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ERYTHROPOEITIN

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ABSTRACT

Erythropoietin (Epo), the first growth factor to be discovered, is an endocrine hormone produced by specialized renal cells. The rate of Epo production is determined primarily by the oxygen demands of these renal cells relative to their oxygen supply. However, Epo production is modulated by various hormones, nutritional factors, cytokines, and the integrity of the erythron. Epo interacts with specific receptors found almost exclusively on erythroid pro-

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genitors. This interaction results in an expansion of the number of the erythroid progenitor and triggers late committed progenitors to undergo terminal maturation when provided with essential nutrients.

Recombinant human Epo (rHuEpo) is commercially available for human use. It is safe, easily administered, and almost universally effective in treating the anemia of patients with renal failure. It has also been successful in treating the anemia of some patients with neoplasms, myelodysplastic syndromes, HIV infection, rheumatoid arthritis, and aplastic anemia. Much remains to be learned about the regulation of Epo production, the physiologic actions of Epo, and how best to use this growth factor in the treatment of anemia.

INTRODUCTION

Although persons living at high altitude already had been determined to have higher red cell counts than those living at sea level, Viault (117) was the first to show that hypoxia stimulates erythropoiesis. In 1906, Carnot & Deflandre (16), influenced by the recent discovery of the first hormone, secretin, postulated that the effect of hypoxia on erythropoiesis was mediated by a hormone that they called hemopoietin. However, because their experimental evidence was, unfortunately, not reproducible, their hypothesis remained unsupported for several decades. In 1950, Reissman (100) reported the results of his classic experiment in parabiotic rats. These animals develop a communicating microcirculation through which the plasma, but not the cellular components of the blood, equilibrate. When one parabiont of each pair was exposed to hypoxia, the rate of erythropoiesis of both parabionts increased, although the hemoglobin oxygen saturation was reduced only in the rat exposed to hypoxia. These results indicated that erythropoiesis is hormonally controlled. In 1953, Erslev (25) reported that injection of plasma from hypoxic rabbits into normal rabbits resulted in an increase in the rate of erythropoiesis of the recipients and concluded that the rate of erythropoiesis is regulated by a plasma factor, which he termed erythropoietin (Epo). The development of a bioassay (22) and, subsequently, of a sensitive radioimmunoassay for Epo (108) facilitated the investigation of the site and regulation of Epo production and of the activity of this factor. The task of extracting pure Epo from biological fluids proved difficult because the methods of protein separation were inefficient and consequently required excessive amounts of crude material. In 1977, Miyake et al (92) extracted several milligrams of homogeneous Epo from a large quantity of urine from patients with aplastic anemia. In 1985, the genomic DNA for Epo was extracted from a human DNA library and transfected into Chinese hamster ovary cells (78), thereby initiating commercial production of recombinant human Epo (rHuEpo). With the 1986 (31, 123) publication of the results of the first phase I and II clinical trials of rHuEpo in patients with anemia of

chronic renal failure, the era of hematopoietic growth hormone therapy was inaugurated.

For rHuEpo therapy to be successful, an adequate supply of iron, folic acid, vitamin B_{12}, and amino acids is required in order to maintain an accelerated rate of erythropoiesis. This form of therapy has led to renewed interest in the nutritional requirements for erythropoiesis and in how these nutrients are processed and delivered to the erythron. In this article, we review the physiology and therapeutic uses of Epo and discuss the effects of nutritional factors on both.

THE Epo MOLECULE AND THE Epo GENE

The human Epo gene, which contains four introns and five exons, is located as a single copy on chromosome 7. The Hep3B human hepatoma cell line, which produces Epo in response to physiologic stimuli in vitro, has served as a useful model for identifying the sites of various functions on the Epo gene (53). A hypoxia-sensitive region and organ-specific regulatory sites have already been identified (99).

The Epo gene codes for a polypeptide consisting of 193 amino acids. During the secretory process, a 22–amino acid leader sequence is cleaved from the N-terminal end, and a C-terminal arginine is removed posttranslationally, leaving a 30.4-kDa glycoprotein that contains 60% protein and 40% carbohydrate. The tertiary structure of Epo is predicted to consist of 50% helical structure comprising four helices in a conformation similar to that of growth hormone. The Epo molecule has three N-linked and one O-linked carbohydrates. The N-linked sugars are essential for in vivo but not in vitro activity of the molecule. They determine the stability and the rate of clearance of Epo by the hepatocytes. The O-linked carbohydrate has no essential role in the biological activity of the molecule, either in vivo or in vitro (76).

SITES OF Epo PRODUCTION

In 1957, Jacobson et al (63) reported that the plasma Epo titer of nephrectomized rats does not rise in response to hypoxia, anemia, or the injection of cobaltous chloride and that this lack of response cannot be attributed to uremia. They concluded that the kidney is the major site of Epo production. The extraction of Epo (20, 43, 107) and of Epo mRNA (14) from the renal cortex confirmed this finding. By in situ hybridization, using radiolabeled riboprobes to detect Epo mRNA, Koury et al (72) identified a peritubular cell of endothelial origin as the site of Epo production.

The initial report (63) showing that the plasma Epo titer of hypoxic rats

does not rise upon exposure to hypoxia was followed by numerous publications on the plasma Epo titers of rats and mice exposed to various hypoxic stimuli. Some investigators detected small but significant increases in plasma Epo titers, which suggested the existence of extrarenal sites of Epo production (26, 51, 64). Production of Epo by extrarenal sites was subsequently confirmed in extremely hypoxic anephric rats (46) and in severely anemic anephric patients (90, 94). Extrarenal sites of Epo production are estimated to produce ~10% as much Epo as renal sites when exposed to comparable stimuli (46). They are probably not responsive to hypoxic stimuli if the kidneys are intact and functioning.

Carmena et al (15) reported that nephrectomy does not affect the ability of neonatal rats to produce Epo. They concluded that Epo is produced by extrarenal sites during fetal and neonatal life and that the site of Epo production switches to the kidney shortly after birth. This observation was subsequently confirmed in several mammalian species (125). The switch from extrarenal to renal Epo production begins late in fetal life and is completed at various times during the perinatal period in mice, rats, and sheep (125). The signal for the switch is still unknown.

The major site of extrarenal Epo production in fetal sheep (126) and in adult rats (39) is the liver. Hepatocytes in the centrilobular areas of the liver express Epo mRNA in severely hypoxic mice (71). Although Epo mRNA is expressed by cells in the liver, Epo has not been found in extracts of hepatic tissue, indicating that it is immediately secreted (40).

Hepatic Epo production increases severalfold under conditions that cause hepatocytes to proliferate. In rats, this increase has been observed following subtotal hepatectomy (95) as well as after exposure to carbon tetrachloride (39). In humans, increased Epo production has been reported in patients with renal failure who develop hepatitis (69). The reason for the increased production of Epo by regenerating liver cells is not known. However, it is tempting to speculate that the proliferating hepatocyte has a feature in common with the fetal hepatocyte that the resting hepatocyte lacks.

The carotid body, the salivary glands, and various other organs have been proposed as sites of extrarenal Epo production, but these proposals have not been supported by subsequent studies. Macrophages obtained from the marrow express Epo mRNA (102) and are potential sources of paracrine Epo production (102). Hermine et al (61) reported that Epo mRNA could be detected by polymerase chain reaction (PCR) in extracts of human marrow cells. Using antisense oligonucleotides, they demonstrated that Epo was produced by multipotential hematopoietic stem cells and acted in an internal autocrine manner to induce erythroid differentiation. The physiologic significance of these observations requires clarification.

THE ROLE OF Epo IN REGULATING ERYTHROPOIESIS

Erythrocytes are enucleate cells that are unable to synthesize proteins. They therefore have a finite lifespan of ~120 days. Accordingly, ~0.8% of the red cell mass is destroyed daily, and a similar amount must be produced to maintain the red cell mass at a constant level. Under normal conditions, the rate of erythropoiesis is set at a level sufficient for replacement of the daily loss of red blood cells (RBCs). However, if the erythron is functioning normally and receives sufficient nutrients, the rate of erythropoiesis can increase five- to eightfold in response to anemia, hypoxia, or an increase in the rate of hemolysis. The rate of erythropoiesis declines in response to hyperoxia, an increase in red cell mass, or a decrease in the rate of oxygen utilization. Shortly after its discovery by Erslev (25), Epo was shown to be the primary regulator of the rate of erythropoiesis under normal conditions and in response to stimuli (50). Current concepts of how Epo regulates the rate of erythropoiesis are reviewed in this section.

Approximately 0.1% of the hematopoietic cells in the bone marrow are pluripotent stem cells predominantly in resting stage (G_0). These cells, which are also found in circulating blood, interact with lectins on the surface of stromal cells to become anchored into specific niches in the hematopoietic microenvironment. Once fixed, they commit to the lymphocytic, erythrocytic, myelomonocytic, or megakaryocytic cell series. Commitment is generally believed to occur in a random, stochastic manner (77), although some evidence suggests that it may be influenced by growth factors (61).

The earliest cell committed to the erythroid series is the burst-forming unit erythroid (BFU-E). The earliest BFU-Es are predominantly in the G_0 phase. All but the most primitive human BFU-Es are fixed in the hematopoietic microenvironment, where they are exposed to a variety of growth factors, including stem-cell factor, granulocyte-macrophage colony–stimulating factor (GM-CSF), and interleukin-3 (IL-3). Interaction of these substances with specific receptors results in recruitment of these cells into the cell cycle and expression of the Epo receptor (104) on their surface. The end stage of this developmental process is the colony-forming unit erythroid (CFU-E). CFU-Es are not morphologically identifiable on routine microscopic examination of bone marrow aspirates or on biopsies. However, they are believed to be only one or two cell divisions upstream from the earliest morphologically identifiable erythroid precursor, the proerythroblast. Almost all CFU-Es are cycling cells that express the maximum number of Epo receptors on their membranes and are therefore the primary target cells for Epo. The interaction of Epo with its specific receptor on the CFU-E determines the daily rate of erythropoiesis, which varies as a function of the plasma Epo titer, the number of CFU-Es, and

the functional integrity of the CFU-E as long as an adequate supply of essential nutrients is provided.

The Epo receptor is a 66-kDa protein that forms homodimers after reaction with its ligand, Epo (124). The Epo receptor belongs to a family of growth factor receptors that have no endogenous tyrosine kinase activity. However, interaction of this receptor with Epo rapidly results in tyrosine phosphorylation of a membrane-proximal cytoplasmic site on the receptor molecule. Recent evidence suggests that this reaction is catalyzed by a member of the Jak 2 family of tyrosine kinases (91). Phosphorylation of the Epo receptor is followed by phosphorylation of several intracellular proteins (21), and c-*myc* is activated shortly thereafter (110). The earliest recorded event, which occurs within 1 min of interaction of the Epo receptor with its ligand, is a rapid influx of calcium. The internalization of Epo ensues within 1 h, and an increase in the synthesis of unspecified mRNAs occurs within 3 h. The specific mRNAs for globin, some erythrocyte membrane proteins, and transferrin receptor first appear after 6 h. Epo receptors are not expressed by early BFU-Es. They first appear on medium BFU-Es and increase in number until the CFU-E stage of development, after which they gradually decline in number as the erythroid cells undergo terminal maturation (122). The specificity of Epo as an erythroid growth factor is underscored by the fact that, except for its expression in low titer on some megakaryocytes (38) and vascular endothelial cells (4), Epo is found only on erythroid progenitors.

The interaction of Epo with its receptor on late BFU-Es and CFU-Es results in (*a*) proliferation of mid- and late BFU-Es (116), (*b*) an increase in CFU-E survival through the inhibition of apoptosis (70), and (*c*) the triggering of CFU-Es to proceed into and through terminal maturation (83). The first two actions result in expansion of the CFU-E population, which in turn amplifies the effect of Epo by increasing the number of Epo-responsive cells. Koury (70) suggested that the primary effect of Epo is to inhibit apoptosis and that if late BFU-E and CFU-E are permitted to survive, then they will express their genetically determined programs in the absence of Epo, i.e. the role of Epo is permissive rather than stimulatory.

Terminal maturation is initiated by the interaction of the Epo receptor on CFU-E with its ligand and terminates with enucleation of the normoblast and the formation of reticulocytes. During the subsequent 3 days, reticulocytes are converted into mature erythrocytes. Reticulocytes normally reside in the marrow for the first 2 days of this transformation, after which they enter the peripheral blood to complete the process of remodeling their cell membrane and removing their nuclear remnants (Howell-Jolly bodies). Under conditions characterized by very high plasma Epo levels, such as severe anemia, reticulocytes may be ejected from the marrow into the circulation immediately or after only 1 day in the marrow. Some investigators have theorized that the

early release of reticulocytes results from the interaction of high-dose Epo with its receptor on the marrow's sinusoidal endothelial cells (4).

During terminal maturation, erythroid precursors must synthesize hemoglobin while undergoing four or five terminal cell divisions, three of which occur after the cell reaches the proerythroblast stage. These two processes must be synchronized in order for the cell to produce enough hemoglobin to reach a normal mean hemoglobin concentration before nuclear maturation impedes additional DNA synthesis and mitosis. Erythroid cells with disordered hemoglobin synthesis undergo an extra mitosis, resulting in microcytic RBCs. Disorders of DNA synthesis cause the cells to reach the targeted mean hemoglobin concentration before they complete the required number of cell divisions and thus result in macrocytic erythrocytes. One hypothesis to explain how cell division is synchronized with hemoglobin synthesis states that DNA synthesis is inhibited when the mean cell hemoglobin concentration reaches its targeted level. Epo initiates but does not regulate terminal maturation of the erythroid precursors. Once under way, hemoglobin and DNA synthesis proceed at a predetermined rate limited only by the delivery of an adequate supply of iron, vitamin B_{12}, and folic acid.

Under normal conditions, all but 1 mg of the iron required for daily erythropoiesis in males and postmenopausal women is derived from the hemoglobin of phagocytosed senescent erythrocytes. Accordingly, only 1 mg/day of iron must be provided by dietary sources. This amount is increased to 2 mg/day in women with average menstrual blood loss and to 4 mg/day in pregnant women. All but the latter requirements are readily supplied by a daily dietary intake of 2000 calories and ~14 mg elemental iron, the content of the average diet worldwide. Consequently, except in infants and children undergoing their growth spurt, iron deficiency is caused by blood loss and not by dietary deficiency. Delivery of sufficient iron to sustain a normal rate of erythropoiesis requires the reticuloendothelial cells to process virtually all of the iron that they harvest daily from the hemoglobin of senescent erythrocytes and to transport it to the cell membrane, where it reacts with plasma-borne transferrin. Transferrin in turn reacts with specific receptors, which are expressed on all proliferating cells but which are found in greatest numbers on the membrane of erythroid precursors that synthesize hemoglobin. The iron-transferrin-receptor complex is internalized, and the iron is transported to the mitochondria, where heme is synthesized.

Decreased hemoglobin production is caused by mitochondrial defects that impair heme synthesis (sideroblastic anemia), by decreased globin synthesis (thalassemia), and by iron deficiency. Of these, iron deficiency is the most common. Iron deficiency can be actual or functional. Actual iron deficiency, which is usually caused by chronic blood loss, results in a decrease in the amount of iron deposited into the stores relative to the amount mobilized for

erythropoiesis. This reduction causes a net daily loss of storage iron, which eventuates in depletion of the iron stores. Functional iron deficiency is characterized by a decrease in the amount of iron supplied to the erythron relative to its needs, despite ample iron stores. This process occurs in association with chronic inflammatory conditions and malignancies, which limit the ability of the reticuloendothelial cells to recycle iron from the senescent erythrocytes, which they phagocytose daily (18). It also occurs when the rate of erythropoiesis is stimulated to exceed the rate of erythrocyte senescence while the iron stores are adequate but relatively unmobilizable.

The mobilizability of iron is receiving renewed attention because of its importance in determining patient response to rHuEpo (32). Iron from phagocytosed RBCs is initially deposited into ferritin, from which it is readily removed for transfer to transferrin. The longer iron remains in ferritin, the more likely it is that the ferritin will be transformed to hemosiderin, from which iron is less easily mobilized (105). Under normal conditions, the amount of iron mobilized from the stores approximates the amount deposited daily. The constant recycling of iron prevents it from becoming immobile. In patients with chronic renal failure, the rate of red cell production, and consequently that of iron turnover, is decreased; therefore, the percentage of iron in a readily mobilizable state decreases. This decrease becomes critical when the rate of erythropoiesis responds briskly to the administration of rHuEpo and causes the requirements for iron to exceed the amount derived daily from senescent erythrocytes. The percentage of iron absorbed from the intestinal lumen increases as the rate of erythropoiesis is increased (34). When the iron mobilized from stores and that absorbed from dietary sources are insufficient to satisfy the requirements of an expanded erythron, then functional iron deficiency ensues.

The nutritional factors that most commonly limit the rate of DNA synthesis during the terminal maturation of erythrocytes are vitamin B_{12} and folic acid, both of which are required for endogenous thymidine production. It is beyond the scope of this article to discuss the metabolism and sources of these two nutrients in detail. However, we emphasize that in adults, folic acid deficiency is the only vitamin deficiency commonly caused by dietary deficiency.

Protein deficiency limits the rate of erythropoiesis of experimental animals at several levels (41). It decreases the rate of regeneration of multipotential stem cells after chemoradiotherapy and reduces the number of CFU-Es and BFU-Es. Its earliest and most profound effects, however, are on Epo production (101). Protein-calorie malnutrition also suppresses erythropoiesis in humans, as indicated by the prevalence of anemia in patients with kwashiorkor (121). However, severe protein depletion in humans is always associated with other deficiencies, making it difficult to study the effects of protein deficiency per se.

REGULATION OF Epo PRODUCTION

Ratio of Oxygen Supply to Oxygen Demand

Epo production by both the liver and the kidneys is regulated primarily by the oxygen supply to the site of production (which is also the site of the Epo sensor) relative to its oxygen requirements (50). The oxygen supply to the kidney is decreased in conditions that cause (*a*) continuous hypoxia to all tissues, which occurs in persons residing at high altitude, in chronic pulmonary disease, in cyanotic heart disease, in anemia, and during increase in the oxygen affinity of the hemoglobin molecule; (*b*) intermittent hypoxia, which occurs during sleep apnea; and (*c*) regional hypoxia, which occurs in patients with hypoxia localized in the kidney.

All of these conditions stimulate Epo production and, consequently, the rate of erythropoiesis. Hyperoxia and erythrocytosis increase the delivery of oxygen to the kidneys, thereby decreasing Epo production and the rate of erythropoiesis. However, a small amount of Epo that is possibly produced constitutively is always detectable in plasma, even in severely polycythemic persons (111). Conditions that increase or decrease the rate of oxygen consumption increase or decrease Epo production in experimental animals, respectively. However, conditions that affect oxygen metabolism in humans often have complex effects on hematopoiesis that may mask their effect on Epo production.

The kidney is an ideal site for Epo production (27) because it is the only organ in which oxygen utilization is closely correlated with blood flow. (Renal oxygen consumption is determined by the rate of sodium reabsorption, which in turn is determined by renal blood flow.) Therefore, the effect of renal blood flow on oxygen delivery is neutralized by its effect on oxygen consumption, and renal Epo production is determined primarily by the oxygen content of the blood.

Epo production is tightly regulated (111). This finding is of teleologic importance because an increased Epo titer results in an increase in the number of circulating erythrocytes, which remain in the circulation for 120 days. Accordingly, if a prolonged lag phase were to occur before the rate of Epo production increased in response to stimuli and if there were a substantial delay in the restoration of basal erythropoiesis after termination of the stimulus, then one would expect large cyclic fluctuations in the rate of erythropoiesis and in the red cell mass. Small diurnal variations in the plasma Epo titers do occur in nonanemic, nonhypoxic persons, and somewhat larger variations have been observed in persons with respiratory abnormalities (89). The precise mechanisms responsible for the tight regulation of erythropoietin production are not known; however, the following observations illustrate some of its effects. First, plasma Epo titers do not rise above the relatively large normal range of the

immunoassay until blood hemoglobin concentration falls below 10.0 gm/dl (111). This apparent lack of response may be the result of rapid compensatory changes in cardiac output and in hemoglobin oxygen affinity, which reduce the stimulus to Epo production, or it may be due to increases in Epo production too small to be discriminated by the immunoassay. Alternatively, it may be caused by a threshold level of hypoxia below which the tissue oxygen sensor cannot detect hypoxia. Second, exposure of experimental animals to continuous hypoxia results in a rise in the Epo content of the kidneys and plasma, which peaks after 8–12 h and then begins to decline (44). Within 24 h of initiating hypoxia, the plasma Epo titer is barely elevated. The reticulocyte count, on the other hand, continues to be elevated until the red cell mass increases. The decline in the Epo titer, with continued hypoxia, can only partially be attributed to compensatory changes that increase oxygen delivery to the tissues. This observation indicates that Epo production is modulated before the red cell mass increases sufficiently to increase the oxygen supply to the kidneys. The nature of this early modulating mechanism is not known, but it is essential for fine-tuning the rate of Epo production. Spivak (111) hypothesized that continuous exposure to hypoxia increases the oxygen sensor's threshold for detection of hypoxia.

Both Epo and Epo mRNA are barely detectable in nonhypoxic kidneys. Accordingly, Epo is not stored in the kidney, and Epo mRNA is produced de novo in response to hypoxia and has a relatively short half-life (111). Exposure to hypoxia initiates a series of transcriptional and translational events that eventuate in the appearance of Epo mRNA after 1.5 h and of Epo after 3 h (73). The number of Epo-producing cells in the kidney increases in proportion to the severity of the hypoxic stimulus, whereas the amount of Epo mRNA per cell is always constant (72). Conversely, in the liver, the amount of Epo mRNA per hepatocyte varies with the intensity of the stimulus (71).

The mechanism by which hypoxia increases the rate of Epo production has been investigated in the Hep3B hepatoma cell line, which produces Epo in a regulated manner in vitro. Based on these studies, Goldberg et al (53) postulated that the oxygen sensor is a membrane-bound, heme-containing enzyme that undergoes a transformational change after becoming oxygenated. This change converts the enzyme to an active form, thus initiating the process of signal transduction, which culminates in the synthesis of a nucleoprotein that activates Epo gene transcription. The putative heme-containing enzyme has not yet been identified, and investigation of the process of signal transduction is still in the early phase. Recently, hypoxia was shown to activate c-*fos* and c-*jun* both in hypoxia-sensitive Hep3B cells and in vascular endothelial cells (54). Both types of cells respond to hypoxia as well as to cobaltous chloride. Hep3B cells respond by producing Epo, whereas vascular endothelial cells produce vascular growth factor. The role of these early-response genes in

initiating or enhancing transcription in response to hypoxia is under investigation. The synthesis of a nucleoprotein, the hypoxia-inducible factor 1 (HIF-1), is induced by hypoxia in Hep3B cells and in other hypoxia-responsive nonerythropoietic cells (106). HIF-1 binds to a transcriptional regulatory element on the 3′ flanking region of the Epo gene and is postulated to be involved in the initiation of Epo gene transcription in response to hypoxia.

In addition to the ratio of oxygen demand to oxygen supply, other factors modulate Epo production. These include hormones, cytokines, the functional integrity of the erythron, and the status of the patient's protein-calorie nutrition.

Hormones

CALORIGENIC HORMONES Calorigenic hormones, such as growth hormone, adrenocorticotrophic hormone (ACTH), and thyroxine, increase the rate of oxygen utilization in experimental animals and the rate of Epo production in response to hypoxia (88). Thyroxine also stimulates Epo production by a noncalorigenic mechanism (87), the nature of which is unclear. It has been speculated that thyroxine activates a steroid/thyroid hormone receptor response element in the enhancer site of the Epo gene.

VASOACTIVE HORMONES Vasoactive hormones, which include renin (3, 51), angiotensin II (42), and adrenergic agonists such as epinephrine (37), increase Epo production by both the kidney and the liver. The effect of renin on Epo production is mediated by angiotensin II. Angiotensin infused in doses ineffective in sham-operated rats stimulates Epo production by the renal remnant of rats made uremic by the surgical removal of five sixths of the renal mass (49). The renal remnants hypertrophy to twice the weight of a normal kidney but do not produce Epo in the absence of angiotensin. Anephric rats produce a significant amount of Epo in response to severe hypoxia initiated a few hours postnephrectomy, but they do not produce a detectable amount if exposure to hypoxia begins 12 h postnephrectomy (49), when tissue angiotensin levels become undetectable (57). Infusion of angiotensin restores the ability of anephric rats to produce Epo in response to hypoxia, even if the hypoxia is initiated 24 h postnephrectomy. This result suggests that nephrectomy, by decreasing angiotensin production, impairs the ability of hepatocytes and renal remnants to produce Epo. In other words, angiotensin plays a permissive role in regulating Epo production (49). This concept is supported by the observation that angiotensin-converting enzyme (ACE) inhibitors suppress extrarenal Epo production in rats (49) and restore normal hematocrits in patients who develop erythrocytosis after renal transplantation (52). In most of these patients, erythrocytosis is caused by inappropriate Epo production by the remnant kidneys (82). This phenomenon can be explained by the hypothesis that

angiotensin must be present for damaged kidneys and extrarenal sites to produce Epo in response to hypoxia. Accordingly, "end-stage" kidneys cannot produce Epo because of damage to both the site of Epo production and the site of renin production. Restoration of renin production by the transplanted kidney restores the ability of the remnant kidneys to produce Epo. Although plausible, this hypothesis is supported only by circumstantial evidence. Pharmacologic doses of angiotensin increase Epo production by intact kidneys of experimental animals (37). This finding may explain the erythrocytosis observed in some patients with Bartter's syndrome.

Other vasoactive compounds that increase Epo production by both renal and extrarenal sites are epinephrine (65) and prostaglandin E_2. In contrast, prostaglandin F_1 inhibits Epo production (35, 49). Other hormones, including prolactin (67) and aldosterone (127), stimulate Epo production in experimental animals, but the physiologic significance of their effects are unclear.

Androgenic Anabolic Steroids

The males of all species studied have higher hematocrits and produce more Epo in response to hypoxia than females (13, 118). Human males excrete a larger amount of Epo in their urine daily than human females (2), although serum Epo levels of normal males, as measured by immunoassay, do not differ from those of females (111). This outcome probably reflects the inability of the immunoassay to distinguish minor differences in Epo titers that fall within the normal range. Injection of androgens into female rats increases their blood counts, whereas castration of male rats (114) and humans (84) reduces their blood counts to the level found in females. This observation indicates that androgenic steroids increase the rate of erythropoiesis. Estrogens have complex and species-specific effects on hematopoiesis.

The metabolism of testosterone results in the formation of the 5βH and 5αH androstanes, of which the latter are more prevalent. Testosterone esters and the 5αH androstanes stimulate both the expression of male sexual characteristics (androgenic properties) and protein synthesis (anabolic properties). The testosterone esters, dihydrotestosterone, and 17 methylated testosterones all have high androgenic to anabolic ratios, whereas 19 nortestosterones have high anabolic to androgenic ratios. All are potent stimulators of erythropoiesis and Epo production (36, 48). The 5βH androstanes, exemplified by the fever-producing steroid etiocholanolone, lack androgenic- and anabolic-stimulating properties. These compounds enhance the response of erythroid progenitors to Epo in vitro and also have been reported to stimulate erythropoiesis in vivo (9, 56), but this is controversial (36). The 5βH androstanes do not affect Epo production.

Androgenic and anabolic steroids act primarily on renal sites of Epo pro-

duction by a mechanism that does not depend on their calorigenic effects. They markedly increase the plasma Epo levels of females rats and, to a lesser extent, those of male rats (45). These steroids have no effect on the Epo titers of hypoxic anephric rats (46, 48), but they increase the plasma Epo titers of patients with anemia of chronic renal failure (23, 30, 45), anemia of chronic disease (103), and aplastic anemia (103). However, they have little or no effect on either the hematocrits or the plasma Epo titers of most but not all anephric patients (23, 30, 45), suggesting that hepatic sites of Epo production are insensitive but not totally unresponsive to these steroids.

Androgenic and anabolic steroids cause both hyperplasia and hypertrophy of the proximal tubular epithelial cells, an increase in renal weight, and transformation of the squamoid cells lining Bowman's capsule into columnar cells indistinguishable from those of the proximal tubular epithelium. The effect of androgens on Epo production correlates with their effect on renal weight (48).

Cytokines

Anemia of chronic disease, which was once believed to be caused primarily by functional iron deficiency (18), now appears to have a more complex pathogenesis that includes the direct effects of inflammatory cytokines on both erythropoiesis and Epo production (74, 86). Ward et al (119) reported that the serum Epo levels of patients with anemia of chronic disease are lower than those of comparably anemic patients with iron deficiency. However, Erslev et al (28) were unable to confirm these observations. More recently, several investigators, using immunoassays for Epo, have observed that anemic patients with rheumatoid arthritis or malignancies have lower plasma Epo levels than comparably anemic patients with iron deficiency (7, 62). The apparent interpretation of these findings is that suppression of Epo production contributes to the pathogenesis of anemias of chronic disease and malignancy. Anemia of chronic disease is usually a mild, nonprogressive anemia in which the blood hemoglobin concentration plateaus at a level that exceeds 9.0 gm/dl (18); more severe anemias occur, but they point to complicating factors. Spivak (111), in a recent review of the physiology of Epo, emphasized that plasma Epo levels of patients with mild anemias do not correlate with the hematocrit and generally fall in or very near the normal range. This result is probably due to the inability of the immunoassay to detect differences between titers that fall in the normal range and the subnormal range. Because the hematocrits of most patients with the anemia of chronic disease are moderately reduced, the Epo titers of a large percentage of patients with this anemia fall into the normal range and do not differ from those of patients with comparable anemias due to iron deficiency. The Epo titers of severely anemic patients with anemia of chronic disease differ from those of patients with iron deficiency. These anemias are most

likely to have complex etiologies. Ward et al (119) found that the plasma Epo levels of patients with rheumatoid arthritis correlated significantly with both serum albumin and transferrin levels. Because both of these proteins are indicators of protein nutrition, protein deficiency may suppress Epo production in some patients. Another possibility is that inflammatory cytokines downregulate the synthesis of Epo, transferrin, and albumin.

Anemia of chronic disease has been attributed to the effects of inflammatory cytokines such as IL-1, IL-6, tumor necrosis factor (TNF), and interferon on both Epo production and responsiveness to Epo production. TNF suppresses CFU-E–derived colony growth in vitro by an indirect mechanism mediated by monocyte-derived interferon (85). The suppression of erythropoiesis caused by interferon can be overcome by high-dose Epo. IL-1 and, to a lesser extent, TNF suppress Epo production by Hep3B cells in vitro and by isolated perfused rat kidneys in vivo (33, 66), whereas IL-6 stimulates Epo production in these models (66). Production of inflammatory cytokines is increased in many of the conditions associated with anemia of chronic disease (58). Although most cytokines are paracrine substances produced primarily in the inflammatory site, serum TNF titers are frequently but not invariably increased in patients with anemia of chronic disease as well as in some patients with conditions such as congestive heart failure that are infrequently associated with this anemia. I am aware of no data that directly correlate the titers of TNF or other inflammatory cytokines with Epo levels and the severity of the anemia of chronic disease. In conclusion, inflammatory cytokines suppress Epo production and CFU-E–derived clonal growth in vitro. They probably contribute to the pathogenesis of anemia of chronic disease, but how and to what extent they do so remain to be determined.

Responsiveness of the Erythron to Epo

In 1959, Stohlman et al (115) observed that the plasma Epo titers of hypoxic rats exposed to sublethal X-irradiation exceed those of hypoxic unirradiated controls. They hypothesized that the clearance of Epo from the plasma of rats is a function of its interaction with its target cells. However, measurements of the plasma half-life of Epo injected into mice with decreased responsiveness to Epo did not differ from those of normal mice (47). An alternative explanation for these observations is that an increase in the responsiveness of the erythron to Epo downregulates Epo production. This hypothesis is supported by observations in WW_v mice, which lack the stem-cell factor receptor and are thus resistant to Epo. These mice have higher plasma Epo titers than comparably anemic nonmutant syngeneic mice. Following transplantation of nonmutant syngeneic marrow cells into WW_v mice, the plasma Epo titer falls to levels comparable to those of nonmutants as their responsiveness to Epo is corrected. These changes do not correlate with changes in the plasma clearance of Epo

(47). The following observations in humans support this hypothesis: (*a*) Hammond et al (59) showed that plasma Epo levels of patients with anemias associated with increased erythropoiesis are lower than those of comparably anemic patients with decreased erythropoiesis; and (*b*) after high-dose chemotherapy or total-body radiotherapy, the plasma Epo level rises independently of changes in the hematocrit (12).

Protein-Calorie Malnutrition

Fried et al (50) showed that the rate of erythropoiesis of starved rats declines within a few days, whereupon their sensitivity to exogenous Epo increases. This observation suggests that an early effect of starvation is to decrease Epo production. After more prolonged starvation, the response to Epo also declines (8).

Whipple (120), in his classic studies on nutrition, noted that a decrease in total protein intake and/or ingestion of proteins deficient in some essential amino acids causes decreased synthesis of specific proteins, which impairs the function of some organ systems. He concluded that organisms prioritize various proteins. Synthesis of some proteins is therefore inhibited by mild protein deprivation, whereas other proteins are synthesized normally until protein deprivation is severe.

In 1934, Orten & Smith (96) reported that the rate of erythropoiesis was decreased in rats fed a protein-depleted diet. These studies were confirmed by Bethard et al (10) using ^{59}Fe incorporation into RBCs as a measure of the rate of erythropoiesis. Subsequently, Reissman et al (101) showed that feeding rats a protein-free diet decreases their ability to produce Epo in response to hypoxia but not their ability to respond to Epo. This result could be due to a decrease in available essential amino acids or to the effect of protein deprivation on oxygen consumption. The decrease in the latter may be a direct effect of protein deprivation or a secondary effect of suppression of thyroid hormone production. Anagnostou et al (5) fed rats isocaloric diets with protein contents that ranged from 0 to 60% of the recommended daily requirement. They found that diets containing 60% of the protein requirements decreased Epo production and that production was further decreased in inverse proportion to the dietary protein content. Rats fed a protein-free diet for only 1 day prior to being made hypoxic exhibited a significant decrease in Epo production. After only 3 days of protein deprivation, Epo production was maximally suppressed but could be restored by feeding a complete protein meal immediately prior to making the rats hypoxic or even 2 h thereafter. Feeding only the individual amino acids cysteine or methionine partially restored Epo production (41). Injection of androgenic or anabolic steroids, or exposure to more intense hypoxia, resulted in an increase in Epo production by protein-deprived rats (41). These studies support the concept that protein deprivation rapidly limits the supply

of amino acids allocated for Epo production. However, this allocation is dependent on the intensity of the stimulus for Epo production. Short-term protein deprivation exerts its effects primarily on renal Epo production. It does not affect Epo production in neonates (79) or anephric rats (6).

It is difficult to study the effects of protein deprivation in humans because conditions associated with protein-calorie malnutrition are usually complicated by a variety of other nutritional deficiencies and/or by infectious diseases. In one study, (121) the plasma Epo levels of patients with kwashiorkor, as measured by radioimmunoassay, did not differ from those of comparably anemic iron-deficient controls. Another study of three normal volunteers fed a diet that was normal in calories but that contained only 17% of the recommended daily protein requirements found that the amount of Epo excreted in the urine after phlebotomy was significantly lower than that measured in the same persons while on normal diets (19).

Little has been written on the effect of carbohydrates on Epo production or erythropoiesis. In one study (17), adding glucose to the drinking water of rats given no other nourishment corrected the decrease in hypoxia-induced Epo production in starved rats.

Miscellaneous

Several reports have implicated the sympathetic nervous system in the regulation of Epo production by experimental animals. The potential clinical and physiologic significance of these findings was underscored by a recent study of patients with primary autonomic failure (11). These patients were anemic and did not have elevated serum Epo levels, and their anemia was responsive to rHuEpo. Therefore, the authors concluded that anemia of autonomic failure likely results from decreased Epo production, implying that the autonomic nervous system plays a physiologic role in Epo production.

Erythrocytosis suppresses Epo production, presumably by increasing the delivery of oxygen to the kidneys. However, it may also do so by increasing the whole-blood viscosity, which suppresses Epo production without affecting the oxygen content of the blood (68, 109). A possible explanation for this finding is that hyperviscosity reduces renal blood flow, which in turn reduces renal oxygen consumption as well as the stimulus for Epo production.

THERAPEUTIC USES OF Epo

Pharmacokinetics, Mode of Administration, and Adverse Effects of rHuEpo

Commercially available Epo for clinical use is produced by mammalian cells transfected with human genomic DNA for Epo (78). Its amino acid and car-

bohydrate composition is virtually identical to that of Epo extracted from human urine. Consequently, it does not stimulate the production of antibodies.

The regimen for administering rHuEpo consists of injecting the compound three times weekly, either intravenously or subcutaneously. Subcutaneous injection is the method of choice in patients without venous access devices, since it is at least as effective as intravenously administered Epo yet easily self-administered by patients.

Several studies have addressed the pharmacokinetics of rHuEpo. Although results vary, in general the half-life for the plasma clearance of intravenous rHuEpo is ~4 h. Subcutaneous rHuEpo reaches peak plasma levels in ~18 h and is cleared from the plasma at a $T_{1/2}$ of ~17 h. The mechanism responsible for clearing Epo from the plasma remains unknown. In experimental animals, <5% of an injected dose of Epo is excreted by the kidneys. The liver, which rapidly removes desialated Epo, has not been shown to play a significant role in clearing rHuEpo. Above we reviewed some of the data indicating that the clearance of Epo is not dependent on interaction with specific receptors on its target cell. To date, no data are available regarding the effect of renal damage or hepatic dysfunction on the pharmacokinetics of rHuEpo; consequently, dose adjustments are not made.

Recombinant human Epo is well tolerated. The only adverse effects attributed to the substance per se are rare transient complaints of bone pain that do not limit therapy. However, some problems have been observed in patients with chronic renal failure who respond rapidly to rHuEpo.

Anemia of Chronic Renal Failure

The plasma Epo titers of patients with anemia of chronic renal failure are invariably lower than expected for the severity of their anemia (93, 97). Recombinant human Epo therapy can serve as replacement therapy in this condition. These patients therefore respond to rHuEpo in a dose-dependent manner. The dose of Epo required to elevate the hematocrit to a target level of 32–36% varies among individuals, but 50–150 mU/kg are usually required three times weekly. Doses of 50–100 mU/kg are required to maintain the target hematocrit. Unresponsiveness to rHuEpo is uncommon and points to complicating factors, including nutritional deficiencies, aluminum toxicity, hyperparathyroidism, infections, and a variety of hemolytic anemias. The most common of these factors is iron deficiency. Patients with renal failure are predisposed to iron deficiency because of blood loss on the dialysis membranes and from uremic gastritis aggravated by the hemostatic defect of uremia. Iron deficiency is characterized by a low serum iron, a total iron-binding capacity (TIBC) saturation of <20%, and a low serum ferritin. These parameters should be monitored prior to initiating therapy; periodically during the early phase of therapy, when the hematocrit is rising; and again if the hematocrit falls during

maintenance therapy. During the initial phase III trials (29) of rHuEpo in patients with chronic renal failure on hemodialysis, almost 40% of patients developed functional iron deficiency characterized by decreased responsiveness to RhuEpo and by a drop in the serum iron and in the saturation of the TIBC. Serum ferritin remained normal or elevated. Responsiveness to rHuEpo was restored in all patients by parenteral iron therapy.

The most common adverse effect of therapy is an increase in blood pressure to hypertensive levels in previously normotensive persons and to even higher levels in patients with preexisting hypertension (29). Hypertension is in most cases readily controlled by antihypertensive medications and rarely requires discontinuation of rHuEpo therapy for more than the time required to control the hypertension. Epileptic seizures (29) occur slightly more frequently during the initial period of therapy, when the hematocrit is rising, but the overall incidence of seizures is no greater in patients who receive rHuEpo than in those who do not. Neither an increased incidence of hypertension nor epileptic seizures were observed in patients treated with rHuEpo for other types of anemias.

In addition to improving the hematocrits of patients with renal failure, rHuEpo also improved these patients' sense of well-being and their exercise tolerance. This outcome was anecdotally apparent to physicians caring for the patients and was subsequently objectively demonstrated by the use of questionnaires (81). Therapy with rHuEpo obviated the therapeutic risks of transfusion therapy, including the diminishing risk of transmitting viral infection, the dangers of precipitating congestive heart failure, and the risk of transfusion reactions. A recent study that compared the cost of transfusion therapy with rHuEpo therapy concluded that the average annual cost of transfusion therapy was $4,630 vs $6,178 for rHuEpo (24). However, these estimates are inexact because of the many assumptions that must be made to determine the full cost of therapy. Nevertheless, the authors concluded that the cost/benefit ratio of rHuEpo justified its use. After the initial success of rHuEpo therapy in patients with chronic renal failure on hemodialysis programs, the efficacy and safety of this compound was also demonstrated in patients with renal failure who were not yet on hemodialysis (32).

Anemia Of Chronic Disease

Anemia of chronic disease was the next disorder selected for clinical trials of rHuEpo therapy (98). This anemia was chosen because of its prevalence and because patients with anemia of chronic disease have lower endogenous plasma Epo titers than comparably anemic patients with iron deficiency, suggesting that decreased Epo production is a factor in the pathogenesis of this disorder.

Patients with rheumatoid arthritis who had hematocrits ≤30% were stratified to receive 0, 50, 100, or 150 mU/kg rHuEpo 3 times weekly for 8 weeks

(98). The targeted effect was a rise in the hematocrit of at least 5 percentage points. There were very few responders until the open phase of the study began. This phase consisted of increasing the amount of rHuEpo by 50 mU/kg every 2 weeks until a response was recorded. Eventually all participants responded, but most required doses ranging from 300 to 600 mU/kg per dose. Investigators concluded that patients with anemia of the rheumatoid arthritis are relatively resistant to rHuEpo but respond to high-dose therapy. A cost/benefit analysis would not likely support rHuEpo as therapy for this anemia except in persons who would benefit from short-term therapy to increase their hematocrits prior to undergoing elective surgery. Nevertheless, the results of these clinical trials were of considerable interest to investigators studying the pathogenesis of anemia of chronic disease because they provided the impetus for studies of the role of inflammatory cytokines in this disorder.

Anemia of Cancer

Anemia of cancer presents a complicated challenge to investigators because this anemia often results from a combination of factors, including anemia of chronic disease; the effects of the tumor on hematopoiesis when it metastasizes to the marrow space; and the effects of chemoradiotherapy on hematopoietic progenitors and, in the case of platinum compounds, on Epo production. The results of several studies indicate that injection of 150–300 mU/kg of rHuEpo 3 times weekly increases the hematocrits of most moderately anemic patients by 5–8 percentage points and also significantly improves their sense of well-being (80, 112). These responses were recorded in patients with a variety of hematologic malignancies and solid tumors treated with various chemotherapeutic regimens, including some that contained platinum compounds. However, the results in patients with more severe, transfusion-dependent anemias were less predictable. In all of the reported studies, parameters of iron availability were monitored, but serum albumin and transferrin levels, which are indicators of protein nutrition, were not reported. Patients with malignancies often become anorectic and have inadequate protein intake, which, when combined with a catabolic state caused by the malignancy, makes them likely candidates for chronic protein deprivation. Protein deprivation, in turn, is likely to affect both their ability to produce and to respond to Epo.

HIV

Patients with HIV infection treated with AZT frequently develop severe transfusion-dependent anemias. A trial of rHuEpo (300 mU/kg three times weekly) demonstrated that patients with pretreatment endogenous plasma Epo levels

lower than 500 mU/ml were likely to respond with a significant rise in hematocrits and a decrease in transfusion requirements (60).

Aplastic Anemia and Myelodysplasia

Aplastic anemia and myelodysplasia are primary disorders of the multipotential hematopoietic progenitors. In the former, little or no terminal maturation occurs, whereas in the latter, a variable proportion of cells undergo disordered terminal maturation, resulting in ineffective erythropoiesis. High-dose rHuEpo therapy increases the hematocrit in ~25–30% of patients (75, 113). Unfortunately, there are no good pretreatment predictors of response, although persons with high pretreatment serum Epo levels are less likely to respond to this therapy.

Use of Epo in Nonanemic Persons

Recombinant human Epo has been used to increase the rate of erythropoiesis of nonanemic persons undergoing therapeutic phlebotomy for hemochromatosis or phlebotomy for the purpose of collecting blood to be used for autologous transfusions during elective surgery. Administration of rHuEpo effectively increases the amount of blood that can be collected (1, 55).

CONCLUSIONS

In the 42 years since Epo was discovered in the plasma of anemic rabbits, we have learned a great deal about its role in the regulation of erythropoiesis and about the complex mechanisms that tightly adjust the rate of erythropoiesis to meet the needs of the vital organs for oxygen. Studies of the regulation of Epo production and of its action have now progressed to the molecular level.

Therapy with rHuEpo has already benefitted thousands of patients with renal failure. Its ability to correct the anemia of some patients with anemias caused by target organ failure or dysfunction suggests that rHuEpo will be more widely used when improved methods of identifying prospective responders become available.

The clinical and laboratory investigations of Epo have led to renewed interest in the nutritional requirements for sustaining an optimal rate of erythropoiesis. They have already changed some concepts of the regulation of iron delivery to the erythron and may very well increase our awareness of the limiting effects of other nutrients, such as protein.

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