MINIREVIEW

Erythropoietin: Physiologic and Pharmacologic Aspects (44183)

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Abstract. **The purpose of this review is to give an update of the recent progress in research on erythropoietin (Epo), the hormone that regulates red blood cell production. Epo is a glycoprotein with a molecular mass of approx 30 kDa, which circulates in plasma of the human with 165 amino acids with three N-linked and one O-linked acidic oligosaccharide side chains in the molecule. Both the α (39% CHO) and β (24% CHO) forms are available for clinical use, and there does not appear to be any difference in the pharmacokinetics of these two forms of Epo. Radioimmunoassays and enzyme-linked immunoabsorbant (ELISA) assays are available in a kit form. Serum levels of Epo in normal human subjects range between 1 and 27 mμ/ml or approx 5 pmol/l. It seems clear that the cells in the adult mammalian kidney which produce Epo are the interstitial cells in the peritubular capillary bed and the perivenous hepatocytes in the liver. Expression of the human Epo gene sequences that direct expression in the kidney are located 6–14 kilobases 5′ to the gene; whereas the sequences that control hepatocyte-specific expression are located within 0.7 KS to the 3′-flanking region and 0.5 KS to the 5′-flanking region. The signal transduction pathways postulated to be involved in the expression of Epo are: kinases A, G and C; both a constitutive factor and a second hypoxia-inducible factor–1 (HIF-1) located in the 5′ end of an hypoxia inducible enhancer region of the Epo gene; and reactive oxygen species. The primary target cell in the bone marrow acted on by Epo is the colony-forming unit erythroid (CFU-E) which has the highest number of Epo receptors. It has been postulated that Epo decreases the rate which Epo-dependent progenitor cells undergo programed cell death (apoptosis). There are two major signal transduction pathways activated by the Epo receptor: the JAK2-STAT5 pathway and the *ras* pathway. Both pathways involve tyrosine phosphorylation. The approved clinical uses of Epo are the anemias associated with end-stage renal disease, cancer chemotherapeutic agents, and patients with HIV infection receiving AZT. Other anemias reported to respond to Epo therapy are anemia of prematurity, rheumatoid arthritis, and myelodysplasia. Other uses of Epo under investigation are in perioperative surgery and preoperative autologous blood donation.** [P.S.E.B.M. 1997, Vol 216]

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The discovery of erythropoietin (Epo), its purification, the cloning of the gene for Epo, and the availability of purified recombinant Epo for the treatment of anemia have been major advances in medicine. Most of these advances have occurred over the past 15–20 years. Carnot and DeFlandre demonstrated over 90 years ago an increase in peripheral red blood cell counts in normal rabbits injected with plasma from rabbits made anemic by bleeding (1). Professor Carnot stated, "we have observed with Mlle De-

Flandre that regeneration of blood after blood letting is under the influence of a humoral process . . . we give this substance the generic name Hémopoiétine.'' The reports by Hjort in 1936 (2), Krumdieck in 1943 (3), and Erslev in 1953 (4) that large volumes of plasma or serum from donor rabbits following a bleeding stimulus, when injected into normal recipient rabbits, produced a brisk increase in peripheral blood reticulocytes were very important in confirming the existence of a humoral factor that controls erythropoiesis. Bonsdorff and Jalavisto (5) gave this humoral substance the name erythropoietin (Epo), which we know today to be the hormone that controls red blood cell production. Reissmann's parabiotic rat experiments carried out in 1950 (6) revived interest in erythropoietin when in these innovative experiments he demonstrated that hypoxia stimulated the production of a factor that regulates red cell production. The seminal finding that nephrectomy prevented the rise in erythropoietin in plasma in animals exposed to bleeding reported by Jacobson and his colleagues in 1957 (7) was very important in elucidating the kidney as a site of production of erythropoietin. Fisher and Birdwell (8) in 1961 carried out studies in which they demonstrated erythropoietin in the perfusate from the isolated dog kidney perfused with blood containing cobalt. Kuratowska *et al.* (9) reported about this same time an increase in Epo production in the isolated rabbit kidney following hypoxemic perfusion. The studies of Nathan *et al.* (10) demonstrating a reduction in erythroid cell precursors in bone marrow of an anephric human subject were also very important in establishing the kidney as a site of Epo production in the human. A major step forward in erythropoietin research was the work of Miyake *et al.,* 1977 (11), in which they purified human urinary erythropoietin to homogeneity. This led to the elucidation of the nucleotide sequence of the human erythropoietin gene in 1985 (12, 13) and to the *in vitro* production of Epo with biological activity in stably transfected monkey kidneys (COS-1) (12) and Chinese hamster ovary cells (CHO) (13). Eschbach and Adamson in 1987 (14) reported the first clinical trials with purified human recombinant erythropoietin in patients with anemia of end-stage renal disease. There have been a number of other advances in erythropoietin research over the past few years, such as: identifying the cellular sites of Epo production in the kidney and liver; the development of immunoassay techniques for erythropoietin; studies on the pharmacokinetics of Epo, especially the plasma clearance of Epo; the finding of diurnal variations in plasma erythropoietin, being highest in the morning and lowest in the evening; signal transduction pathways for regulating erythropoietin production such as G and C kinases and reactive oxygen species; characterization and purification of the erythropoietin receptor; hypoxic regulation of the erythropoietin gene; and the therapeutic uses of erythropoietin now approved by the FDA which are to correct the anemia of end-stage renal disease, for the treatment of patients with anemia of HIV infection treated with AZT, bone marrow suppression due to cancer chemotherapeutic agents, and autologous blood donation. It is the purpose of this review to give an update of the advances in erythropoietin research in the areas of the molecular mechanisms involved with regulating Epo production, cellular sites of production and action of erythropoietin, and the clinical applications of erythropoietin for the treatment of anemia.

Structure of Erythropoietin

Erythropoietin is a glycoprotein produced from a 193–amino acid gene product after an NH_2-terminal leader sequence containing 27 amino acids is cleaved (15). A carboxy-terminal arginine is lost from this 166–amino acid residue during passage into the circulation leaving a circulating hormone with 165 amino acids (Fig. 1). The molecular mass of the erythropoietin peptide is 18 kDa (15). However, the glycoprotein entity as a whole is 30 kDa (17). Erythropoietin is heavily glycosylated with a carbohydrate moiety of approximately 40%. Three N-linked (ASP-24, 38, and 83) and one O-linked (SER-126) acidic oligosaccharide side chains are contained in the molecule (15, 18–21). The carbohydrate constituent of erythropoietin contains fucose, mannose, *N*-acetylglucosamine, galactose, and *N*-acetyl neuraminic acid (22). Human erythropoietin contains 4 cysteine residues that form 2 disulfide bridges between cysteine 7 and 161 and between cysteine 29 and 33 (15). The carbohydrate constituent of erythropoietin is very important in the metabolism of Epo. When Epo lacks the terminal *N*-acetyl neuraminic acid it is rapidly cleared from plasma by the liver primarily because the galactose residues exposed after desialylation bind to galactose binding receptors in the liver (22–25). There are two forms of erythropoietin available for clinical use: the α form, which contains 39% car-

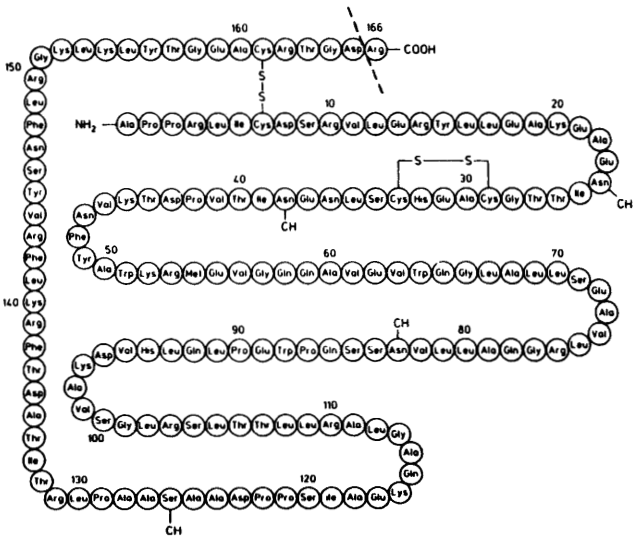

Figure 1. Schematic presentation of primary structure of human erythropoietin. The mature hormone is composed of 165 amino acids, having lost a carboxy-terminal arginyl residue by post-translational modification. There are three N-linked glycosylation cites (CH) at aspartyl residues 24, 38, and 83, and one O-linked glycosylation site (CH) at seryl residue 126. (With permission from Ref. 16.)

bohydrate, and the β form, which contains 24% carbohydrate. Both forms are available for clinical use and there does not appear to be any difference in the pharmacokinetics of the α and β forms. Both are very potent stimulators of early erythroid progenitor cells in the bone marrow.

Assay and Standardization

The polycythemic mouse (ex-hypoxic or transfusion) assay is still regarded as the international reference standard assay for erythropoietin which is an *in vivo* assay measuring radioactive iron incorporation in newly formed red blood cells. This assay is expensive, time-consuming, very insensitive, and requires greater skill and precision than available in most laboratories. The two most common clinical assays for erythropoietin now are the radioimmunoassay (RIA) and the enzyme-linked immunosorbent assay (ELISA). These two assays are available commercially in a kit form. The RIA is very accurate and can be carried out in a short period of time. Serum levels of erythropoietin that cannot be detected by the polycythemic mouse bioassay can be assayed by radioimmunoassay. The ELISA assay has been reported to be easier to handle and more rapid and sensitive than the radioimmunoassay. A high degree of correlation has been reported between a sandwich-type ELISA assay and an RIA for Epo using a monoclonal antibody for Epo (25, 26). A strong correlation has also been reported between the ELISA assay and an *in vitro* bioassay over a wide range of concentrations of Epo (27). A good correlation has been reported between the polycythemic mouse bioassay and a radioimmunoassay for Epo when sera from normal human subjects and patients with several types of anemia were compared (28, 29). The RIA will not distinguish between native Epo, which is active *in vivo,* and asialo Epo, which is inactive *in vivo* (30). Serum levels of erythropoietin in 153 hematologically normal human subjects using an RIA for Epo ranged between 1 and 27 mμ/ml (28). Serum concentrations of erythropoietin in normal human subjects is approximately 5 pmol/l. There does not appear to be a gender difference in serum Epo levels in male and female human subjects in spite of the lower hemoglobin levels in the human female (28, 31). A correlation between hemoglobin concentration (31) or hematocrit (32) and serum levels of Epo has been reported using the RIA for Epo. As indicated in Figure 2, there is a good correlation between serum levels of erythropoietin and hematocrit in anemic patients such as iron deficiency and sickle-cell anemia (32). For example, as the hematocrit is reduced there is an exponential increase in serum levels of erythropoietin. On the other hand, as noted in Figure 2, in the anemia of end-stage renal disease there is no correlation between hematocrit and serum levels of erythropoietin. In addition, serum levels of immunoreactive erythropoietin in patients with anemia of end-stage renal disease are higher than that seen in hematologically normal human subjects (32) (Fig. 2). The best approach to interpreting assays for erythropoietin is to show a correlation between declining hematocrit or hemoglobin

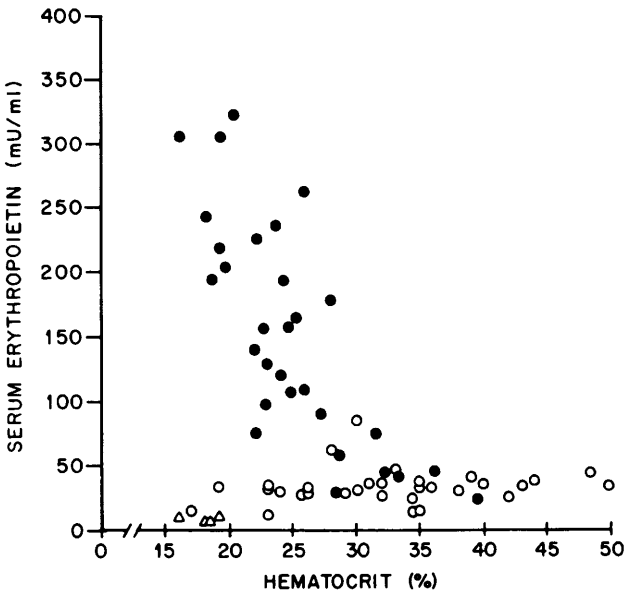

Figure 2. Relation between serum erythropoietin concentration and hematocrit levels in children with normal renal function and varying degrees of anemia due to sickle-cell disease or iron deficiency (●), in children with end-stage renal failure (○), and in anephric children (△). (Based on data from Ref. 32.)

and increasing serum levels of erythropoietin. There is a diurnal rhythm in erythropoietin levels in the plasma, the lowest concentration being at 8:00 AM and the highest level at 8:00 PM (31).

Sites of Erythropoietin Production

Early studies indicate that the liver is the primary site of production of erythropoietin in the fetus, and there is a gradual shift from the liver to the kidney shortly after birth (33, 34). The switch from the liver to the kidney is gradually initiated at 120–140 days of gestation and is completed around 40 days after birth (34). Early studies by Jacobson *et al.* (7) in nephrectomized rats following bleeding indicated that nephrectomy almost completely abolished the increase in plasma levels of erythropoietin when compared with the bleeding response in normal animals. In order to determine whether the nephrectomy experiments merely indicated a "permissive" action of the kidneys, Fisher and Birdwell (8), following cobalt stimulation, and Kuratowska *et al.* (9), following hypoxic stimulation, demonstrated significant increases in erythropoietin titers in the perfusates of isolated kidneys perfused with blood containing cobalt (8) or hypoxemic perfusion (9). It was several years before technology was sufficiently developed to determine the cells in the kidney that produce erythropoietin. Confirmation that the kidney is a primary site of production of Epo in the human was the demonstration by Nathan *et al.* (10) of a low basal level of erythropoiesis in the bone marrow of an anephric patient. In addition, low levels of erythropoietin were found in serum from patients with end-stage renal disease which was restored to normal after kidney transplantation (35). The cloning of the mouse gene for erythropoietin in 1985

provided the technology to study Epo mRNA in mice following a bleeding stimulus (12, 13). After bleeding the Epo mRNA in the kidney was found to be increased up to 1000-fold compared with normal kidneys, whereas only a slight increase in Epo mRNA was seen in the liver (36). The elevation in Epo mRNA in the kidney increased in parallel to the increase in serum levels of Epo, which provided additional support for the hypothesis that the bleeding stimulus induced *de novo* synthesis of Epo rather than the release of erythropoietin from storage sites (37). Koury *et al.* (38), and LaCombe *et al.* (39), demonstrated an increase in Epo mRNA in interstitial cells of the peritubular capillary bed of the kidney. A cell type–specific and hypoxia-inducible expression of the human erythropoietin gene has been demonstrated in transgenic mice by Semenza *et al.* (40). Expression of the human erythropoietin gene sequences which direct expression in the kidney (KIE, kidney inducible element) are located 6–14 kb 5′ to the gene. The sequences which control hepatocyte-specific expression (LIE, liver inducible element) are located within 0.7 kb to the 3′-flanking region and 0.5 kb to the 5′-flanking regions. A negative regulatory element (NRE) has been identified in the human Epo gene that restricts expression only to the 1 nt which resides within 6.5 kb of the 5′-flanking sequences (40). Epo transgenes were prepared spanning LIE and NRE regions (tg Epo 10); the KIE, LIE and NRE (tg Epo 18); and tg Epo 22 isolated from a human genomic cosmid library containing 16.5 kb of 5′-flanking sequences and 2.2 kb of 3′-flanking sequences spanning all of the regulatory elements. As seen in Figure 3, endogenous interstitial cells containing Epo mRNA were in the renal cortex of these anemic transgenic mice with the three transgenes (tg Epo 10, tg Epo 18, and tg Epo 22) (Figs. 3, b, d, and f). No hybridization to the human Epo probe above background was seen in the kidney sections from the tg Epo 10 mice (Fig. 3a); however, in kidney sections from tg Epo 18 and tg Epo 22 mice, hybridization was seen in the same peritubular interstitial cell type with both the mouse and human probes (Fig. 3, c and e). Inducible expression of tg Epo 18, but no expression with tg Epo 10, maps the kidney inducible element to the region 6–14 kb 5′ to the gene. On the other hand, some investigators have reported Epo mRNA in renal tubular epithelial cells (41, 42). In recent studies, Fisher *et al.* (43) have localized Epo mRNA in renal interstitial cells in hypoxic and nonhypoxic monkey kidneys by *in situ* hybridization with either antisense or sense RNA probes generated from a 645 bp *Kpn*I-*Bgl*II fragment of a monkey Epo cDNA (Fig. 4). Epo mRNA was only demonstrated in interstitial cells of the peritubular capillary bed of the hypoxic and normal monkey kidneys utilizing an antisense probe. These studies strongly support the interstitial cells in the peritubular capillary bed of the human as the site of production of erythropoietin. Bachmann and Eckhardt (44), using a combination of high-resolution interference contrast optics, have co-localized Epo mRNA and ecto-5′-nucleotidase in these peritubular interstitial cells, and have

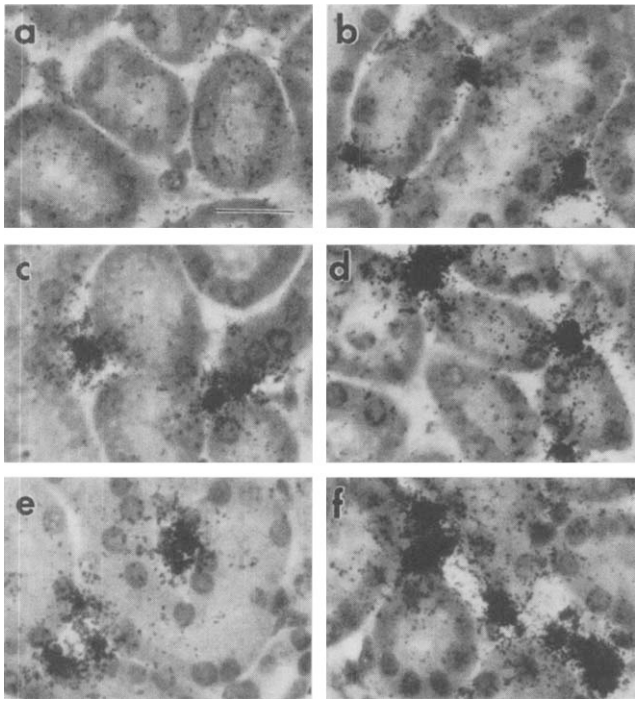

Figure 3. Cell type–specific human Epo transgene expression in kidney demonstrated by *in situ* hybridization. Three-micrometer sections of renal cortex from anemic tgEPO10 (a and b), tgEPO18 (c and d), and tgEPO22 (e and f) mice were hybridized to [^{35}S]-labeled antisense RNA probes specific for human (a, c, and e) or mouse (b, d, and f) EPO RNA. Bar, 20 μm. (With permission from Ref. 40.)

concluded that they were fibroblasts. On the other hand, a recent study by Loya *et al.* (41) using a transgenic mouse model which carries the Epo gene promoter linked to LacZ have reported renal proximal tubular cells as a site of production of erythropoietin. As we have recently explained (43), the reasons for the discrepancy between the study of Loya *et al.* (41) and those of Semenza *et al.* (40) and Maxwell *et al.* (45) could be due to the fact that the transgene used by Loya *et al.* (41) contained only approximately 6 kb of 5′-flanking sequence. In contrast, Semenza *et al.* (40) found interstitial cell expression in transgenic animals having 13.5 and 16.5 kb of 5′-flanking sequence. On the other hand, Maxwell *et al.* (45) used a construct containing approximately 9 kb of 5′-flanking sequences. Thus, it is possible that an important element that controls the cell type–specific expression of Epo in interstitial cells lying between 7 and 9 kb 5′ to the Epo gene was not present in the construct of Loya *et al.* (41). In addition, the construct of Semenza *et al.* (40) contained between 0.3 and 2.2 kb of 3′-flanking sequence. Whereas, the Epo/LacZ construct used by Loya *et al.* (41) contained no 3′ sequence and thus lacked the known hypoxia-inducible enhancer element (46, 47).

The liver is a primary site of erythropoietin production in the fetus (33, 34, 48, 49). Using an *in situ* hybridization technique with anemic transgenic mouse liver sections and a probe that is specific for the expression of human Epo mRNA, Koury *et al.* (38) found transgene expression of Epo

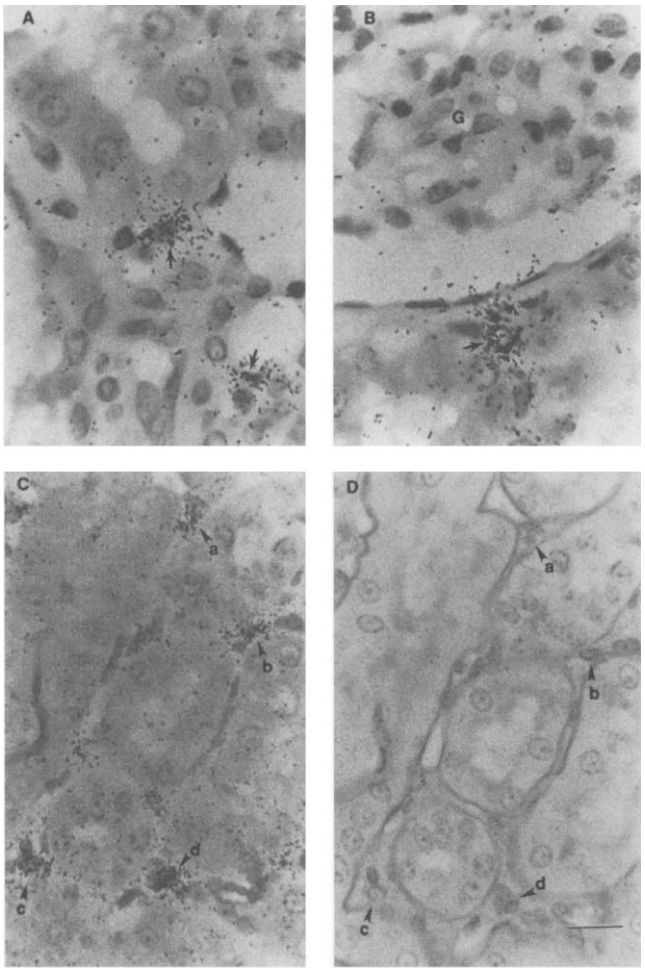

Figure 4. *In situ* hybridization localization of Epo mRNA in a hypoxic monkey kidney. (A) *In situ* autoradiogram of positive (Epo mRNA) interstitial cells in hypoxic monkey kidney. (B) Negative glomerulus (G) and positive interstitial cell. (Magnification of A and B: ×1114.) (C) Higher magnification *in situ* autoradiogram of the hypoxic kidney. (D) Same area of kidney section as C after removal of silver grains from the autoradiogram and staining the section using para-aminosalicylate (PAS). Bar in D, 20 μ*M* for C and D. Arrows A and B, and arrowheads a, b, c, and d indicate Epo mRNA–positive interstitial cells. (With permission from Ref. 43.)

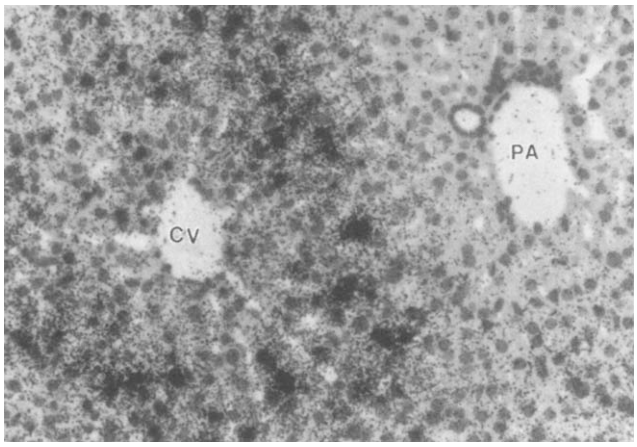

Figure 5. Localization of liver cells synthesizing human Epo mRNA in transgenic mice. *In situ* autoradiogram of a liver section taken from a transgenic mouse bled to a hematocrit of 10% stained with H&E; CV, central vein; PA, portal area. (With permission from Ref. 38.)

mRNA in hepatocytes surrounding central veins of the liver (Fig. 5). The anemic transgenic mouse liver appeared to produce human Epo mRNA exclusively within perivenous hepatocytes (46).

Signal Transduction Pathways and Regulation of Erythropoietin Production

The regulation of erythropoietin production has recently been reviewed by Semenza (46). There have been a number of signal transduction pathways postulated to be involved in the expression of erythropoietin production. Some of these pathways have involved kinases such as kinases A, G, and C (50–55). An autocrine mechanism for the expression of Epo mRNA has also been reported (56). It would appear that cyclic AMP is involved for the most part in amplifying the affect of erythropoietin production requires a prior elevation of erythropoietin messenger RNA by an hypoxic stimulus. Cyclic AMP activation of kinase A has been reported to produce very little effect on erythropoietin production under normoxic conditions but enhances the effects of hypoxia on the expression of erythropoietin (50). Adenosine A_2 receptor activation significantly enhances Epo production in combination with hypoxia and is linked to the activation of adenylate cyclase, increased cAMP, and the stimulation of A kinase (51–53). The most studied recently are G kinase activation with nitric oxide (54, 55) and C kinase (57; Ohigashi *et al.*, unpublished data), and stimulation through free radical production such as H_2O_2 (58). Of interest is the finding that L-NAME, an inhibitor of nitric oxide synthase, the rate-limiting enzyme for the generation of nitric oxide, inhibits hypoxia induced *in vivo* production of erythropoietin in mice (53) and Epo mRNA production in Hep-3B cells (54). In addition, a selective antagonist of cyclic GMP activation of G kinase has been reported to inhibit the increase in production of Epo mRNA in Hep-3B cells exposed to hypoxia (54). It has been postulated that the oxygen-sensing mechanism for Epo gene expression involves a heme protein (59). An interesting study was recently reported which could involve NO in oxygen sensing and in the biological activity of NO (60). Jia *et al.* (60) reported that a dynamic cycle exists in which hemoglobin is 5-nitrosylated in the lungs when red cells are oxygenated, and the NO picked up in the lungs is released during arterial-venous transit. 5-Nitrosohemoglobin in red cells could play a role in signal transduction of NO-induced Epo production. Changes in O_2 tension could regulate NO delivery, for example, by binding NO to hemoglobin in the lungs under oxygenated conditions and releasing NO peripherally by deoxygenation (60).

C kinase has also been reported to be involved as a signal transduction pathway in Epo mRNA expression (57, 58). Calphostin C, an inhibitor of the regulatory head of C kinase, has been reported to inhibit the increase in erythropoietin and Epo mRNA in Hep-3B cells following exposure

to hypoxia (Ohigashi *et al.*, unpublished data). In addition, analogs of diacylglycerol such as OAG and SC-9 have been reported to increase erythropoietin production in Hep-3B cells (Yoshioka *et al.*, unpublished data). The signal transduction pathways for Epo gene expression may involve protein phosphorylation (61).

Studies of DNA-protein interactions involving the 5′ end of an hypoxia-inducible enhancer region of the Epo gene of Hep-3B nuclear extracts revealed both a constitutive factor in Hep-3B cell extracts of both normal and hypoxic cells, and a second factor, which has been named hypoxia-inducible factor–1 (HIF-1) (62). Cyclohexamide, a protein synthesis inhibitor which blocked Epo mRNA production in Hep-3B cells exposed to hypoxia (61), blocked hypoxic induction of HIF-1 production in Hep-3B cells (63). UV cross-linking studies revealed a DNA-binding subunit of HIF-1 which is a protein with an estimated molecular weight of approximately 120 kDa (64). Semenza (46) has postulated that this HIF-1 binds to a 50-nucleotide sequence beginning at 116 nt 3′ to the Epo polyadenylation site. When Hep-3B cells were treated with 2 aminopurine, a protein kinase inhibitor, a marked reduction of hypoxia-inducible factor 1 (HIF-1) activity and Epo mRNA levels were seen in hypoxic Hep-3B cells (47). Semenza (46) has postulated that *de novo* synthesis of an HIF-1 subunit is probably the mechanism of induction of Epo gene transcription. It is also possible that a protein kinase, involved in the phosphorylation of HIF-1, may be necessary for DNA binding activity and hypoxia inducible Epo transcriptional activation.

Hypoxia signal transduction could involve reactive oxygen species such as H_2O_2. Ueno *et al.* (58) have demonstrated in renal carcinoma cell cultures that oxygen-derived metabolites, generated by a xanthine oxidase system, can increase Epo production. When H_2O_2 was scavenged by catalase, the effects of xanthine oxidase in increasing erythropoietin production were blocked. When glucose oxidase was used to generate H_2O_2, erythropoietin production was increased (58). On the other hand, Fandrey *et al.* (65) have found under hypoxic conditions that H_2O_2 suppresses Epo production in a Hep G2 hepatocellular carcinoma cell line. Several reactive oxygen species have been reported to be generated by endothelium to produce vascular smooth muscle activation such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (^-OH), and peroxynitrite ($ONOO^-$) (66). H_2O_2 is well known to activate the kinase C pathway (67). Under hypoxic conditions, followed by reoxygenation in the kidney where oxygenation waxes and wanes, reactive oxygen species such as H_2O_2 are generated that could play a key role in oxygen sensing and Epo mRNA expression. It is quite possible that under hypoxic conditions, as postulated by Semenza (46), there is a decrease in superoxide ion and possibly an increase in reactive oxygen species such as H_2O_2, which could effect transcriptional factors such as Jun and Fos. Much further work is necessary to clarify the link among reactive oxygen species, kinase activation, and Epo mRNA expression.

Action of Erythropoietin

Erythropoietin is known to bind to a specific receptor on the cell surface of erythroid cell membranes (68–72). Epo receptors range from 66- to 78-kDa in molecular mass and may form homodimers following interaction with Epo (68, 69). These receptors have been the most studied in human and murine cells as well as erythroleukemia cell lines (68, 71). The expressed product of the Epo receptor gene in nonerythroid cells has been reported to be 66 kDa (69). The molecular explanation for the observation of a larger 78-kDa Epo receptor in erythroid cells (68) is apparently due to the presence of both additional glycosylation and nontyrosine phosphorylation of the Epo receptor in erythroid cells (70). It has been postulated that this additional post-translational processing gives the most modified form of the Epo receptor the ability to selectively interact with the JAK2 kinase and therefore to transduce an intracellular signal (70). The recent cloning of the Epo receptor gene (71) has facilitated the molecular analysis of the structure and function of the Epo receptor. The burst-forming unit erythroid (BFU-E), the earliest cell in the erythroid lineage, is mostly not responsive to Epo. Low numbers of Epo receptors are seen on the more mature BFU-E, and the density of Epo receptors increases when the BFU-E has matured into the CFU-E (73). The colony-forming unit erythroid (CFU-E) is the primary target cell for Epo, and the highest number of Epo receptors are at the stage of development between the CFU-E and the proerythroblast (73, 74). Reticulocytes and mature erythrocytes do not have Epo receptors. Erythroid cells contain only about 1000 Epo receptor molecules per cell depending upon the response of erythroid cells to Epo (74). The Epo receptors almost completely disappear when the cell reaches the orthochromatic normoblast stage of erythroid cell development (73, 74). When erythropoietin binds to its receptor it is rapidly internalized, undergoing endocytosis and degradation (75, 76). Human BFU-E express only a few erythropoietin receptors, but there is possibly a role for erythropoietin in the survival and differentiation of the BFU-E (73, 77). A sequence of events occurs following binding of erythropoietin to the receptor which includes (78): increased calcium uptake; increased phosphorylation of the Epo receptor and several intracellular proteins (79–82) with the subsequent activation of *c-myc* (83); internalization of the Epo receptor and increased glucose uptake within 1 hr (75, 76); an increase in c-*myc* transcription within 2 hr; an increase in total RNA and unspecified mRNAs; an increase in gene transcription; and increased transferrin receptors after 6 hr; hemoglobin (band 3 and 4 [84]) synthesis begins after 12 hr; and the synthesis of red cell membrane proteins and enucleation begins between 12 and 14 hr. The rapid DNA cleavage in erythroid cells deprived of Epo is an important property of cells undergoing apoptosis (programed cell death) (85). Thus, CFU-E cell

death is avoided by the addition of erythropoietin enabling the CFU-E to survive and differentiate into the nucleated erythroid cell compartment when levels of Epo are high. Koury (86) has proposed a model which postulates that, when Epo levels are increased due to anemia, Epo-dependent progenitor cells that would undergo apoptosis in the presence of normal levels of Epo can now survive and their progenitors then appear in peripheral blood as reticulocytes. Epo has also been demonstrated to stimulate megakaryocyte production by acting on their progenitors, CFU-E-MK (87). An interaction of Epo receptors and stem cell factor (SCF) receptors has also been reported (88, 89). This interaction has been suggested as an explanation for the synergism seen between SCF and Epo in some early hematopoietic cells (88). These investigators speculated that the signal transduction proteins might be recruited to the phosphorylated Epo receptor through SH2 domains such that they would become active either as a result of being phosphorylated by the SCF receptor or merely by being translocated to the cell surface in SCF-treated cells (88). Even though the concept of molecular synergy is attractive, in that only a small fraction of tyrosine phosphorylated Epo receptor co-precipitates with activated c-*kit* (88), the interaction between the Epo-R and c-*kit* requires further experimentation. In addition, it has recently been reported that SCF-dependent phosphorylation of the Epo-R did not initiate an Epo-like intracellular signal (89). These data could mean that there are two distinct pathways for erythroid cell proliferation and prevention of apoptosis in response to Epo. It has also been reported recently that SCF treatment of erythroleukemia cells activated the phosphorylation of the Epo receptor, stimulated proliferation, and appeared to activate the SHC, Ras, ERK-1 Map kinase cascade partly through the Epo receptor (89). However, SCF did not activate the JAK2 kinase or the STAT5-signaling pathway (89). Several mechanisms have been implicated in the signal transduction pathways for Epo. It seems clear that phosphorylation of the Epo receptor and other proteins is involved (79–81). High-affinity, antiphosphotyrosine antibodies have provided good support for this Epo-dependent phosphorylation on tyrosine residues on the Epo receptor as well as a number of other proteins that have not been identified (79). Epo rapidly and specifically induces tyrosine phosphorylation of its own receptor in a human UT-7 Epo-responsive cell line (79). Three Epo receptor subunits were expressed in this cell line, among which only an M, 75,000 subunit was tyrosine phosphorylated following activation by Epo (79). However, with the exception of this cross-linking data, there does not appear to be any other published data to support the existence of more than one Epo-receptor subunit. The tyrosine-specific kinase responsible for the phosphorylation either interacts with an activated Epo receptor after it has been stimulated or else is associated with activation upon binding of Epo to the receptor (78). JAK2 (JAK for JAnus Kinases) has been postulated to be the most important Epo receptor–associated interaction (90, 91). JAK phosphorylation of the receptor probably creates SHC docking sites. Epo induces tyrosine phosphorylation of JAK2 kinase and activates its *in vitro* autophosphorylation, supporting the hypothesis that JAK2 is the kinase that is associated with Epo binding and tyrosine phosphorylation and mitogenesis (90). Others have shown the expression of a dominant negative form of the JAK2 kinase prevented Epo from transducing a proliferation or anti-apoptosis signal (91). Epo has been reported to induce the expression of early response genes c-*jun*, *jun*D, and c-*fos* mRNA and the activation of AP-1/CAT activity in human erythroleukemia K562 cells (92). It was postulated that *jun* and *fos* expression may be relevant in the mechanism of growth control by Epo (92). Epo (93–96) and interleukin-3 (IL-3) (97) have recently been reported to stimulate activation of the JAK2 tyrosine–specific kinase and induce tyrosine phosphorylation and activation of STAT5. The STATs are a class of latent cytoplasmic transcriptional factors (signal transducers and activators of transcription) of which 6 members of this family are currently known (98). Wako *et al.* (93) and Penta *et al.* (94) reported the activation of STAT5 by Epo. Damen and co-workers (95) showed that STAT5 could possibly interact with Tyr 343 in the Epo receptor but that elimination of these tyrosine residues in the Epo receptor still allowed Epo to activate STAT5. It was recently reported that Epo or IL-3 stimulation induces binding of STAT5 to the tyrosine-phosphorylated Epo receptor or IL-3 receptor β subunit, respectively, in IL-3–dependent 32D cells expressing the Epo receptor (97). A model depicting the two signaling pathways activated by Epo is shown in Figure 6. The first pathway is the interaction between the STAT5 SH_2 domain and the Epo receptor cytoplasmic tyrosines, including Y-343, which are phosphorylated by the activation of receptors associated with JAK2 tyrosine kinase. STAT5 is then activated by the phosphorylation of a tyrosine residue on the carboxy-terminal side of the SH_2 domain. Homodimerization of the two STAT5 molecules occurs after phosphorylation. The activated STAT5 homodimers are translocated to the nucleus where the phosphorylated STAT5 activates target genes (97). The second signaling pathway is the *ras* pathway, which is associated with binding and tyrosine phosphorylation of SHC binding sites are also created for the P85 subunit of Pl-3 kinase and for the HCP tyrosine phosphatase. HCP recruitment is thought to result in the negative regulation of the receptor complex. Tyrosine phosphorylation of VAV requires only the membrane proximal receptor domain; similarly, tyrosine phosphorylation of STAT proteins requires the membrane proximal region. Epo activation of the *ras* pathway includes: tyrosine phosphorylation of SHC; the association of SHC within Grb2 (an adaptor protein) as well as with SOS (a G protein nucleotide exchange factor); an increase in *ras* bound GTP activation of RAF-1; tyrosine phosphorylation of mitogen-activated protein kinases (MAPK) (100); and the induction of gene transcription through the activation of transcription factors (TF) (99). Epo receptor phosphorylation may introduce binding sites for the amino-terminal SH_2

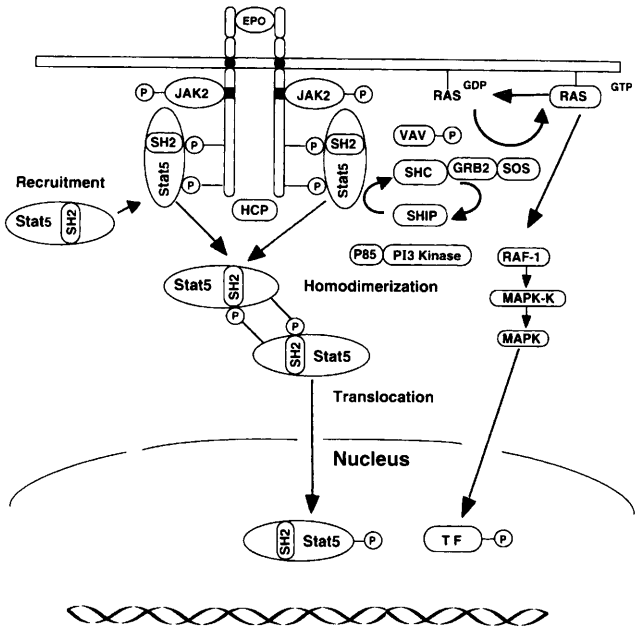

Figure 6. Signaling pathways activated by Epo receptors. The two major pathways activated by Epo binding are the JAK-STAT pathway and the *ras* pathway. The JAK-STAT pathway activation requires the membrane proximal region of the Epo receptor on the erythroid cell, while the activation of the *ras* pathway requires the membrane distal region. Receptor tyrosine phosphorylation of the membrane distal region is required for the association of hematopoietic cell phosphatase (HCP) and is proposed to negatively affect receptor complex activation. The membrane proximal region is required for VAV phosphorylation. Model adapted from Ihle and Kerr (99) and Chin *et al.* (97). Circled P, a phosphorylated tyrosine residue; TF, transcription factors; JAK, Janus kinases; SH2, *src* homology 2 domain; HCP, hematopoietic cell phosphatase; MAPK, mitogen-activated protein kinases; Grb2, an adaptor protein; SOS, a G protein nucleotide exchange factor.

domain of hematopoietic cell phosphatase (HCP) (101). In that deletion of the binding sites in the Epo receptor increases sensitivity to Epo binding of HCP is thought to influence negatively the receptor complex (102). Evidence has been provided to show that Epo induces RAF-1 activation which is required for Epo-mediated erythroid cell proliferation (103). A 145-kDa tyrosine phosphorylated protein that becomes associated with SHC in response to Epo and multiple cytokines may serve to modulate both RAS and inositol signaling pathways (104). Based on its properties, this protein is called SHIP for SH_2-containing inositol phosphatase (104). Barbar and co-workers have recently published further details of Epo receptor signaling through SHC, *RAS,* RAF, and ERK (the MAP cascade) (105). IL-2 and IL-15 produced a dose-dependent tyrosine phosphorylation of SHC; however, Epo failed to activate SHC (105). On the other hand, Epo, IL-2, and IL-15 activated the tyrosine phosphorylation of the adaptor protein, shp2, and the association of shp2/Grb2/cytokine receptor complexes (105). Epo, IL-2, and IL-15 also activated RAF-1 and ERK2, demonstrating that the RAF-1/MEK/MAP kinase was activated (105). Thus, it appears that Epo can activate the RAF-1/MEK/MAP kinase pathway *via* SHC-dependent or SHC-independent pathways. Epo and IL-3 were recently demonstrated to activate tyrosine phosphorylation of cbl and association with crk adaptor proteins in hematopoietic cells suggesting that the inducible cbl-crk association is a proximal component of a signaling pathway downstream of the Epo-R and multiple cytokine receptors (106). Erythropoietin has also been reported to stimulate G protein–coupled phospholipase D in an Epo-responsive cell line (B65Ut.Ep cells) (107) and the induction of metallothionein production in erythroid cells (108).

Clinical Applications of Recombinant Erythropoietin

A major advance in clinical medicine over the past several years has been the use of recombinant erythropoietin in the treatment of patients with anemia associated with end-stage renal disease who are being maintained on dialysis (109). It has enabled these patients to become free of dependence on transfusions and the risks that accompany this intervention. Without a doubt there has been a very significant improvement in the quality of life in end-stage renal disease patients due to the use of Epo in treating their anemia (109). Erythropoietin has also been approved by the FDA to treat anemic patients with HIV infection receiving AZT (110) and cancer patients suffering from anemia associated with cancer chemotherapeutic agents (111, 112). Other uses of erythropoietin under investigation are the anemia of chronic disease such as rheumatoid arthritis (113), anemia of prematurity (114, 115) bone marrow transplantation (BMT) (116) in combination with granulocyte colony–stimulating factor (G-CSF) (117), myelodysplastic patients (118), perioperative surgery (119), preoperative autologous blood donation (120–122), and the anemia of pregnancy (123). The guidelines for the clinical uses of recombinant erythropoietin have been previously reported (124–126).

Therapy with recombinant human Epo is associated with the development of hypertension or else it aggravates a preexisting increase in blood pressure in patients with end-stage renal disease (126–128). This increase in blood pressure is sometimes rapid and severe, and the patient may exhibit hypertensive encephalopathy and seizures (129). This increase in blood pressure occurs almost exclusively in patients with anemia associated with renal disease treated with rhEpo (92). Heidenreich (130) reported that rhEpo produced a direct pressor effect on isolated rat renal resistance vessels and that this contractile response did not require the presence of endothelial cells but disappeared when Ca^{2+} was removed from the medium. rhEpo has been reported to produce a pressor response without increasing the hematocrit in patients with end-stage renal disease (131). These complications may be avoided by reducing the dosage of Epo. Epileptic seizures are seen more often during the initial phase of therapy when the red cells are beginning to increase (126). Several potential mechanisms have been pro-

posed for the increase in blood pressure seen with rhEpo therapy (132) such as increased blood viscosity (133); loss of hypoxic vasodilation (134, 135); direct vascular effect due to increased calcium uptake (136, 137); the activation of receptors for rhEpo on vascular endothelial cells (138); to increase endothelin 1 release (139–141); mitogenic effects (142, 143); and enhanced transient platelet reactivity resulting in a change in platelet aggregability (144–146). There have been no reports of allergic manifestations after either subcutaneous or intravenous administration of erythropoietin. Apparently, it may be necessary to treat some of the patients with a mild increase in blood pressure with antihypertensive agents as well as ferrous sulfate when iron deficiency occurs. Pharmacokinetic studies suggest that the kinetics of erythropoietin conform to a two-compartment model with significant interspecies variation. The average half-life of erythropoietin α (24% carbohydrate) in hemodialysis patients is 9.3 ± 3.2 hr, and the volume of distribution is 5.5% body wt (124). The pharmacokinetics of Epo β (18% carbohydrate) in normal human males is 4.4–11 hr and 6.9 ± 2.9 hr in hemodialysis patients (147).

There are a large number of pharmacologic agents that increase or decrease erythropoietin production (148). The most potent agents for triggering erythropoietin production are cobalt and nickel. Other agents which have been reported to increase plasma levels of Epo in experimental animals include thyroxine, growth hormone, prolactin, serotonin, vasopressin, testosterone, 5α-dihydrotestosterone, prostacyclin (PGI_2), prostaglandin E-1, 6 keto PGE1, angiotensin 2 (through renal vasoconstriction), albuterol (β_2 adrenergic agonists) and adenosine A2 agonists (NECA) (148). Nandrolone decanoate has been reported to produce an improvement in the anemia in selective hemodialyzed patients similar to that achieved by rhEpo but there is no real advantage to using androgens in combination with rEpo in anemia (149). Zidovudine (AZT) has also been reported to increase plasma levels of Epo in HIV-infected patients receiving AZT therapy (150, 151). Agents that have been reported to decrease erythropoietin production in experimental animals are the mercurial diuretics, alkylating agents, β_2 adrenergic blockers, adenosine A1 agonists (CHA), and tumor necrosis factor (148). Theophylline, a nonselective adenosine A1, A2 receptor antagonist, has also been reported to suppress Epo production in patients with polycythemia following kidney transplantation (152–154).

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