EXHIBIT 2

Erythropoietins and Erythropoiesis
ed. by G. Molineux, M.A. Foote, and S.G. Elliott

Studies of erythropoiesis and the discovery and cloning of recombinant human erythropoietin

MaryAnn Foote

Amgen Inc., One Amgen Center Drive, M/S 17-2-A, Thousand Oaks, California 91320, USA

Introduction

Patients who are anemic because of chronic kidney disease, cancer, arthritis, or chemotherapy or radiation therapy often report fatigue [1–6]. Before the introduction of recombinant human erythropoietin (rHuEPO), anemia and its sequelae fatigue were treated with red blood cell transfusion, androgen stimulation of red blood cell production, and/or iron supplementation, among other treatments [7]. While effective in increasing red blood cell counts, both transfusions and androgen therapy have inherent risks [8]. Transfusions of red blood cells can be complicated by blood-borne pathogens, iron overload, immunologic consequences, and lack of or delayed hemoglobin response. Transfusions often improve but do not correct anemia and usually must be given frequently, and androgen therapy can cause viralization or abnormal liver function. rHuEPO is an ideal therapy because it mimics the action of the endogenous hormone by stimulating the production of red blood cells. Patients with chronic kidney disease are unable to produce adequate amounts of endogenous erythropoietin (EPO) to stimulate red blood cell production. Patients with cancer often have damaged bone marrow, with or without the insult of chemotherapy, that does not completely respond to the endogenous hormone.

The cloning of the human *EPO* gene by Fu Kuen Lin and colleagues was a difficult and frustrating endeavor. This milestone and the subsequent creation and production of rHuEPO as a therapeutic option was a breakthrough that has enabled physicians to ameliorate anemia and its sequelae. Patients treated with rHuEPO report a return to more normal lives. rHuEPO is the standard of care for treatment of anemia in patients with chronic kidney failure or receiving chemotherapy, and in other disease settings.

This chapter is a literature review of the history of the early work in erythropoiesis and the discovery and cloning of EPO. Other chapters discuss its commercial production and the clinical uses of rHuEPO.

Early studies in erythropoiesis

Bright [9] is credited with being the first scientist to recognize that anemia was a complication of kidney disease, but Jourdanet [10] has been credited as the first scientist to observe the relationship between altitude and blood viscosity. Jourdanet noted the similarity of symptoms reported by patients with altitude sickness and the symptoms reported by patients who had experienced severe blood loss. Several years later, Viault [11, 12] expanded knowledge of red blood cells and the effect of altitude on them, and quantified the change in red cell counts as altitude increased. On a train trip from the city of Lima, Peru to the high-altitude tin mines of that country, he repeatedly sampled his blood, blood of willing fellow travelers, and blood of a dog, rooster, and llama. Viault noted an increase in his red blood cell count from $5 \times 10^6/mm^3$ to $8 \times 10^6/mm^3$ during the ascent to higher altitude.

Other early scientists continued studies in an attempt to understand the mechanism of erythropoiesis in rabbits [13] and immigrants to the high Alps [14, 15]. One theory proposed at the time to explain the polycythemia seen at high altitudes was that low oxygen pressure directly stimulated bone marrow to increase red blood cell production. This theory held for nearly 50 years.

In 1906, Carnot developed the concept of humoral regulation of erythropoiesis [16–18]. Serum from anemic rabbits was injected into normal rabbits, and caused an increase in the red blood cell counts of the normal rabbits. Carnot suggested that "hemopoietine" present in the serum of anemic rabbits was responsible for the increase in cell numbers. Many other investigators repeated these experiments in anemic rabbits or rabbits raised at high altitudes [19–23].

For almost 30 years, researchers continued to repeat Carnot's work. Because some investigators were successful and others were not, controversy continued about the mechanism of erythropoiesis. Finally, Erslev [24] modified Carnot's original study: He injected large amounts of plasma from anemic rabbits into normal rabbits and found that the number of nucleated red blood cells in the bone marrow, the number of peripheral reticulocytes, and the hematocrit of the normal rabbits increased. This study suggested that red blood cell production is mediated by a humoral factor in rabbits. Four years later, Jacobson et al. [25] demonstrated that this factor, EPO, was produced by the kidney. Progress was being made in understanding the relationship between oxygen supply and demand of the body and EPO and erythropoiesis; however, despite more than 100 years of research, nothing was known about the structure of EPO or the *EPO* gene. Some debate continued whether EPO was produced as an inactive precursor in the kidney that was activated in some other tissue or organ.

The role of kidney and bone marrow in erythropoiesis

In the fetus, the liver is the primary site of endogenous EPO production [26]. In the adult, EPO is produced primarily (i.e., >90%) in the adult kidney [25, 27]. The liver [28, 29] and the brain [30] both synthesize some EPO, but the amount produced by these tissues alone is insufficient to maintain adequate erythropoiesis. Thus, kidney disease causes anemia due to loss of the main source of EPO production.

An oxygen sensor within renal cells detects the oxygen content of the blood and the kidney regulates the amount of EPO released into the blood (Fig. 1). The hormone acts on red blood cell precursor cells in the bone marrow to stimulate their proliferation and maturation and to increase the number of red blood cells in the peripheral circulation. The feedback loop is completed when the kidney cells recognize the change in oxygen delivery secondary to the

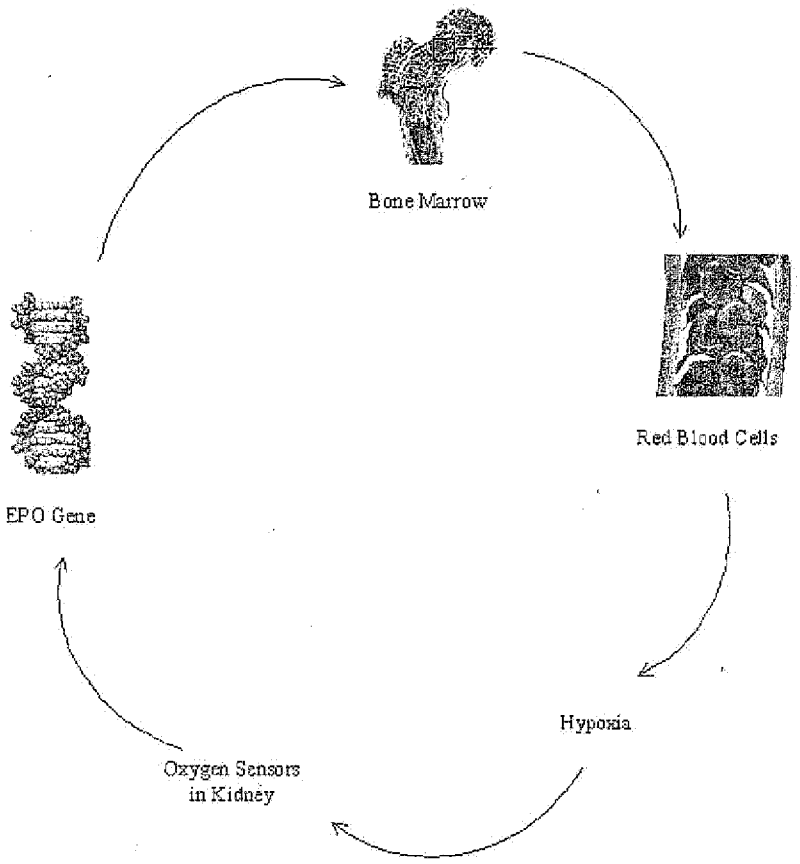

Figure 1. Relationship of kidney and bone marrow in production of red blood cells. (Figure courtesy of Amgen.)

change in the number of circulating red blood cells. Production of new red blood cell slows until the sensor cells recognize a need for increased erythropoiesis. Some EPO, albeit in trace amounts, is always detectable in the circulating blood, even in patients with total kidney failure, suggesting that a subset of cells provides a continuous output of EPO even when oxygen delivery is normal.

Isolation of EPO from urine

Even though the amounts of endogenous EPO increase under conditions of hypoxia or anemia, EPO represents a minor fraction of the total protein in the blood. A significant obstacle to the development of EPO as a therapeutic agent was the difficulty in isolating and purifying adequate amounts of the hormone to allow for its characterization. Several groups attempted to purify human [31, 32] and sheep [33, 34] EPO. The results were inconclusive, with the purity of the product questionable or produced in insufficient amounts to allow chemical characterization.

In 1977, Miyake, Kung, and Goldwasser succeeded in isolating and purifying milligram amounts of EPO from 1500 L of urine from patients with aplastic anemia [35]. Patients with aplastic anemia characteristically overproduce EPO, however the amounts present are still small. Large volumes of urine were essential for the recovery of a sufficient amount to purify EPO.

Miyake et al. [35], using a seven-step process that included ion exchange chromatography, ethanol precipitation, gel filtration, and adsorption chromatography, produced a preparation with a potency of 70,400 units/mg protein, 21% yield, and a purification factor of 930. This method allowed the production of enough material to partially characterize the hormone. With a source of EPO now available, a strategy for the cloning and expression of the human gene was devised.

Cloning of the *EPO* gene

In the 1980s, the nucleic acid sequences for cynomalogous monkey and human EPO were finally isolated and characterized, each by a different method. Several hurdles needed to be overcome, including the limited amount of data about the primary structure of human or monkey EPO, lack of a known source of mRNA, no information about the genomic structure of the gene, lack of simple tests to confirm that the cloned sequence encoded the *EPO* gene, and controversy about the induction mechanism for EPO production due to hypoxia. The difficulty in cloning the gene was further complicated by an inability to determine which step in the process was responsible for the failure.

In 1981, Goldwasser presented the first 26 amino acids of the protein at a meeting. Sue and Sytkowski [36] published the sequence in a paper that

described the development of polyclonal antibodies to EPO. The Sue and Sytkowski paper was subsequently shown to have two errors, unknown at the time, in the amino acid sequence. Another group [37] published a putative amino acid sequence for the first 31 amino acids of EPO, but it was proved to be erroneous, as it had the same two errors reported by Sue and Sytkowski as well as three additional errors in the amino acid sequence, again unknown at the time of publication. Goldwasser had provided Lin and colleagues at Amgen Inc. with the sequence he and his colleagues had obtained for the first 26 amino acids of human EPO, but of course, none were aware at the time of the errors. They were aware of the possibility that the available peptide sequence may overlap the intron-exon boundary of the *EPO* gene thereby preventing successful cloning using oligonucleotide sequences based on the peptide sequence. Earlier, Goeddel et al. [38] had successfully sequenced, cloned, and produced recombinant human insulin. This protein has only 51 amino acids, compared with EPO's 165 amino acids, and required nearly 10 years of work to sequence. With newer biotechnology techniques, it was naively thought that the sequencing of EPO would be easy. Lin needed not only to isolate the gene with no knowledge of its structure or of a simple way of confirming that the gene was in hand, but also to express the gene in a suitable host cell to provide a product with the proper structure, including the carbohydrate and polypeptide components of the molecule.

Lin used many approaches, including the standard gene-cloning routes known at the time, all of which failed. He persisted and eventually succeeded only because he used a technique far more complex than any technique tried earlier. This novel approach involved the use of multiple sets of fully degenerate oligonucleotide probes to screen a human genomic library. Two small pools of oligonucleotides corresponding to short fragmented samples of EPO amino acid sequences were used. Both pools, one of 20 nucleotides and the other of 17 nucleotides, had low codon degeneracy. Because of the degeneracy of the genetic code, the same amino acid can be encoded by more than one codon; Lin and colleagues accounted for every possible codon that encoded these putative amino acid sequences necessitating 128 different probes in each pool. The probes were labeled with radioactive phosphorus to identify any matches of a single probe with the human genome. The gene library on which the probes were tested consisted of the total human genome, fragmented into pieces 10,000 to 20,000 nucleotides long. Lin and colleagues found that probes in both mixtures hybridized with four of the 1.5 million clones in a human fetal liver genomic library [39]. Analysis of these clones showed that at least one contained the entire coding region of the human gene for EPO and it was the basis for developing the expression system using transfected Chinese hamster ovary (CHO) cells. In parallel with this effort, cDNA from the kidneys of anemic monkeys was prepared using mixed probes based on the human EPO peptide sequences [40] and the monkey gene also was cloned.

After Lin and his colleagues successfully cloned the gene, Jacobs and colleagues [41] also cloned the human *EPO* gene using degenerate oligonu-

cleotides and a peptide sequence derived from urinary EPO supplied by Miyake. Cloning of additional *EPO* genes from other species used the sequence information from the human and monkey genes and proceeded rapidly. The mouse *EPO* gene was cloned using monkey and human EPO DNA segments as hybridization probes [42, 43]. Subsequently, *EPO* genes from other species were cloned by hybridization or by polymerase chain reaction using probes or primers based on the known EPO sequences, including the genes from the rat [44], pig [45, 46], sheep [45, 47], and cow [48], among other animals.

Expression of the *EPO* gene

Clones of the gene for EPO were inserted into CHO cells, which synthesized the 193 amino acid precursor protein, removed the signal peptide and carboxy-terminal arginine, added *N*- and *O*-linked carbohydrate to glycosylation sites, and released the mature protein into the culture medium. Immunologic, biologic, and biochemical assays showed that the recombinant hormone had the *in vivo* biologic activity and was immunologically equivalent to human EPO, as revealed within the limits of the available assays [39]. EPO expressed by CHO cells has a molecular weight of 30.4 Kd and contains 40% carbohydrate [49].

Discussion

The isolation and expression of the gene for human EPO was a major achievement that capped almost 155 years of exploration into the nature of anemia and the production of red blood cells. EPO is present in minute quantities in the blood, and difficulty in isolating and purifying the hormone in amounts that would allow investigation of its properties posed a significant obstacle to the development of EPO as a therapeutic agent. After Miyake, Kung, and Goldwasser developed a technique to purify urinary EPO, a strategy for the cloning and expression of the human gene could be devised. The innovative approach of Lin et al. allowed the successful isolation, cloning, sequencing, and development of the recombinant protein. Large-scale production began and clinical trials started. The recombinant protein, epoetin alfa, produced dose-dependent increases in erythropoiesis that paralleled the expected response to endogenous EPO. The commercial production of epoetin alfa has been translated into benefits for millions of patients (Tab. 1). Epoetin alfa ameliorates the debilitating symptoms of anemia and allows these patients to have more normal lives. Other chapters in this volume will elaborate on the clinical use, production, formulation, and other important and evolving aspects in the study of rHuEPO.

Table 1. Some benefits of epoetin alfa therapy

- Increased exercise tolerance
- Improved central nervous system function
- Reduced heart enlargement
- Reduced extreme fatigue
- Increased ability to perform daily functions of life
- Reduced risk of alloimmunization in transplant recipients
- Improved coagulation

References

1 Eschbach JW, Abdulhadi MH, Browne JK, Delano BG, Downing MR, Egrie JC, Evans RW, Friedman EA, Graber SE, Haley NR et al. (1989) Recombinant human erythropoietin in anemic patients with end-stage renal disease. Results of a phase III multicenter clinical trial. *Ann Intern Med* 111: 992–1000

2 Volgelzang NJ, Brietbart W, Cella D, Curt GA, Groopman JE, Horning SJ, Itri LM, Johnson DH, Scherr SL, Portenoy RK (1997) Patient, caregiver, and oncologist perceptions of cancer-related fatigue: Results of tripart assessment survey. The Fatigue Coalition. *Semin Hematol* 34: 4–12

3 Demetri GD, Kris M, Wade J, Degos L, Cella D (1998) Quality-of-life benefit in chemotherapy patients treated with epoetin alfa is independent of disease response of tumor type: Results from a prospective community oncology study. Procrit Study Group. *J Clin Oncol* 16: 3412–3425

4 Glaspy J, Bukowski R, Steinberg D, Taylor C, Tchekmedyian S, Vadhan-Raj S (1997) Impact of therapy with epoetin alfa on clinical outcomes in patients with nonmyeloid malignancies during cancer chemotherapy in community oncology practice. Procrit Study Group. *J Clin Oncol* 15: 1218–1234

5 Peeters HR, Jongen-Lavrencic M, Bakker CH, Vreugdenhil G, Breedveld FC, Swaak AJ (1999) Recombinant human erythropoietin improves health-related quality of life in patients with rheumatoid arthritis and anaemia of chronic disease; utility measures correlate strongly with disease activity measures. *Rheumatol Int* 18: 201–206

6 Gabrilove JL, Cleeland CS, Livingston RB, Sarokhan B, Winer E, Einhorn LH (2001) Clinical evaluation of once-weekly dosing of epoetin alfa in chemotherapy patients: Improvements in hemoglobin and quality of life are similar to three-times-weekly dosing. *J Clin Oncol* 19: 2875–2882

7 Foote MA, Colowick A, Goodkin DA (2002) Basics of anatomy and physiology: Red blood cells and anemia. *AMWA J* 17: 13–20

8 Watson AJ (1989) Adverse effects of therapy for the correction of anemia in hemodialysis patients. *Sem Nephrol* 9: 30–34

9 Bright R (1836) Cases and observations illustrative of renal disease accompanied with the secretion of albuminous urine. *Guy's Hospital Reports* 1: 338–379

10 Jourdanet D (1863) De l'anemie des altitudes et de l'anemie en general dans ses rapports avec la pression del l'atmopshere. *Balliere* (Paris) 44

11 Viault F (1891) Sur l'augmentation considerable du nombre des globules rouges dans le sange chez les habitants des haut plateaux de l'Amerique du Sud. *CR Acad Press* (Paris) 111: 917–918

12 Viault F (1891) Sur la quantité d'oxygen contenue dans le sang des animaux des hauts pleateaux de L'Amerique du Sud. *CR Acad Sci* (Paris) 112: 295–298

13 Muntz A (1891) De l'enrichissement du sang en hemoglobine, suivant les conditions d'existence. *CR Acad Sci* (Paris) 112: 298–301

14 Meischer F (1893) Ueber die beziehungen zwischen meereshohe und bescharfenheit des blutes. *Korresp-Bl Schweiz Arz* 23: 809–830

15 Meischer F (1893) Bemerkungen uber eine verbesserte form der mischipette und ihren einfluss auf die genauigkeit der blutkorperzahlung. *Korresp-Bl Schweiz Arz* 23: 830–832

16 Carnot P (1906) Sur le mecanisme de l'hyperglobulie provoquée par le serum d'animaux en renovation sanguine. *CR Acad Sci* (Paris) 111: 344–346
17 Carnot P (1906) Sur l'activite cytopoietique du sang et des organs regeneres au cours des regenerations viserales. *CR Biol* (Paris) 111: 463–465
18 Carnot P, Deflandre C (1906) Sur l'activité cytopoietique du sang et des organs regeneres au cours des regeneration du sang. *CR Acad Sci* (Paris) 143: 432–435
19 Gibelli C (1911) Uber den wert des serums anamisch gemachten tiere bei der regernation des blutes. *Arch Exp Path Pharmark* 65: 284–302
20 Giribaldi G (1920) Sul potere emopoietico del siero di sangue di animali resi anemici con iniezioni sottocutanee di glicerina. *Biochim Terap Sper* 7: 52–65
21 Forster J (1924) Luftverdünnung und Blutregeneration durch "Hemopoietine". *Biochem Z* 145: 309–317
22 Sandor G (1932) Über die blutbildene Wirkung des Serums von Tieren, die in verdünnter Luft gehalten wurden. *Z Ges Exp Med* 82: 633–646
23 Thorling EB (1969) The history on the early theories of humoral regulation of the erythropoiesis. *Dan Med Bull* 16: 159–164
24 Erslev A (1952) Humoral regulation of red cell production. *Blood* 8: 349–357
25 Jacobson LO, Goldwasser E, Fried W, Plzak L (1957) Role of the kidney in erythropoiesis. *Nature* 179: 633–634
26 Congote LF (1977) Regulation of fetal liver erythropoiesis. *J Steroid Biochem* 8: 423–428
27 Mirand EA, Prentice TC (1957) Presence of plasma erythropoietin in hypoxic rats with and without kidneys or spleen. *Proc Soc Exp Biol Med* 96: 49–51
28 Koury MJ, Bondurant MC, Graber SE, Sawyer ST (1988) Erythropoietin messenger RNA levels in developing mice and transfer of ^{125}I-erythropietin by the placenta. *J Clin Invest* 82: 154–159
29 Erslev AJ, Caro J, Kanusu E, Silver R (1980) Renal and extrarenal erythropoietin production in anemic rats. *Br J Haematol* 45: 65–72
30 Marti HH, Wenger RH, Rivas LA, Straumann U, Digicaylioglu M, Henn V, Yonekawa Y, Bauer C, Gassmann M (1996) Erythropoietin gene expression in human, monkey, and murine brain. *Eur J Neurosci* 8: 666–676
31 Espada J, Gutnisky A (1970) A new method for concentration of erythropoietin from human urine. *Biochem Med* 3: 475–484
32 Espada J, Langton AA, Dorado M (1972) Human erythropoietin: Studies on purity and partial characterization. *Biochim Biophys Acta* 285: 427–435
33 Goldwasser E, Kung CKH (1971) Purification of erythropoietin. *Proc Natl Acad Sci USA* 68: 697–698
34 Goldwasser E, Gross M (1975) Erythropoietin: Assay and study of its mode of action. *Methods Enzymol* 37: 109–121
35 Miyake T, Kung CK, Goldwasser E (1977) Purification of human erythropoietin. *J Biol Chem* 252: 5558–5564
36 Sue JM, Sytkowski AJ (1983) Site-specific antibodies to human erythropoietin directed towards the NH_2-terminal region. *Proc Natl Acad Sci USA* 80: 3651–3655
37 Yanagawa S, Hirade K, Ohnota H, Saski R, Chiba H, Ueda M, Goto M (1984) Isolation of human erythropoietin with monoclonal antibodies. *J Biol Chem* 259: 2707–2710
38 Goeddel DV, Kleid DG, Bolivar F, Heyneker HL, Yansura DG, Crea R, Hirose T, Kraszewski A, Itakura K, Riggs AD (1979) Expression in *Escherichia coli* of chemically synthesized genes for human insulin. *Proc Natl Acad Sci USA* 76: 106–110
39 Lin FK, Suggs S, Lin CH, Browne JK, Smalling R, Egrie JC, Chen KK, Fox GM, Martin F, Stabinsky Z et al. (1985) Cloning and expression of the human erythropoietin gene. *Proc Natl Acad Sci USA* 82: 7580–7582
40 Lin FK, Lin CH, Lai PH, Browne JK, Egrie JC, Smalling R, Fox GM, Chen KK, Castro M, Suggs S (1986) Monekey erythropoietin gene: Cloning, expression and comparison with the human erythropoietin gene. *Gene* 44: 201–209
41 Jacobs K, Shoemaker C, Rudersdorf R, Neill SD, Kaufman RJ, Mufson A, Seehra J, Jones SS, Hewick R, Fritsch EF et al. (1985) Isolation and characterization of genomic and cDNA clones of human erythropoietin. *Nature* 313: 806–810
42 McDonald JD, Lin FK, Goldwasser E (1986) Cloning, sequencing, and evolutionary analysis of the mouse erythropoietin gene. *Mol Cell Biol* 6: 842–848
43 Shoemaker CB, Mitsock LD (1986) Murine erythropoietin gene: cloning, expression, and human gene homology. *Mol Cell Biol* 6: 849–858

44 Nagao M, Suga H, Okano M, Masuda S, Narita H, Ikura K, Sasaki R (1992) Nucleotide sequence of rat erythropoietin. *Biochim Biophys Acta* 1171: 99–102
45 Wen D, Boissel JP, Tracy TE, Gruninger RH, Mulcahy LS, Czelusniak J, Goodman M, Bunn HF (1993) Erythropoietin structure-function relationships: high degree of sequence homology among mammals. *Blood* 82: 1507–1516
46 Pearson PL, Smith TP, Sonstegard TS, Klemcke HG, Christenson RK, Vallet JL (2000) Porcine erythropoietin receptor: molecular cloning and expression in embryonic and fetal liver. *Domest Anim Endocrinol* 19: 25–38
47 Fu P, Evans B, Lim GB, Mortiz K, Wintour EM (1993) The sheep erythropoietin gene: Molecular cloning and effect of hemorrhage on plasma erythropoietin and renal/liver messenger RNA in adult sheep. *Mol Cell Endocrinol* 93: 107–116
48 Suliman HB, Majiwa PA, Feldman BF, Mertens B, Logan-Henfrey L (1996) Cloning of a cDNA encoding bovine erythropoietin and analysis of its transcription in selected tissues. *Gene* 171: 275–280
49 Davis JM, Arakawa T, Strickland TW, Yphantis DA (1987) Characterization of recombinant human erythropoietin produced in Chinese hamster ovary cells. *Biochemistry* 26: 2633–2638