Developmental regulation of erythropoietin and erythropoiesis

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Moritz, K. M., Gaik Bee Lim, and E. M. Wintour. Developmental regulation of erythropoietin and erythropoiesis. Am. J. Physiol. 273 (Regulatory Integrative Comp. Physiol. 42): R1829–R1844, 1997.—It is well established that erythropoiesis occurs first in the yolk sac, then in the liver, subsequently moving to the bone marrow and, in rodents, the spleen during development. The origin of the erythropoietic precursors and some factors suggested to be important for the changing location of erythropoiesis are discussed in this review. Until recently, the major site of erythropoietin (Epo) production in the fetus was thought to be the liver, but studies have shown now that the Epo gene is expressed strongly in the fetal kidney, even in the temporary mesonephros. The metanephric Epo mRNA is upregulated by anemia, downregulated by glucocorticoids, and contributes substantially to circulating hormone levels in hemorrhaged ovine fetuses. Other sites of Epo and Epo receptor production, likely to have important actions during development, are the placenta and the brain.

OXYGEN IS REQUIRED for all processes of life at the cellular level (18, 68). Because red blood cells act as the oxygen transporter in blood via the high-affinity hemoglobin molecule, erythropoiesis has to be tightly regulated so as to maintain homeostasis and to meet changes in oxygen supply and demand. Only one type of fish (of the family Channichthyidae) does not have hemoglobin, because of the loss of the β-globin gene some 25 million years ago (see DEVELOPMENT OF ERYTHROPOIESIS IN FISH). The principal factor in the regulation of erythropoiesis is a glycoprotein hormone named erythropoietin (Epo). Although other factors may synergize with Epo (as discussed later), mice that are null mutants for either the Epo gene or its receptor die about day 13 of development (180), demonstrating the critical importance of this one hormone. It is effective at 10−11 M. The hormone is very sensitive to changes in oxygen availability, and its levels are finely tuned by changes in levels of oxygenation, by a classical negative feedback loop. The story of erythropoiesis is therefore very much the story of Epo and its regulation.

Erythropoiesis is the process whereby a fraction of primitive multipotent hemopoietic stem cells becomes committed to the red cell lineage, forming first burst-forming units-erythroid (BFUe), then colony-forming units-erythroid (CFUe), normoblasts, erythroblasts, reticulocytes, and ultimately the mature erythrocyte. The oxygen-carrying pigment of mammalian erythrocytes, or red blood cells, is hemoglobin, four heme molecules attached to four globin chains, which is synthesized in the BFUes. Stem cells require one major growth factor (Steele factor, Stem cell factor, c kit ligand) for their replication and express the c-kit receptor. In the adult, erythropoiesis requires the actions of interleukin-3 (IL-3) and Epo acting on their appropriate receptors.

ERYTHROPOIETIN: HISTORY

The concept that red blood cell production was regulated hormonally was first proposed in 1906 by Carnot and Deflandre (78). Subsequent to that, however, it took nearly half a century before the existence of such a factor, named erythropoietin, was proven conclusively. It was another 20 years before the hormone was purified in amounts sufficient to deduce its amino acid structure and 10 years after that the world was presented with the gene for Epo. In the 12 years since, many advances have also been made in the understanding of the mechanism of action of Epo and its regulation.

There have been many reviews about these topics in the adult (36, 77, 78, 83, 86, 123, 138, 161, 184). This review will concentrate on the development of erythropoiesis in the embryo/fetus/neonate and the sites at which it occurs, as well as the ontogeny and regulation of Epo during development.
ERYTHROPOIESIS: MAMMALIAN

When and where do erythrocyte precursor cells originate? For some time now, it has been known that, in humans, erythropoiesis occurs first in the yolk sac (3- to 4-wk-old human embryo), where relatively large, nucleated red blood cells (megakaryoblasts) are formed, containing predominantly, but not exclusively, embryonic hemoglobin (130). Embryonic hemoglobin (Hb Gower 1) contains 2α- and 2ε-globin chains. There are also small amounts of Hb Gower 2 (αεεε εεεε) and Hb Portland (ζεεε εεεε). By 8 wk, the type of hemoglobin synthesized is predominantly fetal (αεεε εεεε) with small quantities of adult (αεεε εεεε); the liver is the predominant site of definitive erythropoiesis, and the cells are nonnucleated macrocytes. Erythropoiesis begins in the bone marrow by 11–12 wk and ceases in the liver about the time of birth (178).

The sheep has been used widely as a developmental animal model. It is particularly suitable for the study of erythropoiesis, hemoglobin switching, and erythropoietin production, as the sites of erythropoiesis, and the types of hemoglobin produced at different stages, most closely resemble the situation in humans (175). Fetal hemoglobin (αεεε εεεε) can first be detected at 26 days (where term is 145–150 days) and completely replaces embryonic Hb by 37 days, and synthesis of αεεε εεεε terminates around birth. Adult hemoglobin synthesis (αεεε εεεε) begins ~1 mo before birth, but some cells contain fetal hemoglobin until 3 wk after birth. The liver is the major site of fetal erythropoiesis from 26 to 130 days, when the bone marrow becomes the most important site. The fetal spleen does not contain a store of red blood cells until just after birth (17, 134).

In mice and rats the same sites of erythropoiesis occur as in humans, but the embryonic hemoglobin (εεεε εεεε) is replaced by adult hemoglobin (αεεε εεεε) without the formation of fetal hemoglobin (αεεε εεεε). The yolk sac is seeded by hematopoietic precursors by 8 days, and the liver by 10 days, postcoitum. More recently, the questions have concerned whether there are independent cell precursors for embryonic and definitive red blood cells and the origin of the cells that colonize the yolk sac.

By establishing that both primitive and definitive erythroid cells could synthesize, simultaneously, embryonic, fetal, and adult hemoglobins, investigators came to the conclusion that there was no change in hemopoietic cell lineage, but rather a time-dependent change in progenitor cell genetic programming (152). Similar conclusions were drawn from studies on the differentiation, in vitro, of mouse embryonic stem cells (ES; 142). Mouse ES are totipotent cells derived from the inner cell mass of mouse blastocysts, which, if allowed to differentiate, in vitro, can give rise to complex cystic embryoid bodies that may also include visible blood islands by day 8 of culture (34). Even before differentiation begins, these ES cells, in preculture medium, can be shown by reverse transcription-polymerase chain reaction to be expressing Epo and Epo receptor (EpoR) genes (142). By day 8 of differentiation, erythroid BFU

can be detected. When the EpoR was “knocked out,” embryonic primitive erythropoiesis occurred, but definitive cells did not colonize the liver. Homozygous mutants died by embryonic day 13.5 because of failure of definitive erythropoiesis in the fetal liver. However, primitive yolk sac erythropoiesis was not affected until embryonic day 9.5, after which proliferation was impaired and the erythrocytes were smaller than normal (98, 180).

However, the yolk sac is not the only preliver site of development of multipotent hematopoietic progenitors (CFU-S). It is now known that, in the mouse at least, an intraembryonic preliver site of production of CFU-S is a region designated the AGM [dorsal aorta, genital ridge/gonads, and pro/mesonephros (114)]. Using an in vitro organ culture system, these workers were able to show that hematopoietic stem cells initiated autonomously in the mouse AGM region, and they suggested that the in vitro expansion potential of CFU-S from the AGM was greater than that of yolk sac-derived cells. Hence they concluded that the definitive adult cells that colonized the fetal liver most likely derived from the AGM region. The most recent evidence does suggest that the precursors of primitive and definitive erythroid cells may be distinct types of cells (123).

What is the precursor of the stem cell? Some evidence says it is cells that also have the potential to form endothelial cells. Both blood island formation and vasculogenesis do not occur when the vascular endothelial growth factor receptor Flk-1 is knocked out (147). Both endothelial cells and erythroid precursors have EpoRs, although of different affinities and, maybe, structure (4). It has been suggested that endothelial cells express more of the truncated receptor (EpoR-T), which is more common in early erythroid precursors (5).

ROLE OF EXTRACELLULAR MATRIX IN ESTABLISHING LIVER ERYTHROPOIESIS

The family of integrins is composed of cell surface receptors that mediate interactions between cells and the extracellular matrix (71). They are composed of two units (α and β). Hemopoietic progenitor cells adhere selectively to fibronectin, not to collagen, laminin, or proteoglycans (71), and one member of the β1-integrin receptors, very late activation antigen 4, has been shown to be critical for erythropoiesis specifically (63). In mice in which the β1-integrin gene has been deleted by homologous recombination, the hematopoietic stem cell can differentiate but not colonize the liver, although yolk sac production is normal (67).

WHY DOES ERYTHROPOIESIS CEASE IN THE LIVER TOWARD TERM?

There is some evidence that the suppression of erythropoiesis in the liver is due to increasing glucocorticoid concentrations in fetal plasma as term approaches. Decapitation of rat fetuses, which thereby removed the pituitary, at day 17.5 of gestation, resulted in significantly increased liver erythropoiesis at day 21 than in control fetuses (10). These studies were ex-
tended (76), and similar results were obtained in fetuses of bilaterally adrenalectomized mothers and were reversed by cortisol treatment. In vitro, the effect of dexamethasone on the number of CFUe was dependent on the concurrent Epo concentration (14). When Epo values were >25 mU/ml, dexamethasone treat-
iment inhibited CFUe formation. Erythroid precursors, in rat liver, contain dexamethasone-binding sites with characteristics of glucocorticoid receptors at days 15–16 of gestation (113). In chronically cannulated bilaterally adrenalectomized fetal sheep, the amount of α-globin mRNA in the liver at term was significantly greater than in intact controls (Gunnersen and Wintour, unpublished results). Thus there is some support for the idea that increasing levels of glucocorticoids may decrease hepatic erythropoiesis, at least in rat and sheep fetuses. It is likely that there may be other mechanisms that have a role in decreasing liver erythropoiesis and these may differ between species.

ROLE OF EPO/EPOR IN DEVELOPMENTAL ERYTHROPOIESIS

In definitive or adult erythropoiesis, there is an absolute requirement for Epo. The EpoRs first manifest at the BFU-e stage and reach their highest numbers in the CFU-e, after which receptor numbers decline.

Fetal erythroid colonies display different growth characteristics from the adult. There is an accelerated rate of maturation, resulting in erythrocytes that are enlarged compared with adult erythrocytes and which have a shorter life span of 70 days compared with 120 days in the adult. In studies of human umbilical cord blood samples, both BFU-e and CFU-e reached maximal concentrations sooner than adult progenitors (69). Moreover, human fetal BFU-e could be stimulated by Epo alone, whereas the adult BFU-e required additional factors, such as IL-3 or GM-colony-stimulating factor (CSF) (39). CFU-e also have different sensitivities to Epo at different stages of development. CFU-e derived from 8.5-day mouse embryos were more sensitive to Epo than 13.5-day fetal liver and adult bone marrow (187), although this effect was not observed in human progenitors (148). The mechanisms by which these effects occur are not well understood, but could result from different forms of the EpoR being expressed during fetal life or different proportions of more efficient receptors being expressed.

Role of Epo in "fetal" vs. "adult"-type erythropoiesis.

In adult humans, an accelerated fetal-like erythropoiesis occurs when high concentrations of Epo are administered (64), and the mature erythrocytes have a relatively shorter lifespan. Studies in adult rats have shown that recombinant human erythropoietin (rHuEpo) stimulated newborn-like hemoglobin synthesis (1).
disulfide bridges. Although Epo has no significant amino acid homology with any other protein apart from the recently cloned megakaryocytes a factor hormone, thrombopoietin (102), structurally it belongs to a family of cytokines (9, 107). Other members of this family include growth hormone, prolactin, the CSFs, and interleukins. Recently a peptide of 20 amino acids, with no sequence homology to Epo, has been found to activate the Epo receptor (101, 179). This is discussed in more detail later.

**SITES OF SYNTHESIS: ADULT**

Kidney. The studies of Jacobson et al. (75) in 1957 established the kidney as the main source of circulating Epo in the adult. Hybridization histochemistry studies in the mouse to localize Epo mRNA (84, 88) confirmed that the kidney is a primary site of Epo synthesis. The cells that synthesize Epo in the kidney have been shown to be interstitial cells with fibroblast-like characteristics (7) in the vicinity of the proximal tubules in humans (38, 94), monkeys (50), mice (88), and sheep (28).

The other major site of Epo production in the adult is the liver, but hepatic production cannot compensate for loss of the kidneys in cases of chronic renal failure. The contribution of hepatic Epo to circulating plasma levels is 10–15% in anemic nephrectomized rats (41). The hepatocytes are the major cell type producing Epo mRNA in the liver (143), although with the use of a transgenic mouse model (145) it was shown that cells labeled as being Epo producing were hepatic epithelial cells in the area around the central veins (85). Maxwell et al. (112) have since found (1994) expression of Epo in the fibroblast-like Ito cells that share some characteristics with the cells making Epo in the kidney.

Other sites of expression. Expression has also been found in the brain (32, 108, 111, 122, 154, 182), the testis (154), and the placenta (24). Hypoxia can increase mRNA in brain, but to a lesser extent than in kidney. In these sites, particularly the brain and testes, there is a barrier to the free diffusion of blood-borne hormone or locally produced hormone. It is, therefore, quite likely that the local production of Epo is required for a purpose other than the classical one. For both the brain and the testes, there is some evidence of local effects (see discussion in EPO AND EPOR IN THE FETAL BRAIN).

**REGULATION IN THE ADULT: OXYGEN SENSOR**

Regulation of Epo gene expression occurs mainly at the transcriptional level, although it is tissue specific, developmentally controlled, and inducible. There are no intracellular stores of the hormone. Because tissue hypoxia is the major stimulus to Epo production, much interest has centered around how this hypoxia is actually sensed by the body (for review, see Refs. 18 and 137). In 1988, Goldberg et al. (58) proposed that the oxygen sensor may be a heme protein that changed its conformational state when insufficient oxygen was present and thus transmitted the hypoxic signal. Other studies have suggested a cytochrome P-450 protein may be involved (46) or possibly the hydrogen peroxide system (2, 47). A region in the 3′ flanking region of the Epo gene, an area that serves as a hypoxia-inducible enhancer (15, 135), has also been identified. In 1992, Semenza and Wang (146) identified a DNA-binding protein, hypoxia-inducible factor 1 (HIF-1), that bound to part of this region. HIF-1 has now been purified from Hep3B and HeLa cells and characterized (157, 158). HIF is a heterodimer transcription factor containing a 120-kDa HIF-α and a 90-kDa HIF-β subunit. The HIF-β is identical to the aryl hydrocarbon nuclear translocator sequence. The heterodimer HIF-1 binds to a consensus sequence on the 3′ enhancer of the Epo gene, close to where the orphan receptor HNF-4 binds to two tandem consensus steroid hormone response elements, separated by 2 bp. Both HIF-α and HIF-β bind to the transcriptional activator p300 and thus trigger the Epo promoter to transcribe Epo mRNA (70, 87, 144).

There is also evidence for at least one, if not more, negative regulatory element in the 5′ promoter region of the Epo gene. In 1994, with the use of antisense oligonucleotides, Imagawa et al. (72) showed that binding to a 30-bp GATA element stimulated Epo gene transcription even in the absence of hypoxia. This implies that this element is normally a negative regulatory element. More recently, it has been shown that GATA transcription factors can negatively regulate Epo gene expression (73). In addition, in the 3′ enhancer of the human Epo gene, there is a response element for the TR2 orphan receptor, which also suppresses Epo gene expression (89). The TR2 orphan receptor is a protein of 603 amino acids that shares homology with members of the steroid/thyroid hormone receptor family. TR2 and Epo transcripts co-localized in kidney, liver, and brain. Thus Epo gene expression is probably under both positive and negative regulation.

**THE EPO RECEPTOR**

The human and mouse EpoR have been cloned (for reviews, see Refs. 104 and 184). In both, there are eight exons and seven introns (see Fig. 1), encoding a 507/508 amino acid peptide (66 kDa). In the human gene, exons 1–5 encode the 251-amino acid extracellular domain, exon 6 encodes a 20-amino acid membrane-spanning region, and the 236-amino acid cytoplasmic domain is encoded by exons 7 and 8. The exact identity of the ligand-binding domain is not known, but it is thought to be encoded by the 5′ portion of the fifth exon and to stretch, probably, from asparagine 209 on the extracellular domain to the WSXWS (Trp-Ser-X-Trp-Ser) motif (142). The intracellular signaling domain appears to be between Gly 352 and Met 396 (141). In the last 40–90 amino acids of the COOH terminal cytoplasmic domain, there is a negative regulatory domain, removal of which increases the efficiency of the EpoR, such that the dissociation constant (Kd) is now 1 pM instead of 100 pM (183). In fact, in ~30 members of a Finnish family who have a benign elevation in hematocrit, the EpoR gene contains a mutation that truncates the receptor by 70 amino acids from the
COOH terminal (29, 103). One of this family became an Olympic gold medalist in cross-country skiing (103).

At least two other forms of EpoR have been found. One is soluble, as an alternative splicing event introduces a stop codon before the TM sequence. In the mouse, the EpoR may bind Epo, but in the human it is thought not to do so, as it lacks exon 5 (65, 141). There have also been reports of EpoR-T that lacks most of the cytoplasmic region except for 56 amino acids encoded by exon 7 and intron 7 (122, 123). This truncated receptor is predominantly expressed in immature erythroid cells and can transmit a mitogenic signal, but fails to prevent apoptosis, and thus cells expressing EpoR-T may not mature to functional erythrocytes (122). The full EpoR is found on late-stage progenitors (see Fig. 1).

EpoR (R129C) has a point mutation in the extracellular domain of the EpoR (183) in which the arginine at position 129 is changed to a cystine. This leads to a receptor that is constitutively active in the absence of the ligand.

One of the most important breakthroughs in determining the mode of action of Epo in binding to its receptor has come from the recent finding of an Epo mimetic peptide (101, 160, 179). This Epo mimetic is a cyclic (disulfide bonded; cyst p6 to cyst p15) 20-amino acid peptide, which bears no sequence homology to Epo. It functions as a dimer (2 × 2.1 kDa), binds to and causes dimerization of two EpoR molecules, and functions both in vitro (on human bone marrow) and in vivo (in mice) as a biologically active Epo. The K_d for the EpoR is 1,000 times lower (200 nM) than Epo itself (K_d = 200 pM), so that much larger concentrations of the Epo mimetic are required to produce the biological effects of Epo. However, significant progress has been made in understanding how the natural ligand Epo might bind to the EpoR. It is quite probable that in the future more active Epo-mimetics will be produced, which may be of great clinical importance.

**SIGNAL TRANSDUCTION**

In recent years, much has been learned about the subsequent signaling events following receptor dimerization. The signal transduction pathway includes the Janus tyrosine protein kinase 2 (JAK2) and the phosphorylation and nuclear translocation of STAT1 and STAT5 (signal transducing and activators of transcription proteins) (8, 79, 129, 177).

**ONTOGENY OF EPO PRODUCTION IN THE FETUS AND NEONATE**

It had long been thought that, unlike in the adult in which the kidney is the main Epo-synthesizing organ, the liver is the predominant source of Epo in the fetus. This was based on organ ablation studies in fetal sheep (186) and newborn rats (60). More recent studies on Epo mRNA expression in intact fetal sheep have shown that the kidney is a major site of Epo production from very early in gestation (41 days) (95, 174). In the ovine fetus, the Epo gene is expressed in interstitial cells in the vicinity of the proximal tubules in both the permanent metanephric kidney (28) and in the transient mesonephric kidney (174) (see Fig. 2). This is also the site of synthesis in human fetal kidney (94). In all
mammals, there is a period during which these transient mesonephric kidneys and the early metanephric kidneys are present simultaneously and, during this period (18–54 days in the sheep), both kidneys contain Epo mRNA, as shown in Figs. 2 and 3.

By 60 days, the ovine fetal kidney expresses the Epo gene at a level 10-fold higher than in the liver at the same age (95). Taking into account that the liver weighs five times as much as the combined kidney weight, it would seem as if both organs contribute significantly to fetal production. At ~80 days of gestation, expression in the fetal liver begins to decline, but expression remains high in the kidney until after 100 days. By 140 days, Epo mRNA levels in the liver were only 15% of 60-day levels. In the kidney, expression of Epo mRNA at 140 days was 10% of that at 60 days (see Fig. 4).

This change in expression of the Epo gene was reflected in plasma Epo levels (see Fig. 5), with concentrations being significantly lower at 130–145 days than at 100 days (118, 120, 176). In the rat, fetal plasma Epo levels declined from day 17 to day 21 of pregnancy; this decline was accompanied by a large decline in amniotic fluid Epo (22). It should be noted that Epo does not cross the ovine placenta either from fetus to ewe (167) or from ewe to fetus (168), and thus fetal plasma Epo concentrations in the sheep reflect fetal production and clearance. This appears to differ from the mouse where placental transfer was demonstrated by Sawyer et al. (140) in 1989. However, in mice that were null mutants for Epo, because of homologous recombination, the fetus dies at 13.5 days of development, indicating that maternal Epo is not sufficient to maintain adequate erythropoiesis and viability of the developing mouse. Widness et al. (165) have reported that Epo clearance rates are significantly greater in the late-gestation ovine fetus than in the adult, which could account for lower levels in the fetus than in the adult. The sites at which Epo are metabolized/cleared are not known even in the adult. It has been claimed that metabolic clearance rate and/or half-life (t = 1/2) of rhEpo in adult rats or sheep is not affected by the numbers of erythropoietic precursors in the bone marrow (133) or by removal of the kidneys or liver (172). In adult rats, the metabolic clearance rate and t_of rhEpo are not affected by pregnancy (59). One major unanswered question, therefore, is how and where Epo is cleared in the fetus.

In human fetuses, Epo mRNA is also detected in the kidney at midgestation (125), and plasma Epo levels have been reported to increase significantly throughout development (155), although there was no correlation with maternal Epo values (92). However, the samples from early to midgestation were taken by fetoscopy, whereas those at term were from cord blood after delivery. Widness et al. (165) reported that cord blood Epo values were higher following labor when compared with cesarean section. Therefore there may not be a real increase in plasma Epo with gestational age if samples were collected by the same method, e.g., fetoscopy. In fact, other studies (51) showed quite low levels of fetal Epo throughout human development from 20 to 40 wk, levels being generally less than 5 mU/ml.

It is interesting that Epo levels should be relatively low during gestation when erythropoiesis is occurring at a rapid rate. In the ovine fetus, blood volume is increasing at the rate of 7 ml/day over the last third of gestation to keep up with the rapid growth of the fetus (12). Hematocrit is maintained over this period so the fetus must be producing some 50 × 10⁹ extra red blood cells daily. It thus would appear that Epo is either more efficient at stimulating erythropoiesis during fetal development or that some other factor is synergizing with Epo to increase its effectiveness. Also, it may be that Epo acts as a paracrine hormone in the liver as this is both a site of Epo production and erythropoiesis during development. Hepatocyte growth factor (HGF) in the presence of Epo has been demonstrated to stimulate hemopoietic progenitors to form colonies in vitro, an effect that is synergized by stem cell factor (54). The HGF receptor mRNA is highly expressed in embryonic erythroid cells, and HGF mRNA was also produced by the embryonic liver of the mouse (54). A recent report presents evidence that thrombopoietin may have a proliferative effect on erythroid cells in vitro (128). Activin, which is known to be formed by the placenta, can also potentiate the effects of Epo on BFUe and CFUe formation in vitro (115, 185). The IGF family have also been reported to stimulate erythropoiesis in vitro (6, 16), but IGF-1 was not effective in preventing the normal postnatal decline in hematocrit in the neonatal lamb in vivo (119).
Epo concentrations are significantly higher in small-for-gestational age fetuses (92, 151), and this is associated with fetal acidemia and with fetal erythroblastosis. Umbilical cord plasma Epo was also higher in

Fig. 3. Light microscope image of mesonephros at 41 days of gestation in the ovine fetus, hybridized with sense (A) and antisense (B) 35S-labeled riboprobes for ovine Epo. Arrows indicate a group of interstitial cells in the vicinity of the proximal tubules (P) expressing the Epo gene. Light (C)- and dark (D)-field micrographs of a section of ovine metanephros from a 60-day gestation fetus using the same probe. The sites of Epo gene expression are groups of interstitial cells labeled with black dots (arrows in C) or white dots in the dark field. All sections were counterstained with hematoxylin-eosin. Original magnification x400. [From Wintour et al. (174).]

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Fig. 4. Relative levels of Epo gene expression in liver (open bars) and kidney (hatched bars) across gestation in the ovine fetus as determined by competitive reverse transcription-polymerase chain reaction.

Fig. 5. Plasma Epo levels (open bars) and hematocrit (hatched bars) in sheep at various stages of development.
infants of diabetic mothers (169) and in infants of mothers consuming >300 g of ethanol per week (61). Interestingly, infants with renal agenesis have elevated serum Epo concentrations compared with reference values (166). Neonates with renal agenesis have normal values of hematocrit, hemoglobin, and serum Epo. It has also been demonstrated that very low birth weight infants have significantly greater plasma clearance and distribution volume compared with adults (171).

In healthy children, serum Epo decreases after birth (35.6 mU/ml, range 17–56 mU/ml) and reaches a nadir during the first 2 mo (11.5 mU/ml). Epo levels subsequently increase slightly and remain constant between 2 mo and adolescence (18.8 mU/ml, range 7–47 mU/ml) (37). The period of changing Epo levels after birth occurs in other species as well, such as the sheep (176). Neonatal lambs may exhibit a wide range of plasma Epo concentrations in the month after birth although levels are always higher than in the unstimulated fetus or adult (see Fig. 5). After 3–4 wk of age, the plasma Epo concentrations start to decline and by 8–10 wk have reached the basal adult level (176). This occurs despite the well-documented fall in hematocrit that occurs after birth in all species studied. In the neonatal lamb, this decline in hematocrit occurs because of a constant increase in plasma volume with only a slight increase in red cell volume over weeks 3–7 (119). The red blood cells are thus diluted in an increasingly larger plasma volume.

In normal babies, this period is known as the physiological anemia of infancy (27). In premature babies, however, the anemia is more profound and persists longer and can lead to a clinical condition termed “anemia of prematurity” (153), which is characterized by low reticulocyte counts and an inadequate Epo response. These infants frequently require blood transfusions and in recent years there has been a number of clinical trials using rhEpo to prevent or alleviate the anemia. Results of early studies were conflicting, with some supporting the use of exogenous Epo to treat the anemia (11, 40) and other studies finding that Epo treatment had low efficacy and was required at high doses to be effective (62, 148, 173). In a study on rhesus monkeys, it was in fact shown that doses that were effective at increasing Hb values in adults were not effective in infant monkeys (56). A cost-benefit analysis carried out in the United States in 1994 (149) showed that at that time there were no cost savings with routine use of rhEpo with supplemental red blood cell transfusions compared with transfusions alone. This was supported by another study (45) that pointed out that rhEpo can only become a more cost-efficient treatment if the efficacy of rhEpo can be increased and/or the cost of the treatment lowered. A pilot study, however, has shown that the timing of the treatment is important. Infants with very low birth weights had significantly lower transfusion requirement if treatment was begun at 3 days of age (105). Many clinicians remain cautious, however, with respect to widespread use of rhEpo in premature babies.

**EPO AND EPOR IN THE FETAL BRAIN**

It is of great interest that Epo may have an important role in the fetus/neonate during brain development. In adult rats it was shown that whole brain contained small amounts of Epo mRNA (<5% of that in the normoxic kidney) that could be increased by hypoxia, but was insignificant with respect to the 200-fold increase induced by hypoxia in renal mRNA content (154). The rat pheochromocytoma cell line, PC-12 cells, expresses an EpoR, the mRNA of which is indistinguishable from that expressed by rat splenic erythroid precursors, but which has a very low affinity ($K_d = 16$ nM) compared with either of those on rat cells ($K_d = 95$ pM for high-affinity sites; $K_d = 1.9$ nM for low-affinity sites), suggesting that some accessory protein may alter the ligand-binding affinity (111). These PC-12 cells responded to Epo by increasing intracellular calcium ion and monoamine (dopamine, dihydroxyphenylacetic acid, and homovanillic acid but not 3-methyl-dopamine) concentrations.

Primary cultured cells from the septal region of 15-day embryonic mice responded to high levels (5–10 U/ml) of rhEpo with a 50% increase in choline acetyltransferase activity 2 days later (82). Comparable effects occurred in the cholinergic hybridoma cell line SN6.10.2.2. What was of even greater significance, potentially, was that in vivo both low dose (4 daily injections of 12.5 U/injection) or high dose (4 daily injections of 125 U/injection) of rhEpo injected into the brains of rats with unilateral fimbria/fornix resections increased the survival rate of septal cholinergic neurons (82). In the brain, the Epo produced was slightly smaller than renal Epo (30.3 vs. 30.5 kDa), which is thought to be due to less sialylation of brain Epo, and this Epo, in vitro tests, was more potent than serum Epo at stimulating erythropoiesis, but less potent as a neuronal growth factor. As a neuronal growth factor, Epo worked in the nanomolar range, whereas one-tenth or less stimulated erythropoiesis. Epo production in the brain and placenta probably has more relevance to fetal development than to physiological actions in the adult (111). Specific Epo-binding sites were found in mouse brains, with the highest intensity of labeling occurring in the internal capsule, corpus callosum, zona incerta, fimbria hippocampus, and mammillothalamic tract (32). Moderate labeling also occurred in the hippocampus, pyramidal cells of the cortex, brain stem, mesencephalon, lateral posterior thalamic nuclei, and granular layer of the cerebellum. Both Epo and EpoR mRNAs were detected in these mouse brains. Very recently, human and monkey brains have also been shown to express both Epo and the EpoR (108), and it was also demonstrated that mouse astrocytes are a source of Epo that can be increased >100-fold by hypoxia. The sites at which human brain tissue seemed to contain Epo and EpoR mRNA were the temporal cortex, amygdala, and hippocampus. However, brain capillary endothelial cells also contain abundant (10,300...
Because the AT4 receptor appears to be involved in globin precursor of LVV-hemorphin 7 in the brain, speculate that Epo may control the production of the pus, and nucleus caudatus (108), it is tempting to and cerebellar cortex contained immunoreactive glo- 

The synthesis of globins, by mouse neurons, has been reported (116). It was claimed that neuronal cells is indeed the natural ligand for the ANG IV receptor- globins (116). It has been suggested that this peptide b and has an identical sequence to part of the sheep 

When the human EpoR gene was incorporated into the exact cellular expression sites of Epo and EpoR mRNA. When the human EpoR gene was incorporated into the genome of transgenic mice at relatively low levels (4.7 × 10^{-4} to 17.6 × 10^{-4} ng/µg mRNA), human EpoR transcripts were detected in the brains of the adult mice. No endogenous (mouse) EpoR mRNA was detected in adult brains, but very high levels (4.1 × 10^{-2} ng/µg mRNA) were found in the brains of mouse embryos at embryonic day 10, which decreased with development, disappearing by embryonic day 16 (100). This work suggested strongly that the Epo/EpoR sys- tem might have a more important role in the developing rather than the adult brain. The human fetus at 13–17 weeks of gestation, which covers the period of maximum neurogenesis (33), expresses EpoR mRNA in the spinal cord, tentatively identified as ependymal cells enclosing the central canal (93). Epo protein has been measured by enzyme-linked immunosorbent as- say in the CSF of preterm and term neonates at levels higher than in infants or adults (80). These workers have also reported that Epo and EpoR mRNAs are present in brains of human fetuses from 5 to 24 wk of gestation (81). This is a very exciting new area. The finding that Epo mRNA has been detected in the hippocampus and amygdala of humans, monkeys, and mice and can be upregulated by hypoxia/anemia may have significant potential importance for the fetus, because these areas are all vulnerable to damage by intrauterine hypoxic episodes (106). It has therefore been suggested that Epo may play some role in brain development. Very recently a decapeptide, LVV-hemorphin 7, was isolated from sheep brain; LVV-hemorphin 7 binds to the angiotensin IV (ANG IV) receptor (AT_4) and has an identical sequence to part of the sheep β-globins (116). It has been suggested that this peptide is indeed the natural ligand for the ANG IV receptor-AT_4. The synthesis of globins, by mouse neurons, has been reported (126). It was claimed that neuronal cells of the adult hippocampus, basal ganglia, and parietal and cerebellar cortex contained immunoreactive glo- 

It is of note that the knockout of genes, such as GATA 2 and 3, which arrests erythropoiesis, also leads to brain abnormalities (127). As noted earlier, the normal intracellular signaling of the EpoR involves association of a membrane proximal region with JAK2 that is autophosphorylated. JAK2 is found in embryonic mouse brain, and activation of a JAK/STAT (signal transduc- ers and activators of transcription) pathway leads to proliferation of one form of central nervous system progenitor cells (20).

**EPO AND EPOR IN THE PLACENTA**

Epo mRNA was detected in the human placenta by reverse transcription-polymerase chain reaction, but there was insufficient mRNA to be detected by Northern blot even using 50 µg total RNA loaded (24). It was quite surprising that relatively large amounts of immu-noreactivity were detected by immunohistochemistry on a wide variety of placental tissue-villous cytotrophoblasts, endovascular and intravascular cytotropho- blasts, cytotrophoblast cell columns, and syncytiotropho- blasts. Epo is not stored in the adult kidney or liver (78, 83). The finding that EpoR mRNA is expressed by placental tissues, in amounts detectable by Northern analysis, and that the EpoR antibody labeled all the same tissues as the Epo antibody, makes one wonder whether previously detected Epo immunoreactivity was actually due to Epo antibody binding to Epo protein that was attached to the EpoR (25). Therefore, until good hybridization histochemistry studies are carried out with human placenta, the site of Epo synthesis in this tissue must be regarded as unproven. An independ-ent study showing that endothelial cells of human umbilical cord do contain EpoR mRNA suggests that one site of Epo action, whether derived from a local or systemic source, could be placental vasculature (5). The role of Epo in the placenta is still unknown. It could contribute to the increase in maternal plasma Epo that occurs during normal human and rat pregnancy (26, 30). The increase seems to be due to increased production rather than reduced metabolic clearance rate, at least in rats (59).

**REGULATION OF SYSTEMIC EPO IN THE FETUS**

In the ovine fetus, it has been demonstrated that a standard hemorrhage of 20% fetal body weight over a 10-min period elicits a greater plasma Epo response earlier in gestation (100–110 days) than later in gesta- tion at 140 days (118, 120). If the fetus is nephrecto- mized at ~100 days, basal levels of Epo over the following week do not change. If the fetus is then hemorrhaged, however, the liver is not able to compen- sate fully for loss of the kidneys in terms of Epo production. The plasma level reached at 24 h posthem- orrhage was only 19 mU/ml in nephrectomized fetuses compared with 70 mU/ml in the intact fetuses (97). Expression of hepatic Epo mRNA was increased to the same extent in both groups (see Fig. 6). This indicates that although the liver may produce sufficient Epo for maintaining adequate erythropoiesis in, for example, cases of renal agenesis, it can only increase production to a limited extent during periods of erythropoietic stress. It also demonstrates that the renal mRNA is translated into protein.

Studies have also indicated that levels of Epo gene expression during development may be dependent on fetal glucocorticoid status. Maternal treatment for 2–3 days with a synthetic glucocorticoid, dexamethasone,
at 60 days of gestation, leads to a decline in Epo expression in both the kidney and liver, but does not affect maternal Epo mRNA expression in either organ. At 80 days of gestation, a similar infusion only decreases renal mRNA. Cortisol infusion directly to the fetus for 48 h at 100–110 days also decreases renal Epo mRNA (see Fig. 7). Adrenalectomy in late gestation is associated with a fivefold increase in renal Epo mRNA, an effect that can be reversed by replacement of cortisol

Fig. 6. Epo mRNA/standard ratio in liver (A) and kidney (B) of ovine fetuses at 3 stages of gestation: 75 (4C, 4H), 110 (4C, 4H, 6HN), and 140 days (4C, 4H), where C is control, H is hemorrhaged, and HN is nephrectomized and hemorrhaged. Values are means ± SE. Ratios are corrected for fixed amount of total RNA (10 µg) and standard (10 fg). *P < 0.05; **P < 0.01. [From Lim et al. (97), with kind permission from Elsevier Science Ireland Ltd., Bay 15K, Shannon Industrial Estate, Co. Clare, Ireland.]

Fig. 7. Effect of cortisol infusion (F) into 3 bilaterally adrenalectomized (Adx) fetuses for 5 days preceding tissue collection at 141 days of gestation on Epo gene expression, compared with Epo expression in 6 control and 7 bilaterally adrenalectomized fetuses. Top: Bioimaging analyzer-generated image; Bottom: mean ± SE of ratio of Epo mRNA to standard. **P < 0.01. [From Lim et al. (96), copyright The Endocrine Society.]

Fig. 8. Effect of intravenous cortisol infusion (230 µg/h for 48 h) into 5 ovine fetuses at 100–110 days of gestation compared with 7 control fetuses. Top: Bioimaging analyzer-generated image; Bottom: mean ± SE of ratio of Epo mRNA to standard. **P < 0.01. [From Lim et al. (96), copyright The Endocrine Society.]

Fig. 9. Effect of intravenous cortisol infusion (230 µg/h for 48 h) into 5 ovine fetuses at 100–110 days of gestation compared with 7 control fetuses. Top: Bioimaging analyzer-generated image; Bottom: mean ± SE of ratio of Epo mRNA to standard. **P < 0.01. [From Lim et al. (96), copyright The Endocrine Society.]
to the fetus (96, see Fig. 8). It is possible that the effect of steroids is via an interaction with a GATA transcription factor.

**ROLE OF GATA TRANSCRIPTION FACTORS IN REGULATION OF BOTH ERYTHROPOIESIS AND EPO**

The family of GATA transcription factors contains proteins that bind to the motif TA (GATA) A/G in the DNA of promoter or enhancer of many genes. The family now includes six members (GATAs 1–6), all related by a highly conserved DNA-binding region, comprising two zinc fingers. The first one to be described (GATA1) was first described in chicken erythroid cells as an "erythroid-specific factor" (Eryf 1; 44) and in human erythroid cell lines as NF-E1 or GF-1 (44, 110, 157). It is a basic 38-kDa protein comprising 304 amino acids with zinc fingers between amino acids 110–134 and 164–188 (43). Mammalian GATA1 contains 413 amino acids. The carboxyl finger is thought to be sufficient for DNA binding, with the amino finger possibly specifying location. GATA-1 is found in the hematopoietic system and Sertoli cells of the testes. GATA1 and SP1 (which activates the CCACC box) appear to cooperate with other transcriptional factors to activate or inhibit transcription (49). GATA1 is found in the yolk sac where primitive erythropoiesis is occurring, but the level of GATA1 mRNA vs. globin mRNA is much higher in the fetal liver where definitive erythropoiesis occurs (163). Evidence suggests that GATA1 represses embryonic γ-globin and may also repress fetal γ-globin production (13, 136). In some genes (e.g., mouse Epo), the core promoter contains a GATA motif instead of the consensus TATAAA site (3) and binding of GATA1 can inhibit transcription initiation. Although it was not clearly apparent in early studies of chimeric mice containing GATA1-deleted male embryonic stem cells (131, 132, 159), it is now established that GATA−/− embryos die of anemia at 9.5–10.5 days of embryonic development, and both primitive and definitive cells die at the proerythroblast stage (53). Similarly, GATAs 2 and 3 have been shown to be essential for erythropoiesis (126).

In the 5′ promoter region of the Epo gene, there is a negative regulatory element at −30 bp that contains a GATA site (72). Further studies by this group established the rule of GATA factors in the regulation of Epo (73). Glucocorticoid inhibition of mouse GATA function has been demonstrated by the interaction of the steroid receptor ligand complex and GATA protein (21).

**UNANSWERED QUESTIONS**

The regulation of developmental erythropoiesis is obviously of great interest to both clinicians and scientists alike. This review demonstrates that although our knowledge has increased dramatically over the last 5–10 years, many more questions are constantly being generated. The recent findings of Epo in the placenta and brain lead us to wonder as to its role in these tissues. Very little is still known about how hypoxia induces Epo. What is the oxygen sensor? Why is Epo mRNA present in such a large amount early in gesta-

tion in both liver and kidney and then why does it decline? What is the role of the GATA transcription factors in the regulation of Epo production? Are there forms of the EpoR in the fetus that vary from those in the adult? Is there a greater proportion of a more active EpoR in the fetus and a change to a large proportion of less active EpoR in the neonate?

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