Review

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Erythropoietin and erythropoiesis stimulating agents

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Erythropoietin (EPO) is the main hormonal regulator of red blood cell production. Recombinant EPO has become the leading drug for treatment of anaemia from a variety of causes; however, it is sometimes misused in sport with the aim of improving performance and endurance. This paper presents an introductory overview of EPO, its receptor, and a variety of recombinant human EPOs/erythropoiesis stimulating agents (ESAs) available on the market (e.g. epoetins and their long acting analogs – darbepoetin alfa and continuous erythropoiesis receptor activator). Recent efforts to improve on EPO's pharmaceutical properties and to develop novel replacement products are also presented. In most cases, these efforts have emphasized a reduction in frequency of injections or complete elimination of intravenous or subcutaneous injections of the hormone (biosimilars, EPO mimetic peptides, fusion proteins, endogenous EPO gene activators and gene doping). Isoelectric focusing (IEF) combined with double immunoblotting can detect the subtle differences in glycosylation/sialylation, enabling differentiation among endogenous and recombinant EPO analogues. This method, using the highly sensitive anti-EPO monoclonal antibody AE7A5, has been accepted internationally as one of the methods for detecting misuse of ESAs in sport. Copyright © 2012 John Wiley & Sons, Ltd.

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Introduction

Erythropoietin, a glycosylated protein hormone produced principally by the kidney, is the prime regulator of red blood cell (RBC) production (erythropoiesis). It stimulates the production of red blood cells due to increased survival, proliferation, and differentiation of erythroid progenitors in the hematopoietic tissues (bone marrow).[1]

Recombinant human EPO (rHuEPO) was developed in the 1980s and soon after became the leading drug for treatment of anaemia associated with chronic kidney disease (CKD), virtually abolishing the need for RBC transfusion.[2] Later on it was also used for treatment of anaemia that accompanies critical illnesses,[3] HIV infection, major surgical procedures, and chemotherapy-treated cancer patients.[4,5] Ongoing pre-clinical and clinical trials are exploring the potential use of rHuEPO as a tissue protective agent in the brain, heart, kidney, and in wound healing.[6–9]

RHuEPO has been misused in sport with the aim of improving performance and endurance. Increased RBC mass due to rHuEPO use results in increased oxygen delivery and increased muscle performance.[10] The use of rHuEPO in sport has been banned by the International Olympic Committee since 1990; however, a direct detection method that could effectively differentiate between endogenous and recombinant EPO was developed by Lasne and de Ceaurriz in the decade that followed.[11] The detection method is based on isoelectric focusing (IEF-PAGE) and double immunoblotting using the highly sensitive anti-EPO monoclonal antibody AE7A5[12] and was first used in the Sydney Summer Olympic Games in the year 2000.[13] Among prohibited substances on the World Anti-Doping Agency (WADA) list of 2012 under Class S2 are Erythropoiesis-Stimulating Agents (ESAs) (e.g. rHuEPO, darbepoetin, HIF-stabilizers, CERA, Hematide), and Non-Approved Substances under Class S0 including all drugs under pre-clinical or clinical development or discontinued (e.g. all new or discontinued ESAs).[14]

Besides IEF-PAGE, other methods are used for EPO characterization of biosimilars and copy epoetins. They include ELISA,[15] SDS-PAGE, Sarcosyl-PAGE,[16] and MAIIA (Membrane Assisted Isoform ImmunoAssay).[17]

EPO structure and function

The human EPO gene was cloned and expressed in 1985.[18,19] It is a single copy gene located on chromosome 7 at position 7q22.[20,21] This 3 kb long gene contains five exons encoding a 193 amino acid pro-hormone.[18,19] As far as we know, no abnormal condition in humans has been recognized due to mutations of the EPO gene. EPO gene expression is regulated primarily by hypoxia.[22] Some of the transcription factors involved are inhibitory GATA-2, NF-kappaB and stimulatory HIF-2, HNF-4alfa.[23–25]

The mature EPO protein is a single-chain protein containing 165 or 166 amino acids with molecular weight ranging from 30 to 39 kDa. Different amino acid sequence characterizations of the naturally occurring protein isolated from human urine detected 165[26] or 166 amino acids,[27] whereas the recombinantly produced EPO contains 165.[28] The reason for these differences has never been clearly delineated nor has consensus been reached. During maturation, EPO undergoes several post-translational modifications,

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including cleavage of the N-terminal 27 amino acid signal peptide and intense glycosylation.[27,28] It contains three N-linked (N24, N38, and N83) and one O-linked (S126) oligosaccharide side chains representing approximately 35–40% of the hormone molecular mass. N-glycosylation is essential for EPO's biological activity *in vivo*, including biosynthesis, secretion, receptor affinity, plasma half-life and clearance. However, it is not necessary for its action or receptor binding *in vitro*.[29–31] EPO also contains two intra-chain disulfide bridges between C7-C161 and C29-C33[27] (Figure 1).

In adult mammals, EPO is produced mainly by the renal cortex,[32,33] while in the developing embryo the principal source is foetal liver.[34] EPO is secreted into the blood stream, and its biological effects are mediated by binding to the EPO receptor (EPOR) situated on the cell surface of erythroid progenitors. One EPO molecule binds to two EPOR via two non-identical binding sites that are important for its biological activity.[35–37]

EPO regulates production of up to 2 x 10^{11} RBC per day to maintain physiological conditions. In addition, EPO and its receptor have been identified also in several non-hematopoietic cells and tissue types including the central nervous system, heart, kidney, gastrointestinal system, reproductive tract, endothelium[38–40] and on several types of malignant cells.[41] In many of these tissues, EPO has been shown to be tissue protective via an anti-apoptotic and/or mitogenic manner. EPO has pleiotropic effects, acting not only by an endocrine, but also by an autocrine and paracrine manner.[42]

EPOR structure and signal transduction

The murine EPOR gene was cloned in 1989[43] and the human gene in 1990, 15 years after its ligand EPO.[44] The coding sequence is located on chromosome 19 at position 19p13.3-p13.2.[45] This 6 kb long gene contains 8 exons encoding a 508 amino acid receptor-precursor.[44,46] The EPOR gene has several splice variants: variant 1 encodes a functional trans-membrane protein, variant 2 contains non-coding RNA; [1] and at least one soluble EPOR variant.[47,48] Several known EPOR mutations result in increased or reduced production of RBC causing hematological disorders in humans[49] and, in rare cases, may be the explanation for increased athletic performance.[50–52]

The EPOR is a member of the type I cytokine receptor superfamily which includes a diverse group of hematopoietic factors and growth hormones.[53] The mature human receptor is a single chain protein containing 483 amino acids. During maturation, it undergoes several post-translational modifications such as cleavage of 24-amino acid signal peptide, glycosylation, phosphorylation, and ubiquitination.[44,54,55] The mature receptor consists of an extracellular, a transmembrane, and a cytoplasmic region.[56] The cytoplasmic region of the EPOR does not contain any kinase or other enzymatic domain.[57] Phosphorylation of downstream targets is mediated by Janus protein tyrosine kinase 2 (JAK2) associated with the Box 1 motif in the membrane proximal cytoplasmic region.[58,59] The distal cytoplasmic region contains eight functional tyrosine residues required for signal transduction through several kinases.[60]

EPO binding to the EPOR homodimer triggers the conformational changes in the receptor dimer,[61,62] bringing into close proximity two JAK2 molecules resulting in their auto- and trans-phosphorylation as well as phosphorylation of tyrosine residues in the EPOR cytoplasmic region.[61,63] Receptor activation is followed by activation of several kinases and other signalling molecules. The main signal pathways activated by EPO are JAK2/signal transducer and activator of transcription (STAT) 5 or 3 pathway, mitogen activated protein kinase (MAPK/ERK) pathway, phosphatidylinositol 3-kinase (PI3K/AKT) pathway, and protein kinase C (PKC) pathway.[1,60]

Erythropoiesis stimulating agents (ESA)

Epoetins

Recombinant human EPO (rHuEPO, International Non-proprietary Name [INN]: epoetin) was first produced in the 1980s by transfection of human EPO cDNA or genomic EPO into Chinese hamster ovary (CHO) cells.[18,19] For commercial production, EPO is excreted into the cell growth medium, the medium is collected, concentrated, and the EPO is purified by various chromatographic methods. As N- glycosylation is essential for EPO's biological activity *in vivo*,[29–31,64] only mammalian expression systems enabling sufficient glycosylation are currently used including CHO cells, baby hamster kidney cells (BHK),[65] and the human fibrosarcoma cell line (HT-1080).[66] Diverse production systems result in glycosylation pattern variations and, as a consequence, in potentially subtle differences in each epoetin's biochemical and biological properties. Commercially available rHuEPOs are designated by the INN **epoetin** followed by a Greek letter (alfa, beta, etc.) which reflects the specific glycosylation reported to the WHO (Table 1).

Epoetin alfa was the first commercially available epoetin. It was produced in CHO cells and approved by the US Food and Drug Administration (FDA) in 1989 for the treatment of anaemia associated with CKD. Subsequently, it was approved for use in patients with other anaemia including cancer patients undergoing chemotherapy, HIV patients undergoing antiviral treatment, and pre-surgery patients. Several other epoetins are also produced in CHO cells with some production variations (epoetin beta, theta, kappa and zeta). Epoetin omega is produced in BHK cells.[65] While epoetin delta was the only rHuEPO produced by the human cell line (HT-1080); it has not been marketed since 2009, apparently due to inadequate sales.[66]

Long-acting rHuEPO analogues

One focus of new ESA development has been to improve EPO's pharmacological characteristics, such as a prolonged plasma half-life (fewer intravenous or subcutaneous injections) and the use of non-invasive delivery systems (oral, inhalation). One means to achieve a prolonged half-life is by increasing the molecular mass of the hormone, which has had the effect of reducing EPOR affinity and, thereby, reducing clearance by receptor-mediated endocytosis. Two approaches have been used successfully in commercially available pharmaceuticals: hyper-glycosylation and pegylation.

Darbepoetin alfa (originally 'novel erythropoiesis stimulating protein' [NESP]) is epoetin with five amino acid mutations leading to two additional N-linked glycans (in sum, five N-linked and one O-linked) and an increased molecular mass (Figure 1).[67] This increased glycosylation results in lower receptor affinity, 3-fold longer circulating half-life and greater *in vivo* potency.[68] This allows longer dosing intervals – for example, once weekly *versus* three times weekly used for epoetin alfa. Its use was approved by the FDA and European Medicines Agency (EMEA) in 2001 for the treatment of anaemia associated with CKD and expanded in 2002 to include treatment of anaemia caused by chemotherapy used to treat some types of cancer. Because some clinical trials have shown that Epoetin- and darbepoetin-treated cancer patients

[1] http://www.ncbi.nlm.nih.gov/gene/2057

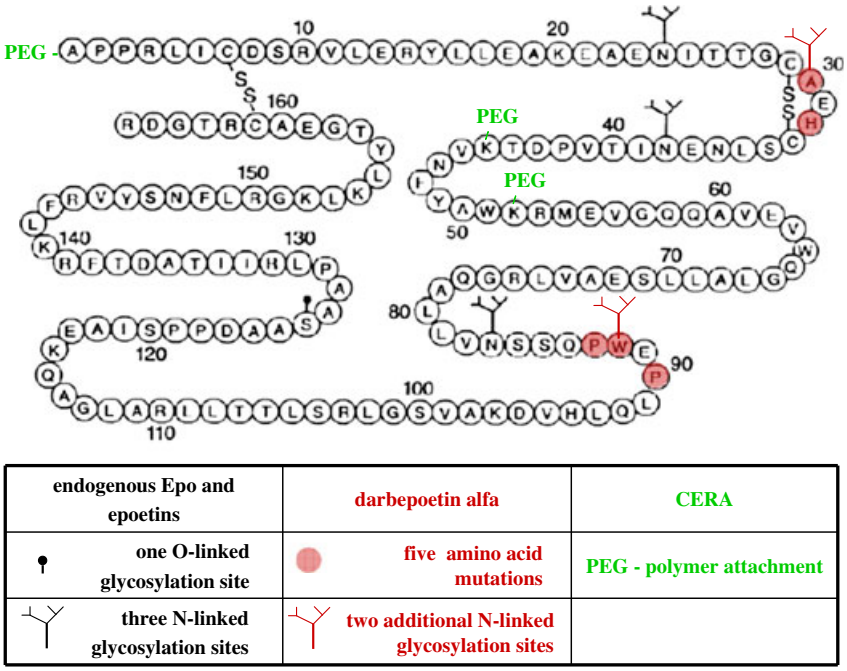

Figure 1. Primary structure of endogenous EPO/epoetin and modifications in darbepoetin alfa and methoxy polyethylene glycol-epoetin beta (CERA) structure. Adapted with permission from Romanowski and Sytkowski.[108]

may risk more rapid progression of their disease, the use of all ESAs in cancer patients has been curtailed markedly.[42]

Continuous erythropoiesis receptor activator (CERA) is pegylated epoetin beta containing a single methoxy polyethylene glycol (PEG) group. After production of epoetin beta by CHO cells, a 30 kDa PEG polymer is chemically bound principally to N-terminal amino acid or K52 or K45 resulting in a molecule of ~60 kDa[69] (Figure 1). This results in a lower receptor affinity, a 7-fold longer half-life and increased *in vivo* activity. It can be administered at longer intervals than darbepoetin, as infrequently as once monthly.[70] It was approved by the FDA and EMEA in 2007 for the treatment of anaemia associated with CKD. Due to patent issues, it is not currently marketed in the United States.

Differences between endogenous EPO and ESAs

As either the human EPO cDNA or genomic sequence is used for the production of rHuEPO, its amino acid sequence, position of disulfide bonds and secondary protein structure are the same as of endogenous EPO.[71] Furthermore, their glycosylation structures are similar except for the slightly greater number of sialic acids in rHuEPO molecule.[72,73] Both endogenous EPO and pharmaceutical rHuEPO are groups of EPO isoforms with differing glycosylation (glycoforms) (Figure 2).[74] EPO's glycosylation pattern can be analyzed by several methods, like isoelectric focusing (IEF-PAGE), SDS-PAGE, and Sarcosyl-PAGE, all using the anti-EPO monoclonal antibody AE7A5.[12] IEF-PAGE analysis allows separation of isoforms according to their isoelectric point. IEF combined with double immunoblotting is a direct detection method that can effectively differentiate endogenous and recombinant EPO isoforms due to subtle differences in sialylation, both in serum and urine.[11,75–77] This method has been accepted internationally for use in detecting misuse of ESAs in sport. The window in which the misuse of ESA can be detected depends on the ESA used and its specific administration protocol (dosage, frequency).[78] Biosimilars and copy epoetins can also be detected with specific ELISA (detection of CERA),[15] SDS-PAGE (detection of epoetin delta and copy epoetins), Sarcosyl-PAGE (detection of CERA, epoetins and serum EPO)[16] and MAIIA (recombinant and endogenous EPO).[79] Several doping detection methods are described in detail in a special article(s) of this issue.

AE7A5: A monoclonal antibody to human erythropoietin used in anti-doping control

The Lasne and de Ceaurriz report[11] of a sensitive double immunoblotting assay to detect EPO in urine and its adoption as the standard method to identify misuse of EPO in sport has brought the monoclonal antibody AE7A5 into prominence and has resulted in some debate about its specificity and usefulness.

AE7A5 was developed as part of a research effort by Sytkowski and his colleagues to use site-specific antibodies to study the structure of human EPO before the crystal structure was known. Using a partial amino acid sequence of the amino terminus of EPO, Sue and Sytkowski prepared rabbit anti-peptide antibodies and showed that they could immunoprecipitate both radiolabelled EPO and biologically active EPO.[80] Furthermore, the antibodies did not neutralize the bioactivity in an *in vitro* assay, leading the authors to conclude that EPO's amino terminus was exposed on the surface of the protein and was not part of the receptor-binding domain.

Using this same synthetic peptide sequence, a series of BALB/c mice were immunized over a six-month period, and fusions of their spleen cells with P3X63Ag8.653 myeloma cells were carried out. Upon screening the supernates from colonies derived from the 30th fusion, designated 'Fusion AE', supernate from well 7A5 was found to have a high titre of anti-peptide reactivity in an ELISA. The cells were recloned and all sub-clones were positive against the peptide. Antibody AE7A5 was shown to immunoprecipitate radiolabelled human EPO and biologically active EPO from human urine and rat plasma.[12] Later, AE7A5 was found to be highly effective in western blotting, a fact that has led to its use today in testing for EPO misuse in sport.

Table 1. List of some commercially available pharmaceutical ESAs (modified from Španinger and Debeljak [74]). The approval date by US Food and Drug Administration (FDA) and European Medicines Agency (EMEA) is indicated (* withdrawn ESA). Other ESAs are being marketed outside the USA and Europe. Novel ESAs in clinical trials (Ct) and preclinical trials (Pt) are indicated

INN	EXPRESS. SYSTEM	MODIFICATION	TRADE NAME (PRODUCER)	FDA	EMEA
Epoetin alfa (α)	CHO		EPOade® (Sankyo)		
			EPOgen® (Amgen)	1989	
			EPOpen® (Esteve)		
			EPOxitin® (Janssen-Cilag)		
			Eprex® (Ortho biologics)	1999	?
			Erypo® (Janssen-Cilag)		?
			Espo® (Kirin)		
			Globuren® (Cilag)		
			Procrit® (Ortho Biotech)	1989	
			Epoetin Alfa Hexal (Hexal AG)		2007
			Binocrit (Sandoz)		2007
			Abseamed (Medice Arzneimittel Pütter GmbH & Co KG)		2007
			EPOstim (Reliance GeneMedix Plc)		2011*
Epoetin beta (β)	CHO		EPOch® (Chugai)		
			EPOgin® (Chugai)		
			Eritrogen® (Roche)		
			Erantin® (formely marked by Boehringer Mannheim)		
			Marogen® (Chugai)		
			Neorecormon® (Roche)		1997
			Recormon® (Roche)		
Epoetin theta (θ)	CHO		Ratioepo® (Ratiopharm GmbH)		2009*
			EPOratio® (Ratiopharm GmbH)		2009
			Biopoin® (CT Arzneimittel GmbH)		2009
Epoetin kappa (κ)	CHO				
Epoetin zeta (ζ)	CHO		Retracrit® (BIOCEUTICALS Arzneimittel AG)		
			Silapo (Stada Arzneimittel AG)		2007
			Retacrit (Hospira UK Limited)		2007
Epoetin omega (ω)	BHK		EPOmax® (formerly marketed by Elanex)		
			Hemax® (formerly marketed by Elanex)		
Epoetin delta (δ)	HT-1080		Dynepo® (formely marked by Aventis Pharma)		2002*
Methoxy polyethylene glycol-epoetin beta (CERA)	CHO	EPO with attached methoxy polyethylene glycol polimer	Mircera® (Roche)	2007	2007
Darbepoetin alfa	CHO	mutated EPO with two additional N-linked glycans	Aranesp® (Amgen)	2001	2001
EPO mimetic peptides			Hematide (Affymax)	Ct II/III	
			Mimetibody (Janssen Pharmaceutica)	Ct I	
EPO fusion proteins				Ct I	
HIF-stabilizers				Ct I	
GATA-antagonists				Pt	
EPO gene doping				Ct I	

Recent developments and trends

Biosimilars and copy epoetins

Since the patents for the original epoetin alfa have begun to expire, several biosimilars have been approved and have emerged in Europe, the Americas, Asia, and Africa, including non-regulated copied rHuEPOs. EPO patents are still in force in the United States. Several epoetin alfa and other biosimilars are being produced using CHO cells, including epoetin alfa, kappa and zeta (Table 1).

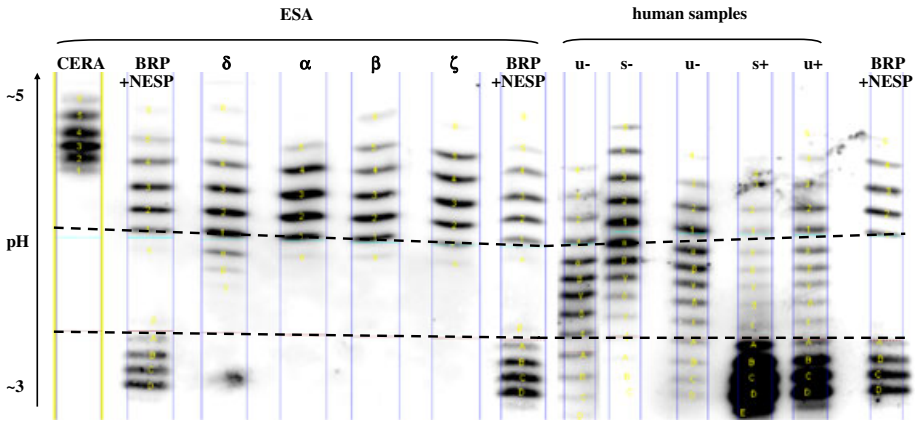

Figure 2. Isoelectric focusing analysis of ESAs (methoxy polyethylene glycol-epoetin beta, BRP + novel erythropoiesis stimulating protein, epoetin delta, alfa, beta, zeta) and human samples (serum, urine); containing only endogenous EPO (u-, s-) or undergoing darbepoetin treatment (s+, u+). BRP is a reference mixture of epoetin alfa and beta. Dotted line indicates the endogenous area. Modified with permission from Španinger and Debeljak.[74]

One problem with biosimilars is that they keep the same designation according to the cell line used, even if the product has a different glycosylation pattern due to differences in expression efficiency or purification. Therefore, the assumption that the bioactivity and other properties of biosimilars is the same as in the originator is problematic.[81]

EPO mimetic peptides (EMP)

EPO mimetic peptides are synthetic peptides that stimulate erythropoiesis in animals and humans. They are described in detail in a special article of this issue.

Hematide™ or peginesatide is a dimer of a 20-amino-acid-long synthetic cyclic peptide, conjugated to PEG polymer, currently in clinical trials for treatment of anaemia associated with CKD and cancer.[82] Erythropoietin Mimetic Antibody Fusion Proteins CNTO 528 and CNTO 530 are IgG linked EMP; CNTO 528 has undergone a Phase I clinical trial.[83,84]

EPO fusion proteins

Numerous approaches resulting in the increased molecular mass of the hormone have been undertaken with the aim of developing a long-acting erythropoietin; including fusion of the N-terminal rHuEPO to a human albumin gene[85] or to the Fc part of a human IgG molecule[86] and fusion of the C-terminal rHuEPO to the C-terminal peptide of human chorionic gonadotropin beta-subunit.[87] The preparation of chemically cross-linked human rHuEPO dimers and trimers[88] or a fusion protein cDNA encoding two EPO sequences with flexible polypeptide linker[89] resulted in molecules with greatly increased half-life. Fusion proteins containing EPO and other growth factors such as GM-CSF and IL-3 have also been prepared.[90,91] Furthermore, several EPO fusion proteins have been designed with the aim of increasing the cell type specificity of EPO action, for example, improved red blood cell specificity or blood–brain barrier transfer.[92,93]

Endogenous EPO gene activators

Several EPO gene expression regulators that enhance endogenous EPO production are in development that can be administered orally. Normally, the expression of endogenous EPO is induced by tissue hypoxia; hypoxia-inducible factor (HIF) is the main transcriptional factor.[23] This factor is composed of two subunits, an oxygen-sensitive HIF-alfa subunit and a constitutively expressed HIF-beta subunit. HIF levels are regulated by the HIF-alfa subunit hydroxylation of its prolylhydroxylase domains (PHDs) and degradation, resulting in down-regulation of EPO expression.[94,95]

HIF-stabilizers are chemical substances that stabilize HIF-alpha transcription factor, mimicking the hypoxic response, resulting in up-regulation of EPO gene expression and erythropoiesis. Several classes of PHD enzyme inhibitors are currently in clinical trials for the development of small molecule-based therapeutics for the treatment of anaemia. Cobalt and nickel are well-known EPO inducers but were withdrawn from clinical use due to toxicity.[96] That would explain why they are not included on WADA's prohibited list. As alfa-ketogluturate is required for PHD activity, several alfa-ketogluturate competitors are being tested.[97] One such molecule, FG-2216, has been tested in a Phase I clinical trial.[98]

GATA-antagonists are non-peptidic organic compounds preventing inhibitory GATA-2 from suppressing the EPO promoter. Several compounds have been tested on animal models including diazepane derivatives K-7174 and K-11706.[99]

EPO gene doping (gene transfer)

An adeno-associated virus (AAV) vector carrying the mammalian EPO gene was used for induction of EPO expression *in vivo* in macaques, mice and cats.[100–102] Severe anaemia developed in many macaques due to an auto-immune reaction.[103] Using a different expression system, a controlled long-term EPO expression was achieved in rhesus monkeys.[104] The use of a vector in which murine EPO expression was directed by an Oxford Biomedica hypoxia response element (OBHRE-EPO) also resulted in a long-term reversal of chronic anaemia in mice.[105]

In humans, EPO gene therapy has been performed *ex vivo* with transfection of dermal cores with an EPO cDNA-CMV promoter-containing vector. Implantation of tissue back into CKD patients resulted in successful transient expression and increased serum EPO levels for up to two weeks.[106]

Human gene doping is still under development; however, it is important to stress that Lasne *et al.* have already proven that gene doping is detectable. The cell type has crucial effects for the characteristics of EPO isoforms even when produced *in vivo*, as the isoelectric pattern of EPO produced by gene-doping in skeletal muscle is different from naturally occurring renal EPO.[107]

Future perspectives

Recombinant EPO has enjoyed great success as a treatment for various forms of anaemia. Moreover, the future likely holds an increasing number of additional applications for the hormone and further commercial success. Given these facts, there continue to be numerous efforts to improve on its pharmaceutical properties and to develop novel replacement products. In most cases, these efforts have emphasized the reduction in frequency or complete elimination of intravenous or subcutaneous injections of the hormone. Fewer injections have been and will continue to be accomplished by prolonging the molecule's *in vivo* half-life and/or by creating slow- or continuous-release formulations. The total absence of injections requires different means of administration of the EPO product or of molecules that can mimic EPO's biological function. As we have seen in this review, such approaches have included new formulations, enhanced glycosylation, alternatives to glycosylation like PEGylation, oligomers and fusion proteins, EPO-mimetic agents including peptides, agents that inhibit HIF-alfa degradation and gene therapy. Each of these approaches has obvious advantages and disadvantages. And all must be judged in light of their safety profiles and how well they can compete with the growing number of biosimilars both in therapeutic efficacy and in cost. The next decade is likely to be an interesting one.

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