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MINIREVIEW

Control of Erythropoietin Production[1] (41646)

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Erythropoietin has been known to be the primary humoral agent which regulates erythropoiesis since the early work in 1906 of Carnot and DeFlandre (1). This hormone was first called "hemopoietine" (1) but has subsequently been named "erythropoietin" (Ep) by Bonsdorff and Jalavisto (2). Reissman's experiments (3), in which one of two parabiotic rats was exposed to an atmosphere of reduced oxygen tension and erythropoietic stimulation was seen in both the hypoxic and nonhypoxic partners, awakened renewed interest in a humoral control of erythropoiesis. Erslev (4) confirmed the Carnot experiments (1) when he injected large quantities of plasma from phlebotomized rabbits into recipient animals and found erythropoietic stimulation. The kidney was established as the primary site of production of erythropoietin with the finding by Jacobson *et al.* (5) in 1957 that bilateral nephrectomy abolished the erythropoietic response of rats to bleeding. More direct evidence for the role of the kidney was provided in the reports by Kuratowska *et al.* (6) and Fisher and Birdwell (7) in 1961 when these investigators demonstrated that erythropoietin could be produced in the isolated perfused kidney following hypoxemic perfusion of the rabbit kidney (6) and the isolated dog kidney perfused with blood containing cobalt (7). This was confirmed by Reissmann and Nomura (167) and by Pavlovic-Kentera *et al.* (221) who demonstrated increased Ep levels in the hypoxemic perfused dog (221) and rabbit (167) kidneys. Fisher and Langston (8) demonstrated later that cobalt and hypoxemic perfusion of the isolated perfused dog kidney produced an apparent synergistic effect by enhancing erythropoietin production in the kidney. The mechanism for the control of kidney production of erythropoietin is not well understood but is postulated to be regulated by the oxygen level in a critical renal sensor cell, and has been the subject of several investigations and reviews (9–17). The purpose of this review is to summarize the present state of knowledge of the many physiologic, pathophysiologic, and pharmacologic factors which may play a role in the control of erythropoietin production.

Purification, Assay, and Standardization. Erythropoietin is a glycoprotein hormone which has recently been purified by Miyake *et al.* (18) who estimated its molecular weight to be in the range of 39,000. However, Espada *et al.* (19), Dorado *et al.* (20), Sytkowski (21), and Rosse (22) have estimated the molecular weight for human urinary erythropoietin to range between 23,000 and 27,000. It is possible that these various erythropoietin preparations are a different molecular species or a desialylated form of erythropoietin.

The exhypoxic (23) or hypertransfused polycythemic mouse assay for erythropoietin remains the International Reference assay for erythropoietin and all *in vitro* and *in vivo* assays for erythropoietin should be standardized against this assay using the International Reference Preparation Erythropoietin as the standard. The International Reference Preparation Erythropoietin (24–26) is provided by the Division of Biological Standards, National Institutes of Medical Research, Mill Hill, London, England. The polycythemic mouse assay is expensive, time consuming, cumbersome, and requires technical experience which few laboratories possess. *In vitro* assays utilizing the fetal mouse liver culture system with tritiated thymidine incorporation into DNA, tritiated deoxyuridine incorporation into RNA, and ^{59}Fe incorporation into heme (27, 28) have been used to assess erythropoietic activity in test samples. However, substances in these samples which may not be erythropoietin but support erythroid cell growth or enhance the effects of erythropoietin in this system, as well as factors which stimulate fetal

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liver production of erythropoietin, are pitfalls which must be recognized in utilizing the fetal mouse liver assay. The hemagglutination inhibition assay for erythropoietin has been used effectively by the originators of this assay (29–31), although some investigators have reported difficulties in correlating this assay with the standard polycythemic mouse assay and other assays for erythropoietin (32, 33).

In reviewing the various assays for erythropoietin, normal human serum levels of erythropoietin in immunological assays and the polycythemic mouse assay have been reported to be in the range of 3.9–36 mu (milliunits)/ml. Hemagglutination inhibition assays (29) show levels between 7–36 mu/ml, and Ep measurements using radioimmunoassays (34–39, 218, 219) range between 13.3–29 mu/ml. Erslev *et al.* (40) and Caro *et al.* (41) report lower values in the range of 3.9–19.0 mu/ml, using the polycythemic mouse assay following the concentration of 240 ml of plasma. Fetal mouse liver (42, 44) and adult mouse bone marrow (45) assays show higher values ranging from 29–50 mu/ml (42–44) up to 790 mu/ml (45). Lange *et al.* (29) summarized several studies of *in vitro* and *in vivo* assays for erythropoietin estimating normal human serum levels to be approximately 30 ± 10 mu/ml depending upon the method of assay used. The lower values found in concentrated specimens may be due to losses incurred in the extraction and boiling techniques used. On the other hand, the higher values which have been reported in fetal mouse liver and normal mouse bone marrow cultures could represent other factors which support the growth of erythroid cells *in vitro*. Cotes and Brozovic (220) have reported a marked diurnal variation in immunoreactive erythropoietin in a healthy male subject during a 4-day control period and following venesection which was the highest around midnight and lowest around noon. In contrast there was no diurnal pattern found in a female subject studied in the same manner (220). DeKlerk *et al.* (43) have developed a technique utilizing radioactive-iron incorporation into heme in the fetal mouse liver *in vitro* assay where a correction factor can be applied to account for changes in serum iron in the samples being analyzed. The radioimmunoassay (RIA) for erythropoietin (34–39) is probably the most sensitive and potentially the most useful assay for routine use in clinical and research laboratories. The recent report (46) of the production of a monoclonal antibody to human erythropoietin may be an important advance in providing a source for a more uniform antibody for use in the radioimmunoassay and other immunoassays of erythropoietin.

Model for the Role of Hypoxia and Prostanoids in Erythropoietin Production. The physiologic and pathophysiologic control of erythropoietin production is still not clearly understood. Hypoxia appears to be the fundamental stimulus which triggers erythropoietin production at renal and extrarenal sites. Our current model for the role of the kidney in the control of erythropoietin production (47) is shown in Fig. 1 and involves, for the most part, an oxygen deficit created by anemia, hypobaria, or ischemia. It is postulated that hypoxia creates a decrease in the oxygen level in a critical renal sensor cell, perhaps in the glomerular tuft (48–52), which eventually leads to the production of prostaglandins and prostacyclin (53, 54) by glomerular tuft cell(s). It is possible that the endothelial (55), epithelial, and mesangial cells (53) in the glo-

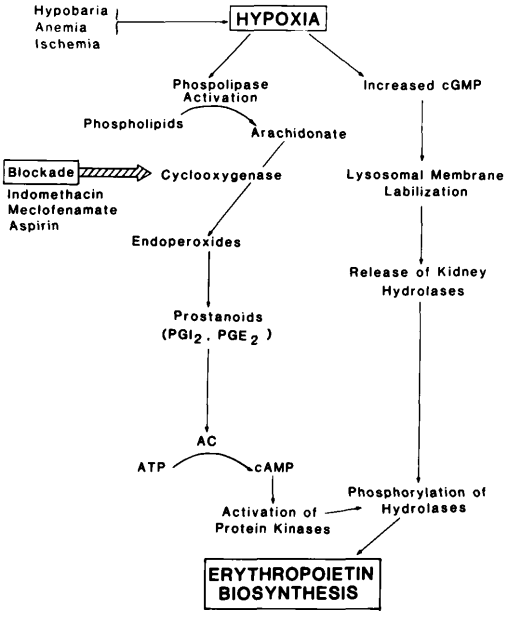

FIG. 1. Schematic Model for Hypoxic Stimulation of Kidney Production of Erythropoietin.

merular tuft, could respond to this oxygen deficit to produce prostacyclin and trigger erythropoietin production. Recent studies (56) on prostaglandin synthesis is human isolated glomeruli and human glomerular epithelial and mesangial cultured cells indicate that the most abundant prostanoid synthesized by these cells was 6-keto-$PGF_{1\alpha}$, a metabolite of prostacyclin (PGI_2). PGI_2 has also been reported to be produced by vascular endothelial cells (55). The mechanism by which hypoxia may initiate the synthesis and/or release of prostaglandins and prostacyclin in the renal cell has not been elucidated.

Neutral proteases and lysosomal hydrolases, documented triggers of erythropoietin production, are also elevated in the kidney after cobalt or hypoxia (57–60). The mechanism of labilization and release of these enzymes from the renal lysosomes has been postulated to be a cholinergic event related to increases in cyclic GMP levels in a renal cell (61, 62). Hypoxia is known to cause the release of renal lysosomal hydrolases (57–60) which then undergo phosphorylation through activation by protein kinases (63, 69, 70) following prostanoid stimulation of renal adenylate cyclase to generate cyclic AMP (9, 64–70), resulting in increased biosynthesis of erythropoietin. ATP depletion has been reported in both myocardial (71, 72) and renal (73) cells following exposure to hypoxia and has been postulated as the cause of the consequent loss of intracellular homeostasis (71, 72). DL-propranolol, a beta adrenergic antagonist, blocked this ATP depletion induced by ischemic hypoxia in the myocardial cell (72). Furthermore, propranolol is known to inhibit the effects of hypoxia on erythropoietin production (74).

Hypoxia has been demonstrated to cause the activation of phospholipase (75, 76) and increased release of prostaglandins from the kidney (77), which may be important in relating increased prostanoids (75, 76) with erythropoietin production. Most species of phospholipase A_2 are absolutely dependent upon calcium ions (76). Calcium has also been demonstrated to enhance phospholipase activity (75) while EDTA inhibited this enzyme (76). Calcium was the most potent of several bivalent cations to stimulate PGE_2 formation in rabbit kidney slices (70), and appears to stimulate phospholipase A_2 activity (78). Hypoxia ($PO_2 < 25$ mm Hg) resulted initially in a decrease in calcium entry followed by an increase in calcium uptake after 30 min in the arterially perfused interventricular septum of the rabbit heart (79). The calcium entry blocker verapamil has been demonstrated to enhance erythropoietin production in rats in response to hypoxia (80) suggesting a link between calcium and erythropoietin production. Calcium deprivation and the calcium transport inhibitor methoxyverapamil (D600) have also been demonstrated to cause a marked increase in renin release from isolated rat glomeruli (81), and calcium antagonists increase renin release from the isolated rat kidney (82). Some investigators (83–85) have postulated an interrelationship between the renin–angiotensin system and renal and extrarenal erythropoietin production. Thus, it is of interest to make certain comparisons between renin and erythropoietin secretion. Renin release has been demonstrated to be stimulated by prostacyclin (PGI_2) in isolated rat glomeruli (86). PGI_2 and its metabolite 6-keto PGE_1 also stimulated erythropoiesis in polycythemic mice (87). In that both renin and erythropoietin production are influenced by calcium and prostanoids, it is important to consider the relationship of prostanoids and calcium. The effects of trifluoperazine (TFP), a specific inhibitor of the calcium regulator protein calmodulin, has been found to increase renal glomerular synthesis of PGE_2 and $PGF_{2\alpha}$ which was associated with the release of arachidonate from two specific phospholipid pools, phosphatidylcholine and phosphatidylethanolamine (88). Whereas mepacrine, a general phospholipase inhibitor, caused a decrease in prostaglandins synthesis by isolated glomeruli suggesting that PG synthesis is associated with a specific arachidonate pool in phosphatidylcholine (88).

It is not known whether the low levels of calcium needed for phospholipase A_2 activation (75, 76) or the high levels of cytosolic calcium seen in cells following hypoxia (79) are related to the increased prostanoid release and erythropoietin production seen following an oxygen deficit.

Arachidonate levels are also elevated in the hypoxic cell following the activation of phospholipase A_2. This arachidonate is acted upon by a cyclooxygenase enzyme leading to an ele-

vation of endoperoxides in renal glomerular cells which could be important in erythropoietin production. Oxygen concentrations of 5–10 μm are sufficient for optimal cyclooxygenase activity (89). Rabbit kidneys perfused *in vitro* convert exogenous arachidonic acid principally to PGI_2, although stimulation of the release of endogenous arachidonate with ischemic hypoxia leads to the appearance of PGE_2 in the renal effluent (90). PGI_2 is probably produced by a vascular wall cell in the glomerular tuft since *in vitro* production of PGI_2 has been demonstrated in the renal vasculature (91). Microvascular endothelial cells have also been demonstrated to synthesize and release the biologically active metabolite of PGI_2, 6-keto-PGE_1 (92) and the intrarenal conversion of PGI_2 to 6-keto-PGE_1 has been reported (93).

The finding that hypoxia of the isolated perfused kidney leads to an elevation in erythropoietin and the prostacyclin metabolite 6-keto $PGF_{1\alpha}$ (14, 94) supports the hypothesis that prostacyclin and/or its intrarenal metabolite, 6-keto PGE_1 (95, 96), may actually be involved as the trigger for the adenylate cyclase system to initiate erythropoietin production. When the erythropoietic activity of prostacyclin and its metabolites 6-keto $PGF_{1\alpha}$ and 6-keto PGE_1 were studied in exhypoxic polycythemic mice, prostacyclin showed moderate activity, 6-keto $PGF_{1\alpha}$ was inactive, and 6-keto PGE_1 was very potent, and three to fourfold more active than PGE_1 (14, 87). Yuan *et al.* (97) have purified 9-hydroxyprostaglandin dehydrogenase from rat kidney, which appears to be the enzyme involved in the conversion of PGI_2 to 6-keto $PGF_{1\alpha}$ and 6-keto PGE_1.

Stimuli of renal production of erythropoietin involving renal artery constriction (ischemic hypoxia) (16), beta-2 adrenergic activation (15) of kidney, and apparently renal carcinoma (124, 125) production of erythropoietin, are probably involved more in increasing the production of PGE_2 which triggers kidney production of erythropoietin (Fig. 1).

In summary, it seems clear that prostacyclin or its metabolite 6-keto PGE_1 play some physiologic and/or pathophysiologic role in kidney production of erythropoietin. Prostacyclin, or its metabolite 6-keto PGE_1, may be released in response to hypoxia (87, 92) to trigger adenylate cyclase (66) leading to an initiation of the erythropoietin biosynthetic cascade according to the model shown in Fig. 1.

Extraction, Localization, and in Vitro Studies on Erythropoietin. Attempts have been made in the past to extract erythropoietin (Ep) from kidneys of hypoxic, anemic, and normal animals (98–100); however, these efforts have not been uniformly successful and have resulted in varying yields of erythropoietin. Contrera *et al.* (98, 99) found that a hypotonic phosphate-buffer extract from the kidneys of hypoxic and anemic rats possessed more erythropoietic activity than could be accounted for in the residual trapped plasma. They postulated the existence of a renal erythropoietic factor which has the capacity to produce erythropoietin or become erythropoietically active when incubated with normal rat serum (99). This renal erythropoietic factor (REF, erythrogenin) was found to be distributed throughout the kidney (Cortex, medulla, glomeruli, and tubules) (210). Kuratowska *et al.* (101) subsequently concluded that a plasma protein is required to interact with a kidney erythropoietic factor to generate erythropoietin. Gordon *et al.* (102) suggested that the renal erythropoietic factor is an enzyme which acts upon a plasma factor to produce erythropoietin. On the other hand, Erslev (103) and co-workers have found that isolated perfused hypoxic kidneys can elaborate erythropoietin into the perfusate when the kidneys are perfused with a medium which does not contain plasma proteins.

Fried *et al.* (104) recently demonstrated that large amounts of biologically active erythropoietin were extractable from hypoxic rat kidneys homogenized in an isotonic phosphate-buffer solution. A correction factor was applied to the amount of erythropoietin trapped in the plasma contained in the quartered kidney fragments which were washed at the time of Ep extraction. The Ep trapped in the plasma was not considered to be sufficient to account for the significant amount of erythropoietin extracted. Sherwood and Goldwasser (105) also reported the extraction of significant amounts of active erythropoietin from whole kidney homogenates of normal rats, cattle, dogs, and rabbits.

The recent work by Katsuoka *et al.* (106) indicates that less than 16% of the total extractable erythropoietic activity in unflushed

kidneys from rats treated with a combination of hypoxia and cobalt represents true kidney Ep emphasizing the contribution of Ep contained in the trapped plasma in kidney homogenates. These investigators also reported (106) that cobalt is more effective in stimulating *de novo* kidney erythropoietin production in combination with hypoxia than either alone, and postulated that the potentiating effect of this combination may be due to hypoxia increasing the sensitivity and/or number of cobalt receptors in the kidney.

The above studies indicate clearly that erythropoietin can be extracted from the kidney itself and provides more definitive evidence for a primary physiological role of the kidney in the *de novo* synthesis of erythropoietin which does not apparently require the interaction of a renal factor with a plasma substrate to produce erythropoietin. However, these findings do not exclude the possibility of an interaction of a renal erythropoietic factor (REF), or enzyme, with a protein substrate within the kidney itself to produce erythropoietin *de novo* in the kidney which is then released into the renal circulation.

Erythropoietin has been localized in the glomerular tuft of the sheep kidney utilizing the indirect fluorescent antibody technique (48). The fluorescence was seen in the capillary wall of the glomerular tuft but not in the peritubular capillary beds, juxtaglomerular cells, or capillary walls of the spleen, liver, or lung. This type of fluorescence in the peripheral portion of the anemic sheep kidney glomerulus was confirmed by Frenkel *et al.* (107). Zucali and Mirand (108) also demonstrated fluorescence in the glomerular tuft of the hypoxic rat kidney utilizing a fluorescent antibody technique. Busuttil *et al.* (49, 50) have demonstrated intense fluorescence in the glomerular tufts of anemic human and hypoxic dog kidneys. They localized the fluorescence in the peripheral portion of the glomerular tuft of the anemic human (49) and the hypoxic dog kidney (50) and suggested that the localization was in the epithelial cells of the tuft. Burlington *et al.* (51) produced erythropoietin in renal glomerular cultures and found an overgrowth of epithelial cells in the glomerular tuft. In contrast, Kurtz *et al.* (52) found an increased level of erythropoietin in the culture media of an isolated rat glomerular preparation but the cells predominantly growing in their subcultures were reported to be mesangial cells. Incubation of anti-Ep with highly purified erythropoietin blocked the fluorescence seen in the glomerular tufts of the human (49) and the dog (50) kidneys. In addition, elevating blood levels of erythropoietin in normal dogs (50) with an intravenous infusion of Ep failed to increase the fluorescence in the glomerular tuft indicating that nonspecific trapping of Ep by the glomerulus, when plasma Ep titers are elevated, is unlikely to be involved. Further immunocytochemical and electron microscopic studies are necessary to completely identify the cells in the glomerular tuft showing this fluorescence. New developments in the production of a monoclonal antibody to erythropoietin (46) may provide the improved technology to identify the cells in the glomerular tuft region of the kidney or extrarenal sites, which synthesize erythropoietin.

Several investigators have attempted to correlate changes in granularity of the juxtaglomerular cells in the kidney with stimulation of erythropoietin production (109–114). Donati *et al.* (222) demonstrated a clear dissociation between renin and erythropoietin secretion. It is most likely that changes in granularity of the juxtaglomerular cells following triggers of erythropoietin production, such as renal artery constriction, are not specific for erythropoietin but reflect a regulatory role for renin secretion. However, it is most likely that hypoxia and other erythropoietic stimuli produce changes in granularity of the juxtaglomerular cells in association with increased renin production. An enhancement of renin production leads eventually to elevated plasma levels of angiotensin II which produces renal ischemic hypoxia and could be responsible for the increased biosynthesis of erythropoietin following activation of the renin–angiotensin system (83–85, 165).

In vitro production of erythropoietin has been reported in normal kidney cell cultures (212–215), in isolated glomeruli (51, 52), and in renal carcinoma cells in culture (127, 216). Some of these studies have been reviewed by Ogle *et al.* (217). Even though *in vitro* formation of a protein, believed to be Ep, has been reported in sheep kidney medulla (214), most *in vitro* culture studies of normal kidney cells implicate the renal cortex as the primary site of erythropoietin production (51, 52, 212, 213, 215, 218).

Erythropoietin Production by Tumors. Erythrocytosis has been associated with tumors of the kidney, liver, central nervous system, uterus, adrenal, ovary, lung, and thymus (115). The kidney is the most common location for a neoplasm associated with erythrocytosis (116). The leading kidney tumor found with erythrocytosis is the renal adenocarcinoma (hypernephroma) (117). The reason why only a small percentage of renal cell carcinomas are associated with erythrocytosis (118–122) is not clear. Several mechanisms have been proposed to account for the increased erythropoietin production in renal adenocarcinomas which involve for the most part tumor-induced hypoxia (123). Some of the mechanisms which deal with intrarenal ischemia and hypoxia are as follows: (i) enhanced production of kidney prostaglandins, especially PGE_2, due to ischemic hypoxia (124, 125); (ii) ischemia related to increased pressure caused by extra- and intracapsular tumors; (iii) tumor obstruction of the renal arterial and venous circulation; and (iv) obstruction of the ureter with elevated intrarenal pressure due to hydronephrosis.

In contrast, cancer cells have also been reported to inhibit erythroid progenitor cells (CFU-E) and it was suggested that malignant cells produce an inhibitor of erythropoiesis (126). The low incidence of erythrocytosis associated with renal adenocarcinoma could be due to these mechanisms opposing polycythemia including a tendency for the tumor cell to produce substances which are inhibitory to erythropoiesis.

In considering further the mechanisms for renal tumor production of Ep it seems most likely that renal ischemia and hypoxia in the renal carcinoma patient may lead to the production of prostaglandins such as PGE_2 and prostacyclin which trigger erythropoietin producing cells within the kidney tumor cells as well as metastatic cells. In that metastatic renal carcinoma cells produce erythropoietin in association with erythrocytosis Ep production is apparently autonomous. Prostaglandins have been demonstrated to be increased in renal cell carcinomas (124, 125). Several investigators (127–129, 216) have recently attempted to grow the renal cell carcinomas in tissue culture and in nude mice in order to produce erythropoietin. Further work using recombinant DNA technology to isolate the gene responsible for the production of erythropoietin may lead to large scale production of erythropoietin *in vitro* by these renal carcinoma cells; making more erythropoietin available for clinical use in the anemia(s) of renal failure and other erythropoietin deficiency anemias, use in radioimmunoassays for erythropoietin, and as an investigational tool in basic research.

Erythropoietin Production in Anemias and Polycythemias. Our current model (Fig. 1) summarizes the role of an oxygen deficit in renal and perhaps extrarenal tissues in triggering erythropoietin production. Erythropoietin is produced normally to maintain day-to-day control of erythropoiesis but may also be triggered by pathophysiologic stimuli as well as pharmacologic agents. When erythropoietin triggers the normal bone marrow an adequate supply of iron is needed in order for the normal increase in red cell mass to develop. A number of clinical disorders may be associated with a secondary erythrocytosis due to increased erythropoietin production. The appropriate response to most anemias is an elevation in erythropoietin titers in blood with the exception of the anemia associated with renal disease, hypogonadism, or chronic inflammation. The anemias that are associated with a more profound elevation in erythropoietin levels in plasma include aplastic anemia, pure red cell aplasia, iron deficiency anemia, and hemolytic anemias. The appropriate response to high altitude, cardiac disease, pulmonary disease, and hemoglobinopathies is an elevation in plasma levels of erythropoietin. Inappropriate elevations in blood levels of erythropoietin may occur in several types of renal disease such as renal cysts, hydronephrosis, malignancies, and renal artery stenosis (14). Inappropriate increases in erythropoietin titers may also occur in adrenal hyperplasia, cerebellar hemangioblastomas, hepatomas, and uterine fibromas (14).

The anemia of chronic renal failure (CRF) does not show the appropriate elevation of erythropoietin relative to the severity of the anemia. The kidney in the anemia of renal failure is unable to produce sufficient amounts of erythropoietin to meet the increased demands for new red blood cells created by the hemolysis and shortened red cell life span and blood loss, especially through the dialyzer unit and uremic gut, and the retained uremic in-

hibitors of erythropoiesis. Deficiency of iron and folic acid may also be present in some patients. A secondary factor(s) in the anemia of renal failure is inhibition of erythropoiesis which includes primarily inhibitors of the erythroid progenitor cell compartment (CFU-E, BFU-E) and heme synthesis (130–145). The nephric renal failure patient produces variable amounts of erythropoietin, but usually an insufficient amount to correct the anemia. On the other hand, the anephric renal failure patient depends primarily on extrarenal erythropoietin production which is known to be more resistant to stimulation than renal erythropoietin. Thus, anephric CRF subjects are more severely anemic than the nephric CRF patients because of their inability to produce sufficient amounts of erythropoietin in the residual renal mass.

Erythropoietin titers in plasma of patients with anemia of renal failure have been reported to be increased (30, 31, 34, 39, 41, 145, 147), unchanged (145, 146, 206), or decreased (41, 148, 207) utilizing various bioassays and immunoassays for erythropoietin. Sherwood and Goldwasser (35), Zaroulis *et al.* (39), Koeffler and Goldwasser (36), Garcia *et al.* (34, 38), and Rege *et al.* (37) using radioimmunoassays for erythropoietin reported mean serum levels of erythropoietin which were higher in uremic anemic patients (range 32–240 mu/ml) than the range of mean values reported for normal subjects (14.9–29 mu/ml).

A most interesting new advance in the potential therapy of anemia of renal failure has been the demonstration recently in several animal models (149, 150) that the anemia of renal failure can be corrected, at least in part, by administering exogenous erythropoietin. In a recent sheep model, Mladenovic *et al.* (150) demonstrated that a decrease in renal function, produced by subtotal nephrectomy, resulted in an anemia which was correctable by the administration of exogenous erythropoietin. However, it is clear that this subtotal nephrectomy model for renal insufficiency does not completely mimic the multifactorial anemia in man where uremia is usually associated with a marked reduction in renal function, which causes further suppression of erythropoiesis and a shortening of erythrocyte life span. The work of Essers *et al.* (151) in the anemic uremic human subject indicates that when uremia is superimposed on a compromised erythropoietic function of the kidney, higher doses of erythropoietin are required to produce a reticulocytosis, apparently because of the suppression of erythropoiesis induced by the uremic toxins. The uremic toxins which have been implicated are the polyamines such as spermine and spermidine (139) and parathormone (152–154, 201–205).

Elevated levels of parathyroid hormone (PTH) are nearly always present in patients with end stage renal disease (154, 201). Zingraff *et al.* (154) reported that 18 patients who were on chronic hemodialysis had a significant increase in hematocrit after subtotal parathyroidectomy. Podjarney *et al.* (201) also reported improvement in the degree of anemia after parathyroidectomy in patients with end stage renal disease who were on hemodialysis. Meytes *et al.* (202) reported that 1-84 bPTH (intact PTH/molecule) but not 1-34 bPTH (PTH fragment) significantly inhibited mouse bone marrow and human peripheral blood BFU-E. On the other hand, Dunn and Trent (203) have reported that PTH actually produced a dose-dependent stimulation of erythropoiesis in fetal mouse liver cell cultures at 10–100 times normal serum levels and 240 times normal PTH levels were required to inhibit heme synthesis casting doubt on the hypothesis that PTH is directly responsible for the anemia of uremia. This stimulatory effect of PTH at one dose and inhibitory at extremely high doses was confirmed by Levi *et al.* (204) and Zevin *et al.* (205).

Because of the large number of patients with renal failure throughout the world being maintained on dialysis and requiring frequent transfusions, the most important therapeutic use of erythropoietin would appear to be for treating patients with anemia associated with renal failure. Recent reports (155, 156) indicate that continuous ambulatory peritoneal dialysis (CAPD) is more effective than hemodialysis in improving the hematocrit and hemoglobin levels in CRF patients. The future direction of research in this area should be aimed at using improved dialysis methods to more effectively remove inhibitors of erythropoiesis and to provide sufficient amounts of erythropoietin to treat the anemia of CRF. In addition, several prostanoids such as 15-methyl prostaglandin PGE_2 and 16',16'-dimethyl PGE_2 (14, 47, 87, 157) may be useful

clinically in combination with erythropoietin for the treatment of erythropoietin deficiency anemias such as the anemia of renal failure. These prostaglandin analogues have a longer *in vivo* half-life because of their resistance to inactivation by 15-hydroxyprostaglandin dehydrogenases.

Polycythemia vera is a disorder characterized by excessive production of red blood cells, granulocytes, and platelets. A certain proportion of patients with polycythemia vera have been reported to have a variety of bone marrow chromosomal abnormalities (208, 209). Polycythemia vera patients have erythropoietin values that are either low or undetectable (35, 37); however, the red cell mass was reported to be significantly elevated after bleeding and within the range of that found in normal men after a bleeding stimulus (158, 159). It has been postulated that patients with polycythemia vera probably have two cell populations, one that is autonomous and independent of erythropoietin, and a second normal cell population whose progeny are demonstrated only when erythropoietin production is stimulated by bleeding (158, 159). On the other hand, Zanjani *et al.* (160) have suggested that one of these population of cells in the polycythemia vera patient is more sensitive to low levels of circulating erythropoietin than normal cells. Alexanian *et al.* (161) measured erythropoietin excretion after multiple phlebotomies in 10 patients with polycythemia vera and in 8 patients having been bled to anemic levels. The erythropoietin values at a specific hematocrit were similar in the polycythemia vera group to those which have been found in normal man or in patients with chronic anemia from bone marrow failure. Therefore, there is ample evidence to conclude that an appropriately elevated erythropoietin production occurs in patients with polycythemia vera after bleeding.

Extrarenal Erythropoietin Production. Extrarenal erythropoietin production is apparently one of the most important sources of erythropoietin for the maintenance of erythropoiesis in patients with anemia of chronic renal failure (162). Nathan *et al.* (163) have found suppressed erythropoiesis in the marrows of bilaterally nephrectomized human subjects awaiting kidney transplantation and on hemodialysis. However, a basal level of erythropoiesis was still present even in the absence of the kidneys. Extrarenal erythropoietin production has been extensively reviewed by Fried and Anagnostou (162). Extrarenal erythropoietin was reported to be very similar to kidney erythropoietin in rats (164) in that it is capable of stimulating heme synthesis in bone marrow cultures and can be neutralized by erythropoietin antiserum. Continuous infusion of subpressor doses of angiotensin II results in an increase in renal (85) as well as extrarenal (165) erythropoietin production. The liver is reported by most investigators to be the primary site of extrarenal erythropoietin production (166). Reissmann and Nomura (167) observed that erythropoietin is detectable in the perfusates of the hypoxemic perfused isolated liver suggesting that the liver is a site of extrarenal erythropoietin. Hepatectomy has been reported (166) to prevent the plasma erythropoietin levels from increasing in nephrectomized adult rats exposed to intense hypoxia. The liver has been demonstrated (168) to be the primary site of production of erythropoietin in the fetus and it is not surprising that the liver retains its capability of producing extrarenal erythropoietin in the adult when there is an extreme demand for red blood cells. In addition, Zucali *et al.* (169) have reported that erythropoietin can be generated in mouse fetal liver cells grown in tissue cultures. Zanjani *et al.* (168) have provided more definitive evidence to support the view that at approximately 4 weeks of age, the kidneys become the primary site of erythropoietin production in the rat. In addition, these workers (168) have shown that nephrectomy in fetal sheep *in utero* does not affect the ability of the fetus to produce erythropoietin. Partial hepatectomy also reduced erythropoietin production in the fetal sheep *in utero* (168). The liver Kupffer cell has been suggested as the hepatic site of extrarenal erythropoietin production (170). Peschle *et al.* (171) have reported an enhancement of plasma levels of erythropoietin following exposure of anephric rats to hypoxia by prior induction of reticuloendothelial system hyperplasia with colloidal carbon or zymosan. In addition, Rich *et al.* (172) have reported *in vitro* production of Ep by macrophages and have postulated that macrophages are involved not only in extrarenal erythropoietin production but also in the possible short-range regulation of hemopoiesis. Even though immunocytochemical

studies have demonstrated that erythropoietin localizes on the cell membranes of nucleated erythroid cells (basophilic and polychromatophilic erythroblasts) in human bone marrow (211) there is no evidence that erythropoietin is actually produced by these erythroid cells. It seems clear that the intensity of the erythropoietic stimulus necessary to trigger extrarenal erythropoietin production must be greater than that required to stimulate renal erythropoietin. In support of this hypothesis, two anephric patients have been reported (173), one whom was surgically anephric and the other who was functionally anephric. Erythropoietin was undetectable in the plasma of these two patients when assayed 9 days prior to a hemolytic episode at a time when the hematocrits were in the range of 16–20% (173). Following a hemolytic episode resulting from a copper intoxication, serum levels of erythropoietin were approximately 420 mu/ml, which is significantly higher than that in sera of normal human subjects (173).

Pharmacologic Agents and Erythropoietin Production. Pharmacologic and humoral agents which are known to stimulate erythropoietin production include cobalt (7, 8, 174), glucocorticoids (175), corticotropin (ACTH) (176), thyroid hormones (175, 176), growth hormone (176, 177), prolactin (178), serotonin (5-HT) (179), vasopressin (180), testosterone (175, 181), 5α-androstanes (182), cyclic nucleotides (65, 68–70, 183, 184), beta-adrenergic agonists (66), renin-angiotensin II (83–85, 165), and prostanoids (64, 185). All of the above substances act by increasing erythropoietin production but some also stimulate erythropoiesis directly in the bone marrow. The agents listed above which have been reported to stimulate both erythropoietin production and to enhance the effects of erythropoietin on the erythroid cell compartment are glucocorticoids (175, 186, 187), thyroid hormones (176, 188), growth hormone (177, 189), testosterone (175, 180, 181, 190, 191), cyclic nucleotides (65, 183, 192), β-adrenergic agonists (80, 193, 194), and prostanoids (64, 185, 193–196). 5β-H steroids probably exert most of their erythropoietic effects directly on the bone marrow (197–200). Several of these agents have potential use in combination with erythropoietin in the therapy of Ep deficiency anemias.

Clinical Implications of Erythropoietin Measurements. Erythropoietin measurements either by the exhypoxic polycythemic mouse assay (23) or the more recently developed radioimmunoassay (34–39, 218, 219) for erythropoietin can provide very useful data in the diagnosis of numerous erythropoietic disorders. First, erythropoietin in plasma of polycythemia vera patients is usually undetectable or reduced below normal levels in most assays (36, 38, 39). On the other hand, secondary polythemias are related to an appropriate increase in the production of erythropoietin which are usually elevated above normal values. Therefore, erythropoietin measurements in plasma can be helpful in establishing the diagnosis of polycythemia vera. The mean serum erythropoietin levels in normal human subjects has been reported to be between 13.3 and 29 mu/ml (34–39, 218, 219). Serum erythropoietin levels in aplastic anemia patients and patients with pure red cell aplasia may range between 800 and 20,000 mu/ml (37). In the radioimmunoassay of polycythemia vera patients sera erythropoietin values were below normal and in the range of 8–18 mu/ml (36–39). Larger supplies of erythropoietin purified to homogeneity (18, 19) are needed to provide sufficient amounts for use in radioimmunoassay. It would appear that the radioimmunoassay offers the best clinically useful assay for erythropoietin in the future. The potential usefulness of erythropoietin is in the therapy of anemia of renal failure and its use in radioimmunoassay making it possible for routine clinical laboratories to utilize the erythropoietin assay in diagnosing various erythropoietic disorders.

Summary. A model has been presented for the role of the kidney in the physiologic and pathophysiologic control of erythropoietin production. The model involves an oxygen deficit created by anemic or hypobaric hypoxia resulting in the release of prostacyclin and its metabolite 6-keto PGE_1 and the release of PGE_2 with ischemic hypoxia. Prostacyclin, 6-keto PGE_1 or PGE_2 activation of adenylate cyclase, an increase in cyclic AMP, activation of a protein kinase and the phosphorylation of a hydrolase leads to increased biosynthesis of erythropoietin. The site within the kidney where erythropoietin is produced is still not clear. Evidence has been presented in support of a cell in the glomerular tuft of the kidney

for the production of prostacyclin which triggers a cascade of events leading to the production of erythropoietin by another glomerular cell. Erythropoietin has been purified to a high specific activity by both Miyake *et al.* (18) and Espada and colleagues (19). Molecular weight studies of purified erythropoietin have been presented which indicate that the molecular weight of one species of erythropoietin is in the range of 23,000–27,000 (19–22) and another species with a molecular weight of 39,000 (18). The International Reference Standard assay for erythropoietin is the polycythemic mouse assay and all assays, *in vitro* and *in vivo,* should be standardized against this biological assay. Radioimmunoassays for erythropoietin have been developed by several laboratories (34–39, 218, 219) utilizing purified erythropoietin. There is a great need for a commercial radioimmunoassay kit available to both basic science and clinical investigators to enhance research on erythropoietin. A large number of pharmacologic agents and hormones are known to trigger kidney production of erythropoietin. Some of these chemical agents act directly to increase kidney production of erythropoietin in addition to enhancing the effects of erythropoietin on the erythroid progenitor cell compartment. Several of these agents, not heretofore used clinically, should be considered as therapeutic agents in anemia, either alone or in combination with Ep. Tumors of the kidney and several other organs are known to increase inappropriate erythropoietin production. Other pathophysiologic triggers of erythropoietin are hypoxic hypoxia due to pulmonary insufficiency and ischemic hypoxia resulting from renal artery stenosis. Renal carcinoma cells have been demonstrated to produce erythropoietin *in vitro* and *in vivo* and may involve some malignant transformation to cause the renal cells to produce increased amounts of Ep.

A model for the anemia of chronic renal failure has been presented postulating erythropoietin deficiency as the primary cause of this anemia. In addition, uremic toxins which inhibit erythropoiesis in the bone marrow and cause shortening of the red cell life span may be one of the causes of this multifactorial anemia. The uremic toxins which have been demonstrated to be primarily involved in the anemia of renal failure are polyamines including spermine and spermidine. Even though some investigators have reported a relationship between the role of parathormone (PTH) and suppressed erythropoiesis in renal disease further work is necessary to clearly establish the role of PTH in the anemia of renal failure. Extrarenal erythropoietin in the human subject is more resistant to erythropoietic stimuli and both fetal erythropoietin and extrarenal erythropoietin in the adult appear to be produced primarily in the liver. Large amounts of purified erythropoietin are needed for use in radioimmunoassay and for the treatment of erythropoietin deficiency anemias such as the anemia of chronic renal failure. A possible source of increased amounts of erythropoietin is through new developments in recombinant DNA technology.

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