Novel Erythropoiesis-Stimulating Agents: A New Era in Anemia Management

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Nearly two decades ago, recombinant human erythropoietin transformed the management of chronic kidney disease anemia by allowing a more sustained increase in hemoglobin than was possible by intermittent blood transfusion. The treatment was highly effective, but because of the fairly short half-life of the molecule at approximately 6 to 8 h, injections usually had to be administered two to three times weekly. A second-generation erythropoietin analogue, darbepoetin alfa, was then created, with a longer elimination half-life in vivo that translated into less frequent dosing, usually once weekly or once every 2 wk. More recently, another erythropoietin-related molecule has been produced called Continuous Erythropoietin Receptor Activator with an even greater half-life, and other molecules are in development or are being licensed, including biosimilar epoetin products and Hematide. The latter is a synthetic peptide-based erythropoietin receptor agonist that, interestingly, has no structural homology with erythropoietin, and yet is still able to activate the erythropoietin receptor and stimulate erythropoiesis. The search goes on for orally active antianemic therapies, and several strategies are being investigated, although none is imminently available. This article reviews the latest progress with these novel erythropoietic agents in this new era in anemia management.

Many nephrologists can still recall the days when large numbers of dialysis patients were transfusion dependent, requiring repeated red cell transfusions every few weeks to increase the hemoglobin concentration from approximately 6 g/dl to a transient value of approximately 8 or 9 g/dl, before falling once again to baseline levels. The advent of recombinant human erythropoietin (EPO; epoetin) in the late-1980s transformed this desperate situation, restoring patients’ ability to use their own bone marrow for red cell production, with a dramatic reduction in the number of blood transfusions used in dialysis centers. Epoetin therapy was found to be highly effective in the vast majority of patients who had anemia of chronic kidney disease (CKD), and adverse effects were uncommon or easily managed.

Because of the fairly short circulating half-life of plasma EPO (approximately 6 to 8 h) (1), however, patients required two or three injections a week. Thus, there was a clinical need for longer acting erythropoiesis-stimulating agents (ESAs), and several of these have been developed or are under development. To date, all ESAs licensed for clinical use are protein based, bearing some structural resemblance to EPO itself. Thus, for agents such as darbepoetin alfa or Continuous EPO Receptor Activator (CERA), modifications have been made to the EPO molecule to allow it to have a longer duration of action in vivo. Protein-based therapies have a number of disadvantages, notably immunogenicity (pure red cell aplasia caused by anti-EPO antibodies), storage and stability (must be stored at temperatures of approximately 4°C), and administration (all currently licensed products are administered intravenously or subcutaneously). Various strategies have been devised to circumvent the limitations of the currently available products (Table 1), and these are discussed in this review. The strategies include the potential development of orally active ESAs, perhaps through stabilization of hypoxia-inducible factor (HIF), although the HIF stabilizers have suffered a recent setback that has seriously jeopardized their ongoing clinical development.

Protein-Based ESA Therapy

The original recombinant human EPOs (epoetin alfa and epoetin beta) have now been in clinical use for nearly 20 yr. Both products are synthesized in cultures of transformed Chinese hamster ovary (CHO) cells that carry cDNA encoding human EPO (2). The amino acid sequence of both epoetins is therefore identical, and the major difference between these products lies in their glycosylation pattern. Thus, it is recognized that human EPO exists as a mixture of isoforms that differ in both glycosylation and biologic activity (3). Other epoetins that have recently become available or are still being developed include epoetin omega (4–6) and epoetin delta (7–9), as well as the copy products of epoetin alfa and other biosimilar epoetins (reviewed by Schellekens [10] in this issue of CJASN). Again, all of these products share the same 165-amino acid sequence as for epoetin alfa and epoetin beta, as well as the endogenous hormone. The cell culture conditions, however, vary. With epoetin omega, baby hamster kidney (BHK) cell cultures are used for the manufacture of this prod-
Epoetin delta is another recombinant EPO that has been used for treating patients with CKD; it was approved by the European Medicines Agency in 2002 and first marketed in Germany in 2007 (7–9). Epoetin delta is synthesized in human fibrosarcoma cell cultures (line HT-1080). The product is also called gene-activated EPO because the expression of the native human EPO gene is activated by transformation of the cell with the cytomegalovirus promoter (11). In contrast to CHO or BHK cell–derived recombinant human EPO, epoetin delta does not possess N-glycolylneuraminic acid (Neu5Gc) because, in contrast to other mammals, humans are genetically unable to produce Neu5Gc as a result of an evolutionary mutation (12). The implications of a lack of Neu5Gc residues in synthetic recombinant EPO, if any, are not clear at present.

Darbepoetin alfa
The development of darbepoetin alfa arose from the recognition that the higher isoforms (those with a greater number of sialic acid residues) of recombinant human EPO were more potent biologically in vivo as a result of a longer circulating half-life than the lower isomers (those with a lower number of sialic acid residues) (3) (Figure 1). Because the majority of sialic acid residues are attached to the three N-linked glycosylation chains of the EPO molecule, attempts were made to synthesize EPO analogues with a greater number of N-linked carbohydrate chains. This was achieved using site-directed mutagene-
sis to change the amino acid sequence at sites not directly involved in binding to the EPO receptor (13,14). Thus, five–amino acid substitutions were implemented (allowing darbepoetin alfa to carry a maximum of 22 sialic acid residues, compared with recombinant or endogenous EPO, which support a maximum of 14 sialic acid residues). The additional N-linked carbohydrate chains increased the molecular weight of epoetin from 30.4 to 37.1 kD, and the carbohydrate contribution to the molecule correspondingly increased from 40% to approximately 52% (13,14).

These molecular modifications to EPO confer a greater metabolic stability in vivo, with the elimination half-life in human after a single intravenous injection of darbepoetin alfa increasing three-fold (25.3 h) compared with epoetin alfa (8.5 h) (15). The half-life after subcutaneous administration is doubled from approximately 24 h to approximately 48 h. This latter characteristic has allowed less frequent dosing, with most patients receiving injections once weekly or once every other week (16). Further extension out to once-monthly dosing with darbepoetin alfa may be possible in some patients, but it is not clear what dosage penalty this incurs. Furthermore, this is possible only in selected patients, generally those who are clinically stable and who do not yet require dialysis.

CERA
The strategy used to synthesize CERA was to integrate a large methoxy-polyethylene glycol polymer chain into the EPO molecule via amide bonds between the N-terminal amino group of alanine and the ε-amino groups of lysine (Lys45 or Lys52) by means of a succinimidyl butanoic acid linker (17). Because the mass of the polymer chain is approximately 30 kD, this doubles the molecular weight of CERA to approximately 60 kD, compared with EPO (30.4 kD). As with other pegylated therapeutic proteins, the half-life of circulating CERA is considerably prolonged compared with that of epoetin: at approximately 130 h (18). Thus, less frequent dosing regimens of once every 2 wk and once every month have been tested in Phase II and Phase III clinical trials (19,20), and the product recently received a
license in both the US and Europe. As with the previous erythropoietic agents, CERA is still administered intravenously or subcutaneously, and adverse events seem to be similar to those associated with the epoetins or darbepoetin alfa. It is possible that the metabolic fate of CERA is different from the existing products, with less cellular internalization after interaction with the EPO receptor, but further experimental work is required to confirm this. As also occurs with darbepoetin alfa, the binding affinity for the EPO receptor is less than for natural or recombinant EPO, but the benefits of the greater stability in vivo far outweigh this minor biologic disadvantage.

In addition to CERA, other pegylated molecules, including epoetin alfa (21) and an epoetin analogue (22), have been tested for their efficacy in experimental animals. These products have not yet entered clinical trials.

Other Protein-Based EPO Derivatives
Several other EPO-like molecules and derivatives are in preclinical or clinical trials. A further hyperglycosylated analogue of darbepoetin alfa was synthesized, with additional carbohydrate residues (AMG114). Although this analogue was found to have an even longer circulating half-life in vivo compared with darbepoetin alfa, the EPO receptor binding affinity was too low to develop this molecule further as a therapeutic agent, and clinical trials have now ceased. Another novel product is synthetic erythropoiesis protein (SEP), which was manufactured using solid-phase peptide synthesis and branched precision polymer constructs. A 51-kD protein-polymer construct was synthesized containing two covalently attached polymer moieties (23). As with darbepoetin alfa and CERA, this polymer stimulates erythropoiesis through activation of the EPO receptor, and with a longer circulating half-life than for EPO alone. The erythropoietic effect of synthetic erythropoiesis protein has been shown to vary in experimental animals depending on the number and type of the attached polymers (24). Recombinant EPO fusion proteins that contain additional peptides at the carboxy-terminus to increase in vivo survival have been expressed (25). Large EPO fusion proteins, of molecular weight 76 kD, have been designed from cDNA encoding two human EPO molecules linked by small flexible polypeptides (26,27). A single subcutaneous administration of this compound to mice increased red cell production within 7 d at a dosage at which epoetin was ineffective (26). Another dimeric fusion protein incorporating both EPO and granulocyte macrophage colony-stimulating factor (GM-CSF) has been created, with the rationale that GM-CSF is required for early erythropoiesis. This EPO-GM-CSF complex proved to be able to stimulate erythropoiesis in cynomolgus monkeys (28) but was later found to induce anti-EPO antibodies, causing severe anemia (29). Yet another approach is the genetic fusion of EPO with the Fc region of human IgG (Fc-EPO) (30). This molecular modification promotes recycling out of the cell upon endocytosis via the Fc recycling receptor (31,32), again providing an alternative mechanism for enhancing circulating half-life. The same effect may be achieved by fusing EPO with albumin.

Another molecule currently undergoing development is CTNO 528, which is an EPO-mimetic antibody fusion protein with an enhanced serum half-life but no structural similarity to EPO (33). Rats that were treated with a single subcutaneous dose of CTNO 528 showed a more prolonged reticulocytosis and hemoglobin rise compared with treatment with epoetin or darbepoetin alfa. Phase I studies in healthy volunteers showed a similar effect after a single intravenous administration of CTNO 528, with a peak reticulocyte count occurring after 8 d, and the maximum hemoglobin concentration being seen after 22 d. None of the 24 participants in this study developed antibodies against the molecule (34).

Interestingly, an Fc-EPO fusion protein has been successfully administered in a Phase I trial to human volunteers as an aerosol, with a demonstrable increase in EPO levels associated with an increase in reticulocyte counts (35). In addition to the EPO derivatives administered by aerosol inhalation, other delivery systems for EPO have been investigated, including ultrasound-mediated transdermal uptake (36) and orally via liposomes to rats (37). Mucoadhesive tablets containing EPO and an absorption enhancer (Labrasol; Gatetfosse, Gennevilliers, France) for oral administration have been studied in rats and dogs (38). Theoretically, this preparation is designed to allow the tablet to reach the small intestine intact. Preliminary experiments in beagle dogs were conducted with intrajejunal administration of a single tablet containing 100 IU/kg recombinant human EPO, with a corresponding increase in reticulocytes 8 d after administration (38). It is too early to say whether this strategy could have any clinical relevance in the treatment of anemia in patients with CKD.

Small-Molecule ESAs
Peptide-Based ESAs
Just over 10 yr ago, several small bisulfide-linked cyclic peptides composed of approximately 20 amino acids that were unrelated in sequence to EPO but still bound to the EPO receptor were identified by random phage display technology (39,40). These small peptides were able to induce the same conformational change in the EPO receptor that leads to JAK2 kinase/STAT-5 intracellular signaling (40), as well as other intracellular signaling mechanisms, resulting in stimulation of erythropoiesis both in vitro and in vivo. The first peptide to be investigated (EPO-mimetic peptide-1) (40) was not potent enough to be considered as a potential therapeutic agent in its own right, but the potency of these peptides could be greatly enhanced by covalent peptide dimerization with a PEG linker. Thus, another EPO-mimetic peptide was selected for the development of Hematide (Affymax, Palo Alto, CA), a pegylated synthetic dimeric peptidic EPO that was found to stimulate erythropoiesis in experimental animals (41). The half-life of Hematide in monkeys ranges from 14 to 60 h, depending on the dosage administered. Further studies in rats using quantitative whole-body autoradioluminography have shown that the primary route of elimination for the peptide is the kidney (42).

A Phase I study in healthy volunteers showed that single injections of Hematide caused a dosage-dependent increase in reticulocyte counts and hemoglobin concentrations (43). Phase II studies have demonstrated that Hematide can correct the anemia associated with CKD (44), as well as maintain the
hemoglobin in dialysis patients who are already receiving conventional ESAs (45). Dosages in the range of 0.025 to 0.05 mg/kg seem to be therapeutically optimal in this patient population (44), and at the time of writing, four Phase III studies are about to be initiated. Hematide may be administered either intravenously or subcutaneously, and dosing once a month is effective (44).

The potential advantages of this new agent are greater stability at room temperature, lower immunogenicity compared with conventional ESAs, and a much simpler (and cheaper) manufacturing process, avoiding the need for cell lines and genetic engineering techniques. Antibodies against Hematide do not cross-react with EPO, and similarly anti-EPO antibodies do not cross-react with Hematide (46). This has two major implications: First, even if a patient does develop anti-Hematide antibodies, these should not neutralize the patient’s own endogenous EPO, and the patient should not develop pure red cell aplasia. Second, patients with antibody-mediated pure red cell aplasia should be able to respond to Hematide therapy by an increase in their hemoglobin concentration, because Hematide is not neutralized by anti-EPO antibodies. This latter hypothesis has already been confirmed in animals (46). Rats that received regular injections of recombinant human EPO were shown to develop anti-EPO antibodies. Injections of Hematide were able to “rescue” these animals and restore their hemoglobin concentration, in contrast to the vehicle-treated group (46). A clinical trial examining this issue in patients with antibody-mediated pure red cell aplasia was also recently performed (47).

Other peptide-based ESAs are in preclinical development. A compound made by AplaGen (Baesweiler, Germany) has linked a peptide to a starch residue, again demonstrating prolongation of the circulating half-life of the molecule (48). Indeed, altering the molecular weight of the starch moiety has been shown to alter the pharmacologic properties of the compound.

Non–peptide-Based ESAs

Several nonpeptide molecules that are capable of mimicking the effects of EPO have also been identified, after screens of small-molecule nonpeptide libraries for molecules with EPO receptor–binding activity (49,50). One such compound was found, but this bound to only a single chain of the EPO receptor and was not biologically active. The compound was ligated to enable it to interact with both domains of the EPO receptor, and this second molecule was shown to stimulate erythropoiesis (49). Further development of nonpeptide EPO mimetics could lead to the production of an orally active ESA in the future.

Other Strategies for Stimulating Erythropoiesis

Prolyl Hydroxylase Inhibition (HIF Stabilizers)

Under normoxic conditions, EPO gene expression is suppressed physiologically as a result of inactivation of the hypoxia-inducible transcription factors (HIF). This inactivation is mediated by HIF-alfa prolyl- and asparaginyl-hydroxylatation. The HIF-alfa hydroxylases require not only oxygen for their catalytic action but also iron and 2-oxoglutarate (51). Thus, HIF-alfa hydroxylation can be prevented either by iron depletion or by the administration of 2-oxoglutarate analogues. These latter molecules have recently been termed HIF stabilizers, and these compounds have been shown to promote EPO expression in cell cultures, as well as in animals and humans.

Interestingly, these compounds were originally developed for their inhibitory action on collagen prolyl 4-hydroxylases, which also need 2-oxoglutarate as a co-factor. The primary aim of the initial studies was to develop drugs for the treatment of fibrotic diseases (52,53); however, the 2-oxoglutarate analogues were found to stimulate erythropoiesis in vivo (54). HIF stabilizers have already been administered to healthy control subjects (55) and to patients with CKD (56) in clinical trials investigating novel strategies for the treatment of anemia. After their administration, increases in plasma EPO levels were found, with a concomitant increase in reticulocyte count. Phase II studies of the first candidate molecule, FG-2216, demonstrated correction of anemia in patients with CKD, in contrast to placebo (56). These agents have the advantage of being orally active, and they also seem to upregulate other genes involved in the process of erythropoiesis, notably those that improve iron utilization.

Unfortunately, several concerns have seriously jeopardized the future application of these orally active agents in the treatment of anemia in CKD. First, at least 100 other genes are upregulated by inhibition of the prolyl hydroxylases, not only genes that promote erythropoiesis but also other hypoxia-sensitive genes, such as vascular endothelial growth factor (57). Although there may be attempts to create HIF stabilizers that upregulate only erythropoiesis genes and not other HIF-sensitive genes, it will take some time to persuade clinicians that there is no risk for potentiation of tumor growth as well as other unwanted adverse effects arising from such ubiquitous gene upregulation.

In mid-2007, there arose another serious barrier to further development of these agents in the treatment of anemia. During one of the Phase II clinical trials of FG-2216, a female patient developed fatal hepatic necrosis that was temporally related to the introduction of this compound (58). Although investigations regarding causality are ongoing, the Food and Drug Administration has for now suspended any further clinical trials with HIF stabilizers.

GATA Inhibition

The GATA family consists of six transcription factors, GATA 1 through 6. Dame et al. (59) reported that GATA-4 is critically involved in EPO gene expression and may be responsible for the switch in the site of EPO production from the fetal liver to the adult kidney. In addition, GATA-2 inhibits EPO gene transcription by binding to the GATA sequence on the EPO promoter, thereby leading to downregulation of EPO mRNA expression and subsequent EPO synthesis (60). GATA-2 therefore acts as a negative regulatory molecule of EPO gene expression. Disrupting this negative signal is therefore a potential future strategy in the management of renal anemia. Several molecules are under investigation, including K-11706, which has been
shown to enhance EPO production both in vitro and in vivo. Oral administration of K-11706 restored the hemoglobin concentrations, reticulocyte counts, EPO levels, and numbers of CFU-E induced by IL-1beta or TNF-alfa in a mouse model of anemia (61). These results raise the possibility of using orally administered K-11706 in the treatment of renal anemia, but clinical trials are not yet under way.

**Hemopoietic Cell Phosphatase Inhibition**

Another strategy with the potential for enhancing erythropoiesis is targeting the src homology domain 2–containing tyrosine phosphatase-1 (SHP-1), also known as hemopoietic cell phosphatase (HCP) (62). This protein tyrosine phosphatase is located in the cytoplasm of hemopoietic cells and was originally identified in human breast carcinoma cDNA (63). SHP-1 binds to the negative regulatory domain of the EPO receptor via its src-homology 2 domains and causes dephosphorylation of JAK-2, thereby functioning as a negative regulator of EPO intracellular signal transduction (64). The potential importance of this molecule in mediating responsiveness to EPO therapy was studied in CD34⁺ cells derived from a population of hemodialysis patients who were responding poorly to EPO (65). Compared with an EPO-responsive group, CD34⁺ cells from EPO-hyporesponsive patients showed increased mRNA and protein expression of SHP-1. Furthermore, treatment of the CD34⁺ cells from EPO-hyporesponsive patients with an SHP-1 antisense oligonucleotide decreased SHP-1 protein expression and upregulated STAT-5, resulting in the partial recovery of erythroid colony formation (65). The gene for SHP-1 has been cloned, and SHP-1 inhibitors have been identified. In vitro inhibition of SHP-1 resulted in a dosage-dependent erythroid proliferation (65). As with the GATA inhibitors, the HCP inhibitors have not yet been tested in humans, and it is therefore not clear whether they would have any role in the management of CKD anemia. These orally active agents, however, could potentially be used as adjuvant therapy to enhance the response to other ESAs or even to enhance the patient’s own endogenous EPO.

**EPO Gene Therapy**

With increasing concern that high dosages of erythropoietic products may be harmful, the ability to generate lower but continuous levels of EPO as a result of gene therapy is a potentially attractive area of research. It does not seem to matter by which cells and at which site EPO is released into the circulation, and a number of delivery systems have been investigated, such as injection of naked DNA (66), adenovirus transfection (67), use of artificial human chromosomes (68), and transplantation of autologous or allogeneic cells manipulated ex vivo (69,70).

As with all gene therapy, there are many hurdles to overcome before this could be used in humans. Not only would there need to be reassurance regarding the absence of oncogenicity, but it would also be imperative to show that tight control of the activity of the transferred gene can be achieved. This may be possible using a number of pharmacologic strategies or potentially by exposure to a rare antigen, when the transgene is expressed in a specific B cell clone (71). Interestingly, animal experiments have shown that linking the EPO transgene to a hypoxia-responsive DNA element (the HIF binding site) can establish an oxygen-dependent feedback regulation of the transgene, similar to that of the endogenous EPO gene (72).

**Conclusions**

As the molecular mechanisms that control red cell production have been elucidated, so, too, have new targets and strategies been developed for stimulating erythropoiesis and treating anemia. After the introduction of recombinant human EPO, attempts were made to modify the molecule and produce longer acting erythropoietic agents, such as darbepoetin alfa and CERA. Other modifications to the molecule, such as the production of fusion proteins, are being explored, as is the potential for EPO gene therapy. The concept that smaller molecules such as peptides or even nonpeptides may be able to bind to and activate the EPO receptor is also being investigated, and the first such molecule (Hematide) is currently in Phase III of its clinical development program. Other strategies, attempting to create orally active agents, such as inhibition of prolyl hydroxylase, GATA, or HCP, remain in the laboratory but may yet translate into future therapeutic agents for the management of CKD anemia.

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Anemia Management with Novel Erythropoietic Agents


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