ERYTHROPOIETIN AND ERYTHROPOIESIS:

APPLICATIONS IN CLINICAL MEDICINE

Internal Medicine Grand Rounds
University of Texas Southwestern Medical School

February 25, 1988

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It is generally accepted that erythropoietin (Epo) is the primary growth factor responsible for the control of erythropoiesis (1). It functions, essentially, by promoting proliferation and differentiation of early committed erythroid precursors in a concentration dependent fashion (2). Thus, the bodies need for oxygen delivery is physiologically regulated, in part, by mechanisms which effect the rate of Epo synthesis and release.

Although a considerable body of knowledge had been developed regarding Epo synthesis and function, the expansion of data in these areas has rapidly progressed since the purification of the Epo molecule, the cloning of the human gene and synthesis of Epo by recombinant technology. Significant quantities of pure and biologically active recombinant human Epo (rHuEpo) are now available permitting both scientific exploration of its function and mechanisms controlling its production, as well as therapeutic application at the bedside.

Structure of Erythropoietin

Human Epo was purified to homogeneity from urine in 1977 in Goldwasser's laboratory (3). The amino acid sequence of the protein was elucidated by Lai et. al. (4). Based on this data, two groups utilized mixed synthetic oligonucleotide probes to isolate the genomic DNA from a human fetal liver DNA library. The cloned gene was then inserted into expression systems and the recombinant product was synthesized (5,6). The Epo gene is composed of 5 exons and 4 intervening sequences. Southern blot analysis using a cDNA probe and human-hamster cell hybrids or human lymphocyte metaphases has located the gene on the long arm of chromosome 7 (q11-22) possibly at band q21 (7). There appears to be no gene duplication or any pseudogenes.

Human Epo is a sialyl-glycoprotein comprised of 166 amino acids with an apparent molecular weight of approximately 34kD (8). The protein portion is about 18kD. There are three N-linked and one O-linked glycosolation sites which may be necessary for assembly and intracellular stability prior to synthesis (9). Themolecule contains two internal disulfide bonds with no free sulfhydryl groups. The carbohydrate portion has terminal sialic acid residues. The asialoprotein has no in vivo activity because of rapid hepatic clearance due to exposed galactose groups (10,11). However, in vitro biologic activity does not require the carbohydrate portion, and actually, the asialoprotein has greater in vitro activity mole for mole (10,12). Epo appears to be composed of 2 tightly packed domains linked by a protease sensitive region. The biologically active site may be located in the linking region (13,14). The predicted amino acid sequence of rHuEpo is identical to that purified from human urine. There appears to be no significant homology between Epo and any other sequenced hematopoietic growth factor or other known protein

ERYTHROPOIETIN STRUCTURE

GLYCOPROTEIN WITH APPARENT MW 34 kD
PROTEIN PORTION 166 AMINO ACIDS MW 18,399 D
TWO INTERNAL DISULFIDE BONDS
NO FREE SULFHYDRYL GROUPS
TWO DOMAINS LINKED BY PROTEASE SENSITIVE REGION
BIOLOGICALLY ACTIVE SITE IN LINKING REGION
THREE N-LINKED AND ONE O-LINKED GLYCOSOLATION SITES
GLYCOSOLATION NECESSARY FOR ASSEMBLY AND STABILITY
TERMINAL SIALIC ACID GROUPS (SIALYL-GLYCOPROTEIN)
ASIALYL-GLYCOPROTEIN HAS NO IN VIVO ACTIVITY
FULL IN VITRO ACTIVITY IN ABSENCE OF CARBOHYDRATE

(3,5,6). The rEpo behaves identically to the purified human urinary Epo in in vitro and in vivo bioassays as well as in the radioimmunoassays with antibody raised to the natural Epo. The carbohydrate moieties also appear to be identical. Finally, the rHuEpo is capable of stimulating erythropoiesis in normal mice and uremic rats in a dose dependent fashion (15).

Site of Erythropoietin Synthesis

The classical studies of Jacobson et. al. suggested that the kidney was the primary site of Epo production during most of post-natal life (16). A number of lines of evidence convincingly support this conclusion. Anephric animals or humans as well as those with renal insufficiency have inadequate levels of circulating Epo (17). Although Epo cannot be extracted from kidneys of normal experimental animals, it has now been successfully isolated from kidneys of rodents undergoing appropriate stimulation to increased synthesis (1). Because of the initial inability to extract Epo from kidneys, a popular hypothesis was that the kidney synthesized a precursor substrate which was activated to Epo by a plasma enzyme. This theory has now been nullified with the demonstration that Epo mRNA can be extracted from hypoxic kidneys and that the mRNA can be translated into Epo in a cell free system (18-20). The site in the kidney responsible for Epo synthesis remains controversial. The two primary candidates for this function have been the glomerulus or the renal tubular cells/interstitium. Immunofluorescent studies using anti-Epo antibodies have shown localization to the glomeruli (21). Renal mesangial cells in tissue culture purportedly synthesize Epo (albeit a non-glycosolated Epo which would be biologically inactive in vivo) (22). Glomerular cultures in vitro are reported to synthesize Epo (23). Using an Avidin-biotin-complex method, an antibody to a peptide sequence of Epo that neutralizes its in vitro activity has been localized to the glomerular epithelial cells (24). In contrast, when the glomerular and tubular fractions of rodent kidneys were separated, more Epo activity by RIA was found in the tubular fraction (25). A similar distribu-

tion of extracted Epo mRNA has also been found (20). Finally, using a mouse Epo genomic probe, in situ hybridization has demonstrated localization over the proximal renal tubular cells of bled rats and baboons (26). Renal cell carcinomas, felt to be tubular in origin, and renal cysts, lined by tubular epithelium, occasionally produce Epo with a resulting erythrocytosis (1). Epo mRNA has been isolated from renal cell tumors, and using mRNA from a renal tumor as template, cDNA was synthesized that expressed Epo gene activity in a recombinant system (27,28). Two recent studies, may shed some light on these conflicting observations. Both studies utilized in situ hybridization techniques, one with an Epo gene probe and the other an Epo mRNA probe. In both studies, glomerular and tubular cells were negative. One study concluded that the activity was in a relatively rare cell type, possibly an endothelial cell (29). The second study also concluded that the activity was in the cortex in peritubular capillary cells, probably endothelial (30).

The kidney is not the only site of physiologically relevant Epo synthesis (31). During fetal life, the liver appears to be the prime site of Epo production. The switch over to renal predominance seems to begin in the third trimester of pregnancy and is completed in the first few weeks post-natally. The data is consistent with a genetically determined event (32,33). Continued extra-renal Epo synthesis probably continues thereafter, but becomes physiologically relevant only in the circumstance of severe renal injury or the anephric state. Estimates in rodents suggest that 5-10% of basal Epo production is extra-renal, but that this may be induced to increase to nearly 20% in the appropriately stimulated setting (34). Clearly, extra-renal Epo synthesis cannot reach levels equivalent to that of the normal kidneys as demonstrated in anephric animals and humans (35). Here also, the cells responsible for hepatic Epo synthesis are not definitely delineated. Macrophages (Kupffer cells) have been proposed (36). Epo production can be demonstrated in cultures of these cells (37). Increased Epo synthesis has been demonstrated in the setting of experimentally induced Kupffer cell hyperplasia (31). On the other hand, increased Epo production in a) regenerating livers after hepatectomy or carbon tetrachloride induced liver injury when exposed to hypoxia; b) occasional patients with hepatocellular carcinoma; and c) during acute hepatitis in patients with the anemia of renal disease suggest that the hepatocyte itself is the responsible cell (38-40). Epo is synthesized by a hepatoblastoma cell line in tissue culture (41,42).

Control of Erythropoietin Synthesis

Epo synthesis and excretion is a highly sensitive system modulated by factors that effect oxygen availability to the Epo producing tissue (43). This contention is supported both by

experimental studies in animals and humans as well as by observations in disease states. However, there are certain qualitative and quantitative differences in Epo response to various stimuli that remain unexplained. Blood loss is a potent stimulus to increased Epo production and clearly is quantitatively related to the magnitude of the hematocrit change as well as the pre-phlebotomy hemoglobin levels (34,44-47). This indicates that oxygen carrying capacity is a major factor governing Epo production. This can be further demonstrated in experimental hemolysis (34) and iron deficiency (48) as well as by exposure of animals to carbon monoxide (44,49). Likewise, transfusion induced polycythemia or the post-hypoxic state are associated with a reduction in Epo release as well as a blunted response to a subsequent challenge to Epo synthesis (44,45,50). Clinical counterparts to these experiments exist. Patients with anemia and normal renal function have serum Epo levels that are elevated andcorrelated in a logarithmic relationship to the magnitude of the anemia (51). As little as a 75 ml. phlebotomy has been said to induce an increase in blood Epo levels (46). Also, patients with polycythemia vera often have Epo levels below the normal range (52) and increased levels of carboxyhemoglobin seen in tobacco smokers may be associated with erythrocytosis (53). Blood oxygen tension also modulates Epo production. Exposure to hypobaric hypoxia is associated with an abrupt rise in Epo levels in blood, and there is a correlation between the degree of response and the magnitude and duration of the hypoxia (44,45,-49). However, the degree of enhancement of Epo synthesis and/or release appears to be different between alterations of oxygen carrying capacity and oxygen tension, anemia being a quantitatively greater stimulus (44,45). Clinically, persons at high altitude have erythrocytosis related to the distance above sea level. Also, erythrocytosis is a common, but not universal, accompaniment of chronic pulmonary disease with hypoxemia and cyanotic congenital heart disease (54,55). Increase of the oxygen affinity of hemoglobin (decreased p50) produced experimentally by exposure to CO or cyanate induces increased Epo production (47,49). Likewise, exchange transfusion with high oxygen affinity blood produces a similar response (1,47). In humans, it is well documented that persons with abnormal hemoglobins with increased O2 affinity have erythrocytosis and those with low affinity hemoglobins are "anemic" (56). Additionally, the oxygen requirement, as measured by oxygen consumption, is a determinant of Epo control. Experimental starvation, and panhypopituitarism are associated with a hypo-Epo state and uncouplers of oxidative phosphorylation, such as DNP, produce an Epo response. Hypothyroidism, panhypopituitarism and protein malnutrition are human examples of similar hypo-Epo states and the experiments with DNP have been reproduced in human subjects (1).

The observations delineated above all suggest that both oxygen availability to, as well as oxygen requirements of, the Epo synthesizing cells are critical determinants of Epo homeo-

stasis. Certain unique features of the renal circulation and oxygen utilization have been suggested to be, on a teleologic basis, optimal for the control of Epo production (57). On the other hand, other features of Epo regulation remain unexplained. Although chronic anemia is associated with a logarithmically correlated increased level of blood Epo, this relationship is not universal in terms of all stimuli or conditions. Acute single episodes of blood loss, hemolysis or hypoxia are associated with an abrupt rise in blood Epo levels, but just as quickly, the rise is followed by a return of Epo levels to near baseline values well before there has been any change in the oxygen carrying capacity or hypoxemic state (1,34,45,47,49). Although this early decline in Epo levels is only partial, the steady state values are often back to within the normal range. This phenomenon, as will be seen later, has made the use of the Epo assay a less than reliable clinical tool. This pattern of Epo response is not only a feature of experimental conditions, it is also seen physiologically. On exposure to high altitude, the same rise and fall in Epo levels are seen prior to a rise in RBC values, unless the altitude is at major extremes. Mountain climbers going to 4500 meters above sea level demonstrate this pattern of response, while climbers remaining at 6300 meters have a persistent Epo elevation (58). Under all of these experimental or physiologic conditions, a further Epo response can be elicited by a second superimposed stimulus. Although a number of hypotheses have been formulated, it is clear that this is truly a function of changes in the rate of de novo Epo synthesis and not merely increased utilization at the bone marrow level or the presence of Epo inhibitors. Other studies tend to exclude increased bone marrow utilization or feedback inhibition by Epo itself as explanations (1). By measuring renal Epo and mRNA content, it has been shown that the phenomenon is a result of an early abrupt rise in both and a subsequent parallel fall (1,20,25). Adaptation factors have been postulated, such as increased blood flow and a "shift to the right" of the oxygen dissociation curve. These do play some role, but clearly do not explain the entire pattern. Some interrelationship between the response of the erythron to the increased Epo production and the degree of sustained Epo release may also exist. Where marrow function is experimentally or clinically ablated, the magnitude of continued Epo production may be greater for a given degree of stimulus (59). These various observations have led to the hypothesis that an initial high Epo level is necessary to initiate increased erythroid proliferation, but that once induced, a lesser level is sufficient to sustain the required expansion of the erythron. As will be seen, this has been proposed to be related to the relative differences in sensitivity of Epo responsive cell types in the marrow.

Erythropoietin Kinetics

The kinetics of Epo generation and metabolism have been assessed in both animals and humans. The response to a variety of hypoxic stimuli is rapid. Serum Epo reaches peak levels from 8-24 hours in animal models, depending upon the type of stimulus (20,45,60). In humans exposed to high altitude, the peak levels are seen at 1-3 days (58). The subsequent decline that occurs prior to the achievement of significant changes in RBC values has been discussed in the preceding section. At the tissue level, in rodents, Epo mRNA increments can be detected as early as 1 hour after exposure to blood loss, and renal Epo increments, indicating de novo synthesis, are seen by 1-2 hours (20). Serum levels can reach 20-30 times baseline within 5 hours (60). The metabolism of Epo has been studied almost exclusively in animals which demonstrate species variation. The volume of distribution has either been limited to the plasma volume or occupies an additional portion of the extracellular fluid. The subsequent T1/2 after equilibration ranges from 1-2 hours up to 7-10 hours. Also, the role of renal metabolism and excretion varies from none to nearly 10%. It is agreed that extrarenal mechanisms for degradation and/or excretion predominate and that the metabolic clearance is sluggish compared to non-glycosolated hormones (12,61,62). Delineation of the fate of native Epo in humans will await studies utilizing labelled rHuEpo in vivo.

Mechanism of Action of Erythropoietin

Data regarding the action of Epo are derived from both in vitro and in vivo studies. The relevance of some of the in vitro observations, in physiologic terms, remains open. The end result of Epo stimulation, where adequate numbers and function of bone marrow precursor cells exist, and where appropriate availability of nutrients, including transferrin bound iron, are available, is an increase in circulating numbers of mature erythrocytes. In

CHARACTERISTICS OF ERYTHROPOIETIN ACTION

BINDS TO SPECIFIC RECEPTORS ON RESPONSIVE CELLS
HIGH AFFINITY RECEPTORS MOST FUNCTIONAL (Kd = 0.09nM)
LOW RECEPTOR DENSITY (±300 PER CELL)
FULL ACTIVITY WITH FEW RECEPTORS BOUND (±180 PER CELL)
EPO-RECEPTOR COMPLEX INTERNALIZED FOR FUNCTION
ACTIVITY IS CONCENTRATION DEPENDENT
MAINTENANCE OF CELL VIABILITY (BFU-e AND CFU-e)
INDUCTION OF PROLIFERATION (BFU-e, CFU-e AND ? LATER)
PROMOTION OF DIFFERENTIATION (CFU-E AND ? LATER)

vivo, Epo stimulates an increase in circulating reticulocytes and an expansion of the recognizable erythroid precursor pool within 1-3 days (63). The delineation of events preceding this

state results from studies utilizing in vitro cell culture systems of bone marrow and peripheral blood. The present model of factors controlling hematopoietic cell development and differentiation has been recently reviewed (64). Utilizing this model, existing evidence indicates that Epo responsive cells are not immediate descendants of the pluripotent stem cell (2). Presently, cells that are targets of Epo action are defined as cells capable of forming recognizable erythroid colonies in in-vitro culture systems rather than cells possessing Epo specific receptors. In this context, stages of erythroid precursor cells have been defined based upon the size and appearance of colonies they form, time to colony development, and relative Epo sensitivity, as well as their responsiveness or dependence upon non-Epo growth factors (65,66). At least three distinct stages of erythroid progenitors are so defined. These include burst forming units (BFU-e) which are in turn separated into early and late forms, as well as the colony forming unit (CFU-e). The earliest BFU-e are dependent upon eythropoietin for proliferation and differentiation, but can be maintained in a viable Epo responsive state in the absence of Epo in the presence of other growth factors (67). These growth factors, in turn, can enhance burst formation, in terms of numbers and size, that result from Epo stimulation. This action of these growth factors is collectively termed burst promoting activity (BPA). At present, two purified growth factors do have clear BPA, GM-CSF and multi-CSF (Interleukin-3) (68-71). The later BFU-e are more Epo dependent and cannot be maintained in a viable state by BPA in the absence of Epo (67). The late BFU-e are also more Epo sensitive than the earlier progenitors (66). CFU-e proliferation is not facilitated by other growth factors and they is absolutely Epo dependent. Most are normally in cell cycle and die after one division in the absence of Epo. Lower levels of Epo (in the Epo suppressed state) maintain CFU-e viability but reduce the proportion in cell cycle. In addition to promoting mitogenic activity, Epo is also required for terminal differentiation. Again, this is concentration dependent and CFU-e are more Epo sensitive than BFU-e (2). It is not clear whether differentiation requires the continued presence of Epo or follows as a secondary consequence of Epo induction of proliferation. Therefore, in simple terms, the physiologic actions of Epo include maintenance of cell viability, induction of proliferation of Epo responsive cells and promotion of erythroid cell differentiation. Each of these functions is, in turn, concentration dependent. This in vitro model would suggest that an initial high level of Epo activity would be necessary to expand the size of the proliferative pool by initiating proliferation of BFU-e. Subsequently, a lower concentration of Epo would be capable of maintaining and promoting CFU-e viability, proliferation and differentiation. This is consistent with the observations of regulation of Epo synthesis after perturbations of oxygen availability. An alternate hypothesis is that following increased Epo stimulation, responsive cells up-regulate their receptor density and become

more sensitive to a given Epo concentration. This, in turn, could be mediated via other erythroid growth promoting factors such as a recently cloned molecule termed erythroid-potentiating activity (71a). The biologic relevance of the other glycoprotein growth factors (BPA) in erythropoiesis is not yet known. Likewise, the requirement for other cell types comprising the hematopoietic microenvironment, both in terms of cell-cell interactions and production of factors that facilitate erythropoiesis, is a subject for further investigation (72,73).

Studies at the cellular level, both in vitro and in vivo, have attempted to further delineate the events that occur with Epo stimulation of responsive cells. It is now clear that Epo action is mediated via specific membrane receptor(s). The characterization of these receptors is not complete. Recently, however, a number of studies have begun to shed light on the receptor(s) and the cellular consequences of Epo binding. A number of earlier studies provided indirect evidence for the presence of an Epo receptor on murine bone marrow cells (2,74). Perhaps 1-2% of mouse bone marrow cells bind Epo (75). However, a large quantity of rather pure erythroid committed cells are necessary for assessing the presence and characteristics of a receptor directly. Infection of mice with Friend anemia virus (FVA) results in a massive proliferation of Epo dependent erythroid lineage cells in the spleen at the CFU-e stage. Using this model, the specific binding of Epo could be demonstrated (76). Initially, a single class of specific receptors was delineated with a high binding constant, low density (600-700 receptors per cell) and the requirement for only a small number of Epo molecules to be bound per cell (approximately 10) to induce terminal differentiation. Subsequent studies, using rEpo with a higher specific activity of radiolabel, demonstrated two classes of receptors. The high affinity receptors (approximately 300/cell) appeared to be biologically relevant. A higher, but still relatively very low, number of Epo molecules bound (approximately180) were necessary for maximal biologic activity (77). These studies also documented that the Epo/receptor complex was internalized in the process of inducing Epo mediated events (78). In this system, the high affinity receptor, isolated from membranes of FVA infected cells has a MW of about 85kD. Other studies have confirmed the presence of Epo specific receptors on hamster yolk sac erythroid cells and murine CFU-e (79,80). Although the quantitative results were somewhat different, the pattern of a low level of receptor density and the requirement for only a small number of sites to be occupied for biologic activity was confirmed. Internalization of Epo in the murine CFU-e system has been demonstrated. The data also support the contention that Epo molecules that can be bound but not internalized are biologically inactive (81). Recently, a novel method for a high degree of purification of human CFU-e in large numbers has been developed. Specific binding of Epo to these cells has been demonstrated as well as the dose response relationship to biologic activity.

These studies also showed a progressive decline in Epo binding of cells beyond the CFU-e stage of differentiation (82).

In summary, there is clear evidence for the presence of specific Epo receptors on Epo responsive cells. They appear to be present in relatively small numbers (a feature that seems to be common to several hematopoietic growth factors) and only a small quantity of bound Epo is necessary to induce biologic activity. Epo function appears to require internalization, but the subsequent fate and mechanism of intracellular action remains to be determined.

A sequence of metabolic events has been documented to occur at the cellular level following exposure of Epo sensitive cells to the hormone, using in vitro and in vivo systems. Increases in RNA polymerase followed by RNA transcription occurs early, with out apparent initial requirements for protein or DNA synthesis (2,83). Subsequently, globin mRNA and globin synthesis, and DNA synthesis are found to be increased, ie evidence for induction of both differentiation and proliferation (84). In vitro, very early changes (within minutes) of intracellular calcium concentration (85) and decreased phosphorylation of a 43kD membrane protein (86) are seen. The latter is both time and concentration dependent. The interrelationships and relevance of these events are unresolved. This subject is reviewed in more detail in reference 2.

Other naturally occurring hormones have been implicated in the control of erythropoiesis in addition to the previously discussed growth factors (BPA) and Epo. Clearly, thyroid hormone regulates Epo synthesis in relation to oxygen utilization. The hormones which have been the subject of the greatest amount of study are the androgenic steroids. There is strong evidence that, in vivo, certain classes of androgens stimulate erythropoiesis by increasing or facilitating Epo synthesis by the kidney, and to a lesser extent, by extrarenal sites. The androgens, via this mechanism, act in concert to enhance the Epo response to a variety of hypoxic stimuli. This fact has led to the utilization of androgen therapy in the anemia of renal disease and other hematologic disorders. There is also some evidence, often not reproducible and usually derived from in vitro studies, that androgens also enhance the action of Epo on cells such as BFU-e and CFU-e (87).

Clinical Applications of the Erythropoietin Assay

As described above, the Epo synthesizing system is highly sensitive to changes in adequacy of oxygen supply to the Epo producing cells. It is also rapidly responsive to these perturbations. Therefore, it would seem reasonable that measurement of circulating levels of Epo could be useful in the differential

diagnosis of certain clinical disorders such as erythrocytosis or identifying anemias secondary to inadequate Epo production. Initially, investigation of this hypothesis was hindered by the problems with Epo assays themselves. Early measurements were accomplished by either in vivo or in vitro bioassays. These were tedious and expensive and suffered from the inability to detect circulating Epo in the normal state. These problems were a consequence of not having purified Epo to perform more sensitive immunologic assays (1). Following the purification of Epo, a number of immunoassays were developed but also were unsatisfactory. Subsequently, refinement of these techniques using purified Epo in radioimmunoassays (RIA) has permitted the evaluation of Epo levels that are normal or decreased with acceptable degrees of sensitivity. Antibodies have generally been raised in rabbits using partially purified Epo as the immunogen and purified Epo as the reagent for competitive binding (88-94). These RIA have shown good correlation with Epo levels measured in bioassays (51,88,89,91). Nevertheless, even with these assays, the apparent normal levels of Epo vary from one laboratory to another with mean normal values reported from 15 to 29 mu/ml (using International Epo standards) and ranges varying from 4.2-36 to <18-81. Thus, at present, interpretation of a result for serum or plasma Epo requires carefully determined normal values for the assay being employed. Very recently, a large survey of serum Epo levels was carried out using an assay system where rHuEpo was utilized as both the antigen for raising a polyclonal antibody as well as the competitive binder. This will probably prove to be the standard for future assay systems. Over 2000 assays were carried out in 1200 hospitalized patients. The normal level was 12.0 +/- 0.8 mu/ml in non-anemic males and 11.0 +/- 0.7 in non-anemic women. Using a single lot of antibody, the serum Epo levels did not fluctuate over time in normal subjects. Epo was undetectable in only 6 of 2000 assays. Five were non-anemic normals and one was a patient with polycythemia vera (94).

Based on results using many of these varied assay systems, it is clear that most anemias are characterized by Epo levels that are increased logarithmically in relation to the degree of the anemia (43,51,52,59,91,94). Certain mechanisms of anemia, however, appear to be accompanied by inadequately increased Epo production relative to the predicted response and seem to share impaired Epo synthesis as part of the mechanism of the anemia. These include 1) anemia associated with starvation and protein malnutrition. This may be due to the decreased metabolic rate in this setting representing a decrease in oxygen requirements (1).2) The anemia of hypothyroidism. This also appears to be a physiologic anemia in the sense that it too is a consequence of decreased oxygen utilization (1). 3) The anemia of chronic diseases. This anemia is found in many patients with infection, inflammation or malignancy. It is a complex pathogenetic process which includes a mild decrease in RBC life span, a decrease in erythropoiesis secondary to defective reutilization of iron from

senescent red cells (and stores) and, in several studies, decreased Epo production (95).The reason for decreased Epo production is not clear, but as will be seen in the section on Epo therapy, it may be a relevant feature in some patients with this condition. 4) The anemia of renal disease. Clearly, the most important contributing factor to anemia in nephric or anephric patients with renal disease is the reduction or inadequate production of Epo (17). (See section on Epo therapy). 5) Alcohol has been observed, in one study, to severely depress serum Epo levels (94). The significance of this observation is not yet clear.

The Epo assay has proven to be less helpful than was hoped in the evaluation of erythrocytosis. In many patients meeting established criteria for the diagnosis of polycythemia vera (PCV) the Epo level is suppressed (90-94,96-99). Rarely, however, is it undetectable, particularly in very sensitive RIA's (94). Thus, Epo suppression is incomplete in this disease. Review of data from several reports suggests that approximately 60% of patients with PCV will have Epo values below the normal range whereas nearly 40% will have normal values. Only 1-2% of patients with PCV will demonstrate elevated levels. In contrast, patients with secondary erythrocytosis (including causes appropriate to the clinical setting such as hypoxia or inappropriate such as with Epo producing renal tumors or cysts) will have elevated values less than 50% of the time (90-94,96-99). However, it is uncommon (perhaps 5%) for decreased levels to be seen in secondary erythrocytosis. In one study of patients with chronic pulmonary disease and erythrocytosis, 50% had serum Epo levels equivalent to non-polycythemic pulmonary disease subjects (55). These observations tend to parallel the experimental setting. When compensatory erythrocytosis has occurred, the Epo levels then return towards baseline and may be within the normal range.

ERYTHROPOIETIN ASSAY IN ERYTHROCYTOSIS

	# PATIENTS	NORMAL	PERCENT INCREASED	DECREASED
PRIMARY (PCV)	159	42	1	57
SECONDARY	158	51	44	5

On the otherhand, when increased hypoxic stress is then superimposed, or the patients are phlebotomized to normal RBC values, the Epo levels may again become elevated (54,58,91,93,97). This elevation after phlebotomy for erythrocytosis does not appear to generally occur in PCV (91). Likewise, patients with inappropriate secondary erythrocytosis do not appear to have fluctuations of their Epo levels with either phlebotomy or hypoxic stress. In summary, the interpretation of blood Epo values in patients with erythrocytosis is difficult. Decreased values strongly favor a diagnosis of PCV. Elevated levels are uncommonly seen except in secondary erythrocytosis. In nearly one-half of

patients in either category, however, the values will be in the normal range. Perhaps measuring Epo levels before and after phlebotomy may aid in distinguishing between hypoxia induced secondary erythrocytosis and PCV in this subgroup.

Recombinant Erythropoietin as a Therapeutic Agent

The primary clinical goal of applying recombinant technology to synthesize Epo is to produce sufficient quantities to be utilized for the treatment of certain types of anemia. There are some disorders where rHuEpo has already been clearly proven to have a therapeutic effect, and others where a theoretical benefit may exist.

The Anemia of Renal Disease. The pathogenesis of anemia in chronic renal failure (CRF) is complex with a number of factors playing either a central role or an occasional contributing element (17). It is well established that anemia occurs in most, but not all, patients with CRF. In a general sense, there is some correlation between the degree of anemia and measurements of glomerular function, particularly at lower levels of renal function but prior to the development of end stage renal disease (ESRD). However, a wide scatter is present and no predictability

ANEMIA OF CHRONIC RENAL DISEASE

WEAK CORRELATION WITH FUNCTIONAL LOSS
HEMOLYSIS: RBC Lifespan 60-80 Days
HYPOPROLIFERATIVE RESPONSE
ERYTHROPOIETIN DEFICIENCY (Relative or Absolute)
INHIBITORS OF ERYTHROPOIESIS ??
Additional Mechanisms (Occasional):
- IRON DEFICIENCY (Microcytosis)
- FOLIC ACID DEFICIENCY
- OSTEITIS FIBROSA
- HYPERSPLENISM
- ALUMINUM TOXICITY (Microcytosis)

exists for a given patient (100,101). One central factor in the development of anemia is a reduction of the RBC lifespan in these patients, especially at or near the uremic state. However, the degree of hemolysis results in a RBC survival in the neighborhood of 50-60% of normal (102-105). Otherwise normal individuals would totally compensate for such a reduction by increasing erythropoiesis adequately. This is accomplished by increasing Epo production such that blood loss can result in a 2-3 fold increase in RBC production rates and with hemolysis up to 6-8 fold. Nevertheless, in patients on dialysis, the rate of erythropoiesis is only in the range of 80% of normal (103,105). Thus, the feedback system is impaired. With the development of reliable methods for Epo assay, it is clear that the primary problem is

one of a relative impairment of Epo synthesis (35,52,88,89,94,-100-102,106). Epo production is always detectable, even in anephric patients, but it is rarely equivalent to that seen in patients anemic from other causes. The degree of impairment of Epo production does not correlate with the degree of renal functional abnormality (94,100,101). It is of interest that the feedback circuit remains intact. Fluctuations, in the expected direction, of Epo synthesis are seen in the presence of transfusion or acute blood loss, but the magnitude is blunted and inappropriate (94,101,107). A third factor has been implicated as important in the anemia of CRF. Several studies, almost all in vitro, have suggested that there is an impairment of the response of the bone marrow erythroid compartment to Epo. This has been attributed to the presence of erythropoietic or Epo inhibitors (17,101,104,108). The most valid test of this concept would be studies evaluating the response of the anemia to the administration of exogenous Epo. A model of the anemia of CRF was developed in sheep. These studies confirmed the inadequacy of Epo levels for the degree of anemia and therefore the primary role of impaired Epo production in the pathogenesis. These studies also demonstrated that the response, in vivo, of the erythron of uremic sheep to Epo was equivalent to that seen in normal animals to the same dose (109). The initial reports of the use of rHuEpo in patients with dialysis dependent CRF have demonstrated no evidence for physiologically significant inhibitors of erythropoiesis or Epo itself (110,111). In addition, in vitro studies demonstrated that both BFU-e and CFU-e were significantly increased in the bone marrow following initiation of Epo therapy (112).

A number of other factors can contribute to anemia in selected patients with CRF. Severe osteitis fibrosa due to secondary hyperparathyroidism is seen in a few patients. Folic acid deficiency due to dietary problems or dialysis may occur. Some patients lose a significant volume of blood with hemodialysis, and if they are not transfusion dependent, may develop iron deficiency. Hypersplenism has been reported in a rare patient on hemodialysis. A microcytic anemia secondary to aluminum excess is now well described. All of these factors should be considered in any given patient and treated if present (17). Nevertheless, the inability to compensate Epo production adequately in the face of mild hemolysis remains the primary mechanism of the anemia of CRF.

The anemia is often symptomatic, particularly in patients with ESRD. In some patients, the initiation of hemodialysis or peritoneal dialysis results in a partial amelioration of the anemia (100,104,105,108,113). One study suggested that this occurred in patients with a greater residual capacity to synthesize Epo and that possibly regular dialysis lessens the rate of hemolysis (113). There is some evidence that the frequency and duration of dialysis is inversely correlated with the magnitude of

anemia in these patients (104). Nevertheless, even after initiation of dialysis for several months and with attention to iron and folate status, many patients remain transfusion dependent. This is an even greater problem in anephric patients, indicating the minimal capacity for Epo synthesis by extrarenal (hepatic) sites (35,88,89). Chronic transfusion, in turn, is accompanied by problems of alloimmunization and iron overload. Androgen therapy has been utilized in this setting. Androgens induce some improvement in RBC values in some patients through an increase in Epo production and perhaps on Epo interaction with the erythron (87,104,114). Still, many patients retain some degree of symptomatic impairment and side effects are frequent.

ERYTHROPOIETIN THERAPY IN CHRONIC RENAL DISEASE

TRIALS IN TRANFUSION DEPENDENT DIALYSIS PATIENTS
RESPONSE:
- OCCURED IN >95% OF PATIENTS
- DOSE DEPENDENT (Degree and Rate)
- IRON DEFICIENT ERYTHROPOIESIS ENCOUNTERED

ADVERSE EFFECTS:
- HYPERTENSION (32%)
- CLOTTED A-V SHUNTS (2%)
- HYPERKALEMIA
- INCREASED CREATININE

ANTIBODIES TO rHuEPO NOT DETECTED
NO EVIDENCE OF INHIBITORS

The commercial production of rHuEpo has provided material to begin clinical trials of Epo therapy. The anemia of renal disease was the obvious first disorder to study. Dramatic results have been demonstrated (110,111). Prompt increases in RBC values occur. The rate and magnitude of response is, as predicted, dose dependent and complete normalization of anemia has been achieved in many subjects. The maximum calculated plasma levels achieved, assuming distribution is limited to the intravascular plasma volume, are very close to those seen in patients with comparable degrees of anemia where Epo production is adequate. In addition, the Epo therapy was administered as three single pulse injections weekly. As noted above, this seems to eliminate a significant role for endogenous inhibitors of Epo action in the majority of these patients. Transfusion dependence was eliminated in all of the patients treated in the two initially reported trials. Maintenance therapy provided continued support of red cell values to levels desired. Adverse effects were seen in some patients. Five of 28 patients receiving adequate doses for an erythroid response developed hypertension or difficulty control ling treated hypertension. Control of the blood pressure was achieved in all by manipulating their drugs and the hemoglobin level. Two of 10 patients in the English study clotted their A-V shunts. Increases in creatinine and potassium occurred in most of the Seattle patients, but could be managed in the majority by

dietary control and longer dialysis periods . The improved sense of well being was accompanied by increased food intake which may explain the creatinine and potassium elevations. Another interesting observation in the Seattle patients was the development of a state of relative or functional iron deficiency which blunted the Epo response. Iron supplementation overcame this problem. It was recognized only in those patients who had not developed major iron overload from transfusion therapy.

Recently, a larger cooperative trial conducted by nine dialysis centers has been reported in abstract form (115). 247 patients were treated with rHuEpo in a manner similar to the initial trials. Only 6 failed to respond. Five of the six had a microcytic anemia. 55 patients required iron supplementation. Of 125 patients on antihypertensive drugs prior to Epo, 26 (20%) had a significant rise in their diastolic pressure and 32 (26%) required additional medication. Of patients not previously hypertensive, 17% became so. Therefore, overall, 32% of these patients had significant rises in their diastolic blood pressure or required more antihypertensive medication. This problem represents the major adverse effect of Epo therapy in patients with ESRD. Problems with hyperkalemia were not reported. Four patients clotted their vascular access. Antibody formation to the rHuEpo has not been detected. The results of the pilot studies and this larger trial indicate that nearly all patients with anemia secondary to renal disease can be successfully treated with Epo, but will require careful monitoring during the initial phases to deal with the frequent problem of hypertension.

Anemia of Chronic Disease. Anemia occurs in a significant number of patients with a variety of disorders of apparently unrelated etiology. Based on clinical evaluation of the characteristics of the anemias, it is clear that they share several features in common and the entity has been termed the anemia of chronic disease (ACD) (95). The disorders in which this process are seen are generally confined to infections, noninfectious inflammatory diseases and malignancy. The pathogenesis is complex, and probably consists of a series of factors each contributing, to a varying degree, to the final result. There is

ANEMIA OF CHRONIC DISEASE

Associated with:
INFECTION
INFLAMMATION
MALIGNANCY
HEMOLYSIS: RBC Lifespan 60-90 Days
HYPOPROLIFERATIVE RESPONSE
DEFECTIVE IRON REUTILIZATION: (% Saturation TIBC <20)
ERYTHROPOIETIN DEFICIENCY: (Relative)

a mild degree of hemolysis in many of these patients. The magnitude of the shortening of the RBC lifespan is on the order of 60-90 days (normal 120 days) (95,116). A normal Epo response and a normal erythron should be capable of compensating for this. However, a hypoproliferative response is seen. One major, and probably the most important, factor limiting the erythroid response is a defect in the reutilization of iron from senescent RBC's and iron stores. This results in hypoferremia and a functional state of iron lack erythropoiesis (95,116,117). The third factor postulated to contribute to ACD is a relative degree of Epo deficiency. Data on this point are conflicting. Epo levels are measurable by both bioassays and RIA. They usually are elevated above the normal range. Several studies have concluded that they are inappropriately low for the degree of anemia. This has been observed in both the experimental setting as well as in patients (52,94,117-123). Other investigators have been unable to confirm these studies (51,124). An experimental animal model of ACD demonstrated a sub-normal Epo response to hypoxia compared to normal animals (118). In vitro bone marrow cultures from patients with ACD show a normal response of erythroid progenitors to Epo (119,120). The same conclusion has been reached with ferrokinetic studies in vivo (117). Another factor that may contribute to the conflicting observations is that Epo levels seem to be most impaired in the more anemic subjects and the patient population studied will therefore influence the results (123). Finally, there is some question that Epo synthesis is impaired in malignancy as compared to the other disease categories associated with ACD (119). This may, in part, be due to the fact that a number of other mechanisms for anemia can also occur in some patients with malignancy, such as major hemolysis, blood loss and iron deficiency, and myelophthisis (125). A recent study of serum Epo levels and in vitro response of erythroid precursors to Epo in patients with malignancy concluded that inadequate Epo production was a major factor in their anemia (120). On balance, the majority of studies suggest that at least some persons with ACD, when symptomatic, might benefit from Epo therapy. Very recently, the use of rHuEpo in two patients with rheumatoid arthritis and moderately severe ACD, without iron deficiency, was reported. Erythrocyte values were significantly improved in both (126). The clinical response was accompanied by an increase in erythroid precursors in their bone marrows. The role of Epo therapy in ACD will require more clinical evaluation, but will probably prove to be effective in a few properly selected patients. The serum Epo assay may be useful for this determination.

Anemia of Prematurity. The red cell values normally fall in the newborn during the first few months of life. In premature infants, this decline is more acute and more severe and results in a number of clinical problems, often requiring transfusion therapy. This has been termed the anemia of prematurity (AOP). Previous studies have indicated that this fall in circulating

RBC's is associated with a relative or blunted response in Epo synthesis, perhaps due to a problem in switch-over from extra renal to renal Epo production at this early age (127). Recently, a study reported that the circulating BFU-e were equivalent in number and Epo responsiveness in premature infants as compared to that of normal term cord blood (128). These observations suggest that Epo therapy may have a role in ameliorating the problem of AOP and provide an option to transfusion therapy.

Sickle Cell Anemia. Another example of relative underproduction of Epo may exist in patients with sickle cell anemia (SCA). Studies have demonstrated that the Epo level in patients with SCA is clearly increased, but tends to be lower than predicted for the degree of anemia (129-131). One explanation, probably true in part, is that the very high p50 (low oxygen affinity) in SCA red cells permits greater oxygen delivery per gram of hemoglobin than in other forms of anemia. Decreased oxygen affinity is seen in other anemias as well, but more so in SCA due to properties of the hemoglobin S molecule (130). Nevertheless, one study has demonstrated that there is a clear correlation between creatinine clearance and serum Epo levels in SCA (129). Also, the relative impairment of Epo production seems age dependent, declining in adults, suggesting that the ability of the kidney to produce Epo is involved (129-131). Certainly, the pathologic lesions seen in the kidneys of adult SCA patients could conceivably impact more upon the Epo producing apparatus than on glomerular function. In this scenario, levels of renal injury that are not ordinarily sufficient to prevent Epo production that is capable of supporting normal erythropoiesis may be significant when stressed by the increased need for Epo that occurs in SCA. In general, the anemia in SCA is not symptomatic due to the circulatory and intraerythrocytic adaptations that increase oxygen availability. However, with increasing age, and in the presence of other clinical problems, the anemia may become symptomatic. Such patients might be benefitted by Epo additive therapy aimed at a mild to moderate improvement in their circulating hemoglobin content for an amelioration of symptoms. Clinical trials have not been reported.

Other Disorders. Several centers are carrying out trials of Epo therapy in patients with CRF and anemia who are not on dialysis treatment. Because of the experience in dialysis patients, it will be of interest to see whether a similar pattern of adverse effects occurs in this group and whether benefits will exceed potential risks.

Another area that has attracted interest, and in which clinical trials are underway, is in patients who are donors for autologous transfusions. The use of a patient's own red cells for elective surgery has the advantage of both a lack of transfusion related diseases and lessens the burden on donor availability. The primary limitation to this approach is the frequency with

which the donor/patient can be phlebotomized due to the inherent maximal rate of repair of the circulating red cell mass. One restriction is the rate of mobilization of the available iron supply. Iron supplementation can increase the repair rate only modestly. If the maximal level of Epo synthesis is also a rate limiting factor, then, hypothetically, both Epo and iron supplementation may increase the repair rate such that the patient can undergo more phlebotomies prior to planned surgery in a lesser time period.

Theoretically, certain disorders associated with primary bone marrow failure such as aplastic anemia and the myelodysplastic syndromes might potentially benefit, from the aspect of anemia, from Epo therapy. These diseases are accompanied by high levels of circulating Epo. The question is whether, in selected patients, the failure of erythroid differentiation might be due to defective committed stem cells which are unusually Epo insensitive. If pharmacologic amounts of Epo were present, perhaps in conjunction with BPA substances such as GM-CSF or multi-CSF, responsiveness might be encountered. Clinical trials accompanied by in-vitro studies will be necessary to answer this question.

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