis may be separated from the systemic circulation by the blood-brain or blood-testis barrier, even if it has been shown that EPO can cross the blood-brain barrier especially after brain damage (Fandrey, 2004).

2. HISTORICAL NOTES

In 1882 Paul Bert, a pupil of Claude Bernard's school in Paris, described for the first time an increased number of red blood cells and consequently an increased blood O_2 capacity in animals living at high altitudes and considered it as genetically derived (Bert, 1882). He was also the one who showed that the mountain sickness is due to hypoxemia (Bert, 1878). Few years later in 1890, the French histologist Viault showed that red blood cell production was inducible by permanence at altitudes; in particular he noted a significant increase in erythrocytes in his blood during a 3 weeklong expedition to the Peruvian mountains (Viault, 1890). On the basis of these early studies, Friedrich Miescher suggested that low oxygen pressure acted directly on the bone marrow to stimulate the production of red blood cells (Miescher, 1893).

In 1906 Carnot and DeFlandre published an intriguing work where they postulated that a humoral factor, which they called *hémopoïétin*, regulated red blood cell production (Carnot et al, 1906) They carried out experiments which showed a prompt increase in reticulocytes in normal rabbits following the injection of plasma from donor rabbits who have had a bleeding stimulus. In the following years other investigators failed to confirm the results of Carnot and DeFlandre and the existence of a hemopoietic hormone was not generally accepted. However, in 1948 two Finnish scientists, Bonsdorff and Jalavisto continued work on red blood cell production and called the hemopoietic substance "erythropoietin" (Bonsdorff E et al, 1948). The most important works on the existence of EPO were the classical

experiments of Reissman (Reissmann, 1950) and Erslev (Erslev, 1953) on parabiotic rats. Reissmann showed that erythroid hyperplasia in bone marrow and reticulocytosis were induced in both parabiotic animals when only one partner was exposed to hypoxemia: this was an elegant demonstration that a circulating substance was able to stimulate red blood cell production in an animal exposed to a normal atmospheric pressure. Finally, Erslev confirmed the early experiment of Carnot and DeFlandre, by inducing reticulocytosis, and later increased hematocrit, in rabbits repeatedly injected with large volumes of plasma from anemic donor animals. In his work Erslev also predicted the potential therapeutic role of EPO in treating anemia. The following step was the important and laborious work of purification of human EPO from urine of anemic patients by Miyake in 1977 (Miyake et al, 1977). It allowed the successful cloning and transfection in mammalian cells of the EPO gene in 1985 by Lin in Chinese hamster ovary cells (Lin et al, 1985) and by Jacobs in African green monkey kidney cells (Jacobs et al, 1985). It permitted the consequent industrial production of recombinant human erythropoietin for treating patients with anemia.

The questions about the site(s) of production of EPO started to be answered when Jacobson reported that bilaterally nephrectomized animals, subjected to bleeding, failed to produce increased EPO (Jacobson et al, 1957). The same observation was also reported in anemic patients affected by chronic renal failure (Gurney et al, 1957). In 1961, Fisher demonstrated that cobalt enhanced production of EPO in the isolated perfused dog kidney (Fisher et al, 1961). Few months later Kuratowska published an experiment in rabbits showing that the isolated kidney perfused with hypoxemic blood increased EPO production, using a reticulocyte assay for the assessment of EPO activity (Kuratowska et al, 1961). However for many years the issue of kidneys as site of synthesis of EPO was still debated. It was thought that kidney under hypoxic stimulus released an enzyme, "erythrogenin", which was able to activate a hepatic precursor of erythropoietin (Gordon et al 1967). This theory was finally abandoned when several different experiments converged to show that EPO was synthesized in kidneys: erythropoietin activity was quantified in the serum-free medium of isolated perfused kidneys in rabbit (Erslev, 1974) and in renal extracts of rats exposed to hypoxia (Jelkmann et al, 1981). The final striking evidence of renal production of EPO was given by the demonstration of EPO mRNA in renal extracts (Beru et al, 1986; Schuster et al, 1987).

In contrast to the adults, liver is the principal site of production of EPO during fetal life (Zanjani et al, 1974). Even though the kidneys take over as the main site of production after birth, liver continues to contribute to the synthesis of EPO in a minor role (Fried, 1972).

3. STRUCTURE AND MECHANISM OF ACTION OF EPO IN ERYTHROPOIESIS

EPO is a glycoprotein composed of 165 aminoacids and 4 carbohydrate side chains with an estimated molecular mass of 34 kD; 40% of which is carbohydrate (Jekjmann, 2003). There are 3 tetraantennary N-linked and 1 small O-linked acid sugar chains. The molecule forms a bundle of 4 α-helices, which are folded into a compact globular structure (Jelkmann, 2003). The carbohydrate moiety, rich in sialic acid, is critical to molecular stability and full in vivo biological activity. In fact, the form of EPO deprived of sialic acid is rapidly sequestered in the liver (Spivak et al, 1989).

The activity of EPO starts by binding to its receptor EPOR. In the absence of EPOR, EPO does not have any erythropoietic activity. Transgenic mice lacking EPOR develop severe anemia (Kieran et al 1996, Lin et al 1996). Human EPOR has been cloned; it is a 484 amino acid glycoprotein and a member of the type I superfamily of single-transmembrane cytokine-receptors (Wojchowski et al, 1999). EPOR is expressed in a variety of cells and organs including placenta, endothelium, and megakaryocytes but the primary target cells are the erythroid progenitors, Burst–forming Unit (BFU-E) and Colony-forming Unit (CFU-E). These erythroid progenitors are derived from the stochastic differentiation of bipotential or multipotential progenitors, a population of stem cells (Suda et al, 1984). The CFU-E cells are the most sensitive cells responding to EPO. Although the EPOR is expressed in both populations of erythroid precursors, the number is highest in CFU-E cells, where it has a density over 1000 receptors per cell. The number of receptors per cell gradually declines in more mature cells (Broudy et al, 1991). The primary mechanism of action of EPO in regulating erythropoiesis is the suppression of apoptosis; as a consequence, the progenitor cells proliferate and differentiate, resulting in an increased formation of normoblasts and finally reticulocytes (Jelkmann, 2003). It has been shown that CFU-E cells do not survive in vitro in absence of EPO. Since the majority of CFU-E cells are cycling, their survival in the presence of EPO may be tightly linked to their proliferation and differentiation to mature erythrocytes (Sieff et al, 1986). EPO also is necessary for the survival and terminal differentiation of a subset of BFU-E cells, while a second subset of BFU-E cells, presumably less mature, survive in absence of EPO if other growth factors like IL-3 or GM-CSF are present (Sieff et al, 1989). In addition to stimulating the proliferation and differentiation of erythroid progenitors, EPO activates the mitotic division of proerythroblasts and basophilic erythroblasts and it accelerates the release of reticulocytes from the bone marrow (Jelkmann,

1992). After an acute increase of plasmatic EPO it takes generally 3-4 days to detect an increase in reticulocytes (Jelkmann et al, 2001).

After binding of EPO to its receptor, a tighter homodimerization of the receptors ensue and a conformational change of the intracellular domains activates receptor associated Janus kinase 2 (JAK2) by reciprocal tyrosine phosphorylation. Activated JAK2 phosphorylate the EPOR and several distinct intracellular signaling molecules. The most important down-stream protein transmitting EPO signals is STAT5 (Signal Transducer and Activator of Transcription). STATs are latent cytoplasmic transcription factors, as soon as they are activated by JAKs, they dimerize and translocate into the nucleus where they bind to specific DNA sequences and allow the transcription of the respective genes (Jelkmann et al, 2001). Suppression of apoptosis is the main mechanism of action of EPO in erythropoiesis and there is evidence in favor of a role of STAT5 in the anti-apoptotic pathway of EPO. In particular, STAT5 appears to mediate the induction of Bcl-xL, an antiapoptotic gene through direct binding to its promoter (Silva et al, 1999). In fact, anemia and high levels of apoptosis in erythroid progenitors are seen in STAT5 knockout mice (Socolovsky et al, 1999). Homodimerization and activation of anti-apoptotic signaling via JAK2 and STAT5 are shared by other receptors, including those for thrombopoietin, granulocyte colony-stimulating factor, prolactin and growth hormone. Receptors for each of these growth factors has been reported to homodimerize, bind JAK2 and activate STAT5 (Wojchowski et al, 1999).

In addition to the JAK2-STAT5 pathway, other intracellular pathways are activated by EPO. These include activation of voltage-sensitive calcium channels via EPOR; by this mechanism EPO might have a role in modulation of neurotransmitters (Kawakami et al, 2001). Another pathway involves activation of phosphatidylinositol-3 kinase (PI3-K) and Akt (Kashii et al, 2001), which in turn lead to upregulation of Bcl-xL and inhibition of apoptosis in Baf-3 cells (Leverrier et al, 1999) as well as activation of NF-kB, which mediates a variety of anti-apoptotic signaling pathways. These pathways, along with the JAK2/STAT5 pathway may play an important role in neuroprotection (further details are elsewhere in the book and also reviewed in Ghezzi et al, 2004).

In summary, prevention of apoptosis in late erythroid progenitors is the principle mechanism of action of EPO in erythropoiesis. Although EPO can support the proliferation of murine erythroid cells (Miller et al, 1999) and induce the entry of erythroid progenitors into cell cycle if dormant (Spivak et al, 1991), the underlying molecular events are only partially understood (Jelkmann et al, 2001).

4. REGULATION OF EPO SYNTHESIS

Tissue hypoxia is the primary stimulus for the production of EPO. The cells producing EPO appear to respond to changes in the oxygen capacity, tension and affinity of the blood (Jelkmann, 1992). Hypoxia induces expression of the gene encoding for EPO and research in this field has become the prototype of oxygen regulated gene expression. Most of the current knowledge has been derived from in vitro studies utilizing EPO producing human hepatoma cell cultures and it is possible that the mechanism by which human hepatoma cells regulate EPO production differs from the O2 sensing mechanism and control of EPO production in the kidney. In fact, EPO gene expression in liver occurs in a graded fashion, while in the kidney it follows an all or none rule; the increase in EPO mRNA with hypoxia is due to recruitment of additional cells, all of which are maximally active (Kouri et al, 1989). Crucial in the hypoxia-signaling cascade is the transcription factor complex, hypoxia inducible factor-1 (HIF-1). HIF-1 binds to the hypoxia-responsive element (HRE) in the 3'-flanking enhancer of the EPO gene. It is now known that HIF-1 controls more than 50 oxygen dependent genes and it is the major regulator of oxygen homeostasis in the body. It is a protein heterodimer consisting of the two basic-helix-loop-helix proteins, 120 kD HIF-1 α and 91-94 kD HIF-1 β (Wang et al, 1995). The HIF-1 α subunit is present in the cytoplasm and is unstable at high pO_2; while the HIF-1β subunit is more stable and is permanently present in nuclei. The HIF-1 α subunit has two oxygen dependent degradation domains that target the protein for an immediate ubiquitination and proteosomal degradation under normoxemia (Salceda et al, 1997). Binding of the tumor suppressor protein von-Hippel-Lindau (VHL) to the two oxygen dependent degradation domains of HIF-1 α has a critical role in the degradation process (Maxwell et al, 1999). Enzymatic hydroxylation of HIF-1 α at proline residues is necessary for recognition by VHL (Ivan et al, 2001). The mammalian HIF prolyl hydroxylases have been cloned and designated "prolyl hydroxylase domain" (PHD) containing 1, 2 and 3 (Epstein et al, 2001). The activity of PHDs depends on the availability of oxygen and it is likely that these enzymes are crucial in oxygen sensing regulation of EPO gene transcription (Fandrey, 2004). The observations that cobalt ions and iron chelators such as desferoxamine prevent the formation of the VHL complex (Maxwell et al, 1999) may explain why those compounds stimulate the synthesis of EPO. While HIF-1α is undetectable in cells during normal pO2, the HIF-1α/β complex can be demonstrated in nuclei within minutes after the induction of hypoxia in cells (Jelkmann et al, 2001).

In addition to erythropoiesis, HIF-1 regulates genes for neoangiogenesis (for example VEGF, vascular endothelial growth factor) and vascular tone (for example nitric oxide synthases). Erythropoiesis and angiogenesis represent adaptive responses to hypoxia that require several days to develop, but HIF-1 also regulates short-term responses to hypoxia, such as the induction of glucose transporters and glycolytic enzymes. Consequently it is possible to argue that HIF-1 plays a role in the metabolic adaptation to hypoxemia represented by the switch of ATP generation from oxidative phosphorylation to glycolysis (Ghezzi et al, 2004). HIF-1 binding is also induced by insulin and insulin-like growth factor 1 and 2, by interleukin 1 β (IL-1 β), and tumor necrosis factor a (TNF-α); thus it is possible to conclude that there is a synergy in the cellular responses to hypoxia, glucose deficiency and inflammation (Jelkmann et al, 2001). However, the interplay between hypoxia, inflammation and glucose deficiency in regulating EPO production are complex. The ability of IGF-1/2, IL-1 β or TNF-α to increase HIF-1 α / β - DNA binding does not necessarily mean that these stimuli result in increased EPO gene expression automatically. In fact, it has been shown that pro-inflammatory cytokines IL-1 and TNF- α lower EPO production (Fandrey et al, 1994; Faquin et al, 1992) while they upregulate other HIF-1 dependent genes like those encoding VEGF or iNOS (inducible NO-synthase) (Hellwig-Burgel et al, 1999). The mechanism by which EPO transcription is reduced during inflammation, despite the increase of HIF-1 binding is not completely understood, but is likely to be due to lowered HNF-4 (hepatocyte nuclear factor 4), a positive transcription factor), and elevated levels of negative regulating transcription factors, GATA-2 and NF-κB (Jelkmann et al 2001; Fandrey, 2004).

H_2O_2 and other reactive oxygen species are responsible for inhibiting EPO gene expression at high pO_2. It has been proposed that H_2O_2 lower EPO expression by activation of GATA-2 (Imagawa et al, 1996). Binding to the GATA-motif, located in the region relative to the transcriptional initiation site of EPO promoter, GATA-2 inhibits the transcription of the gene and it is one of the main candidates to cause the repression of EPO gene under normal levels of pO_2 (Jelkmann et al, 2001).

5. SITES OF SYNTHESIS: KIDNEY AND LIVER

During the fetal life, liver is the principle producer of EPO, but in the adult, expression of EPO is mainly localized to the kidneys. The mechanism and factors determining the switch and the degree of contribution of the liver to the total body production of EPO are different in different species and not completely understood (Fandrey, 2004). Recently it has been proposed that

GATA-4 may be one of the factors that specifically promote EPO expression in the liver (Dame et al, 2004). EPO expression is present in all lobes of the human liver and the cells producing EPO present an expression pattern that follows the pO_2 distribution: high expression around central veins at lower pO_2 and expression extending to the periportal field in anemia or hypoxia (Fandrey, 2004). Since the hepatic expression is so strictly regulated by the pO_2 gradient, it was thought that changes of this gradient around birth could control the switch of production from liver to kidney. But several experiments including surgical procedures aimed at reducing hepatic or renal oxygenation have shown that it does not affect the switch. Thus it is more likely that tissue factors increasing in kidney during and after birth induce EPO expression while repressive factors are expressed in the liver of the adult (Fandrey 2004).

The EPO producing cells in the kidney have been identified in fibroblast-like type I interstitial cells located in the peritubular space in the cortex and outer medulla (Bachmann et al, 1993; Chandel et al, 1998). However until now the attempts to isolate renal EPO producing cells and to set-up renal cell lines for studying the regulation in expression of EPO gene have failed, thus most of the current knowledge derives from in vitro studies on human hepatoma cell cultures.

6. EFFECTS OF EPO IN ORGANS OTHER THAN HEMATOPOIETIC TISSUES

In recent years, there has been increasing evidence that EPO has multiple effects on many tissues and organs far beyond the regulation of erythropoiesis. In the embryo, EPO is a major regulator of vascular formation and organ growth and EPOR have been found in almost every embryonic tissue. EPO and EPOR are also present in many adult tissues. Based on these observations, the concept of a paracrine and/or autocrine action of EPO has been suggested (Bahlmann et al, 2004).

EPO expression has been found in the brain, reproductive tract, lung, spleen, heart and bone marrow. Of particular relevance for the clinical implications is the observation that EPO has a direct activity on endothelial cells. Studies in vitro have shown that EPO increases endothelial cell proliferation and protects endothelial cells against apoptosis (Anagnostou et al, 1990; Carlini et al, 1999). Also human endothelial lines express EPOR and differentiate into vascular structures when exposed to EPO (Carlini et al, 1995; Ribatti et al, 1999). By stimulating mitogenesis and supporting angiogenesis, EPO can improve tissue oxygenation. Recently bone marrow

derived endothelial progenitors cells (EPCs), which promote vascular regeneration, have been isolated from the blood (Shi et al, 1998). EPCs are considered to originate from CD34+ stem cells, which can differentiate into erythrocytes, thrombocytes, leukocytes and endothelial cells (Bahlmann et al, 2004). Recently, it has been shown that EPO stimulates angiogenesis, partly by increasing mobilization of EPCs from the bone marrow (Heeschen et al, 2003). In patients with myocardial infarction the clinical outcome correlates with the number of circulating EPCs (Vasa et al, 2001). Moreover it seems that even in subjects without clinically evident cardiovascular disease the number of EPCs correlates with endothelial functions and cardiovascular risk factors (Hill et al, 2001).

EPOR has been demonstrated to be present in the embryonic and fetal heart (Juul et al, 1998) and EPO seems to be involved in cardiac morphogenesis (Wu et al, 1999), even if expression of EPO in the heart appears to be low (Fandray, 2004). Some authors have recently demonstrated that EPO prevents hypoxia-induced apoptosis in neonatal rat ventricular myocytes and they have suggested an Akt dependent pathway (Tramontano et al, 2003) Another study involving rodent isolated hearts have shown that intermittent hypoxia or EPO administration protect against post-ischemic injury (Cai et al, 2003). In this study, during intermittent hypoxia, there was an increase in EPOR mRNA in the heart, but EPO mRNA was at the limit of detection. As predicted, renal EPO mRNA expression and plasma EPO levels were significantly induced by hypoxia, suggesting a probable endocrine mechanism of cardiac protection (Cai et al, 2003). This is in contrast to the nervous system, where astrocytes produce EPO and neurons express EPOR in response to hypoxia (Bergeron et al, 2000; Morishita et al, 1997), suggesting a paracrine mechanism of protection (further discussion of neuroprotection in ischemia is elsewhere in the book).

Other sites of tissue protection by EPO involve the skin and reproductive organs. Recently, experiments in rat skin using flap models have raised the possibility of tissue protection from ischemia by EPO (Buemi et al, 2002; Saray et al, 2003). Finally, there is evidence of EPO expression in human endometrium throughout the menstrual cycle, with higher levels in the secretory than proliferative phase (Yokomizo et al, 2002). Functionally EPO appears to play an important role for endometrial angiogenesis and consequently reproductive function (Fandray, 2004).

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Chapter 2

EXPRESSION OF ERYTHROPOIETIN AND ITS RECEPTOR IN THE CENTRAL NERVOUS SYSTEM

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Abstract: Erythropoietin (EPO) is a glycoprotein that is produced mainly by interstitial fibroblasts in the kidney. Released into the circulation, EPO makes its way to the bone marrow where it regulates red cell production by preventing apoptosis of erythroid progenitor cells. Recently, EPO has emerged as a multifunctional growth factor that plays a significant role in the nervous system. Both EPO and its receptor are expressed throughout the brain in glial cells, neurons and endothelial cells. Brain-derived EPO is upregulated by hypoxia, and expression of both EPO and its receptor are specifically modulated during cerebral ischemia. EPO has potent neuroprotective properties *in vivo* and *in vitro* and appears to act in a dual way by directly protecting neurons from ischemic damage and by stimulating endothelial cells, and thus supporting the growth of new blood vessels. EPO eventually also modulates inflammatory responses. Thus, hypoxically upregulated EPO is a naturally self-regulated physiological protective mechanism in the mammalian brain, especially during ischemia. As EPO is also a clinically extremely well studied and tolerated compound, its use in stroke patients is tempting.

Key words: neuron; astrocyte; microglia; endothelial cell; HIF-1; VEGF; IGF-1; angiogenesis; neuroprotection; apoptosis; hypoxia; ischemia; stroke.

1. INTRODUCTION

Clinicians, who are treating patients suffering from the anemia of End-Stage Renal Failure with recombinant erythropoietin (EPO), have often reported an improvement of the cognitive function of their patients (reviewed by Ehrenreich and Siren, 2001a). However, it was never quite