

Recombinant EPO production—points the nephrologist should know

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Why should the nephrologist be familiar with EPOs and biosimilars?

The administration of recombinant human erythropoietin (rhEPO) and its analogues provides enormous benefit in the prevention and reversal of anaemia in chronic kidney disease (CKD), malignancy and AIDS, and it supports autologous blood collection. rhEPO-type substances represent the largest market of a class of biopharmaceuticals, with global estimated sales of $\sim 10^{10}$ euro *per annum*. The key process patents for the first-generation of rhEPOs have expired in the EU and other regions, opening the market for biosimilar follow-on products. In addition, confronted with imminent patent expiration, the manufacturers of the innovative compounds have developed second-generation products with improved pharmacokinetic properties. Thus, the clinician will be faced with a panoply of different erythropoiesis-stimulating biopharmaceuticals, and it will be important for him to be familiar with their molecular biology.

The complexities of EPO formation in mammalian cells

rhEPO is produced with the use of cells transfected with either the human *EPO* gene or EPO cDNA (the coding sequence of the gene) linked to an expression vector ('recombinant DNA'), which are integrated into the genome of the host cell and stably expressed over time. The present therapeutic rhEPO preparations are manufactured in mammalian host

cells, because EPO is a complex glycoprotein of 165 amino acids to which four glycans (carbohydrate side chains) are attached. Transfected bacteria such as *Escherichia coli* are useful as hosts for the production of non-glycosylated recombinant proteins (Figure 1). Yeast and filamentous fungi glycosylate proteins differently from mammalian cells, adding for example high mannose-type N-glycans. The synthesis of human N-glycans is a complicated process. It starts in the cytosol with the addition of distinct sugar molecules to dolichol, from which the glycan is transferred to the growing polypeptide in the endoplasmic reticulum. After several sugars have been trimmed from the protein, it is folded and moved to the Golgi complex, where further mannose elimination occurs before N-acetylglucosamine, galactose and sialic acid (*N*-acetylneuraminic acid) are finally added.

The nomenclature of EPO type substances

With respect to pharmaceutical glycoproteins the WHO identifies the group of substances with a stem, e.g. for EPO type substances: '-poetin' [1]. Eucaryotic cell-derived rhEPO, whose peptide core is identical with that of human urinary EPO [2] is termed 'epoetin'. Changes in the amino acid sequence are indicated by a different prefix (e.g. 'darbepoetin'). Analogues of a given EPO type substance with an altered glycosylation pattern due to production in a different host cell system are classified by a Greek letter added to the name ('epoetin- ω ' vs. 'epoetin- α '). Like endogenous EPO, epoetins possess 3 tetra-antennary N-linked (Asn 24, 38 and 83) and 1 small O-linked (Ser 126) glycans, which exhibit microheterogeneity as demonstrated on electrophoresis, isoelectric focusing, mass spectrometry and NMR spectroscopy [3–5]. Carbohydrates amount to 40% of the total molecular mass of EPO (30.4 kDa). The survival of EPO in circulation requires the presence of terminal sialic acid residues of its N-glycans. Asialo-glycoproteins are rapidly cleared via galactose-receptors of hepatocytes [6]. Epoetins contain up to 14 sialic acid residues per molecule. It is unlikely that sialic acid is removed from blood-borne

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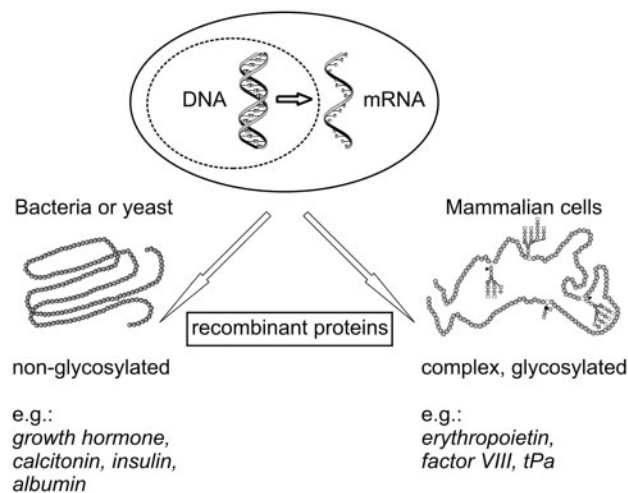


Fig. 1. Host cell suitability for production of non-glycosylated vs glycosylated biopharmaceuticals. The glycosylation pattern of glycoproteins is species-dependent and thus differs between Chinese hamster (genus: *Cricetulus*) CHO, Syrian hamster (*Mesocricetus*) BHK and human cells.

EPO to a major degree, a process which would require action of a neuraminidase.

Differences between current epoetins

In principle, rhEPO can be expressed in any mammalian cell efficiently transfected with the *EPO* gene, but the structure of the glycans and, hence, the *in vivo* activity of the products, differ to some extent [7,8]. Chinese hamster ovary (CHO) cells deficient in the dihydrofolate reductase gene are most commonly used for the large-scale pharmaceutical manufacture of glycoproteins, because gene amplification can be achieved by co-selection in the presence of methotrexate in these cells [9]. The CHO cell-derived products epoetin- α (Epogen[®], Procrit[®], Eprex[®], Erypo[®], Espo[®], etc.) and epoetin- β (Recormon[®], NeoRecormon[®], Epogin[®], etc.) have been clinically used for ~20 years. Clearly, the amino acid sequence of epoetins is identical with that of endogenous EPO, but the glycans exhibit structural and biophysical differences. Epoetin- α is more homogenous and possesses less basic isoforms than epoetin- β [4]. In humans, the plasma half-life of epoetin- α was measured to be shorter than that of epoetin- β by some [10], but not all investigators [11]. Overall, it seems fair to state that the two established products are clinically equivalent.

As to the fine chemical structure of the glycans of the epoetins, their composition depends on the host cell, the transfected plasmid, the culture conditions and the purification process. Accordingly, epoetin- α formulations (Epogen[®], Eprex[®]) from established manufacturers exhibit minor differences in thermal stability, local amino acid environments and the presence of high-molecular aggregates [12]. Thus,

despite their identical INN (International Nonproprietary Name), even these closely related drugs are not identical. Care must be taken that the quality of marketed biopharmaceuticals is not altered when (i) the drugs are manufactured in a new facility, (ii) a new working cell bank is established, (iii) the fermentation process is altered, (iiii) new products, e.g. stabilizers, are introduced or (v) the formulation is changed. Here, an example was the transiently higher incidence of antibody formation towards an epoetin- α formulation (Eprex[®]) in prefilled syringes with uncoated rubber stoppers [13]. The probability of an antibody reaction to therapeutic proteins increases as a consequence of structural differences compared with the endogenous prototype (primary structure and posttranslational modifications), of aggregation of the product or of impurities in the formulation. Pre-clinical studies do not provide reliable information on the immunogenicity of biopharmaceuticals. Furthermore, it is almost impossible to demonstrate an increased immunogenicity of new erythropoietic recombinant drugs in clinical trials. Even during the 'epidemic' phase of antibody-associated pure red cell aplasia (PRCA), the incidence of this immunogenic reaction was very low (18 cases per 100 000 patient-years for the Eprex[®] formulation) and the median duration of treatment before PRCA was diagnosed was as long as 9 months [14].

Novel epoetins and considerations on their potential safety

Since the patents for epoetin- α and epoetin- β have expired recently in the EU and elsewhere, other manufacturers will bring copied products ('biosimilars', 'follow-on biologics') on the market. The European Medicines Agency (EMA) has made efforts to establish guidelines [15] for the development and approval of biosimilar medicinal products containing biotechnology derived proteins, which differ from the regulations for generic chemically defined drugs [16]. According to a recently published supplement referring to EPO, the clinical efficacy and safety of biosimilar rhEPO preparations should be demonstrated in at least two adequately powered, randomized, parallel group clinical trials in comparison to a reference product [17]. Potential differences between biosimilar glycoproteins such as rhEPO and the originator compounds may have implications on immunogenicity, pharmacokinetics and purity, because the transgene, the host cell line, the culture conditions and the purification procedures applied by a follow-on manufacturer cannot be the same as the original. For example, significant cytogenetic variability of CHO cell lines has been documented [18]. Also, the manufacturing process cannot be exactly copied, because important details of the original production process are not in the public domain (Table 1). In addition, safety data for biopharmaceuticals can only come from

Table 1. Production steps influencing product

Establishment of 'Master cell bank'
Sequence of cDNA
Type of vector/plasmid
Accessory DNA elements (promoter, etc.)
Type of host cell
Technique of transfection
Propagation of host cell clone
Establishment of 'Working cell bank'
Maintenance of production cultures
Composition of culture medium
Type of culture vials/bottles
Type of fermenter/bioreactor
Extraction and purification of recombinant product from culture medium
Analysis of product
Formulation

clinical experience and post-marketing surveillance. Physicochemical and functional investigations of copied CHO cell-derived epoetins produced and marketed outside the EU and North America have revealed major isoform differences, batch-to-batch variations in biological activities, as well as endotoxin contamination of some of the products [19].

In some Eastern European, Asian and South American countries, CKD patients were successfully treated with a different rhEPO product (epoetin- ω ; Epomax[®], Hemax[®]), which was expressed in baby hamster kidney (BHK) cells. The amino acid sequence of epoetin- ω is unaltered. However, the expression of glycosyl transferases and glycosidases is species-dependent. In contrast to the CHO cell-derived epoetins, epoetin- ω has an N-glycan with phosphorylated oligomannoside chains and it possesses less O-glycans [20,21].

Another recombinant product newly marketed in the EU is epoetin- δ , which is homologously expressed on gene-activation in a human fibrosarcoma cell line (HT-1080 derivative), into which a DNA sequence was inserted that contains a powerful viral promoter (CMV promoter) to activate the *EPO* gene. Thus, epoetin- δ derives from an endogenous human *EPO* gene that is switched on from its dormant state [22]. Details of the structure of epoetin- δ have not been published. However, an investigation of the N-glycans of another rhEPO of human host cell origin (lymphoblastoid RPMI 1788 cells) has revealed structural differences when compared with human urinary EPO [23]. Epoetin- δ possesses less *N*-glycolylneuraminic acid residues (Neu5Gc) than CHO cell-derived epoetins [24]. This difference is probably not of major relevance with respect to immunogenicity, because the Neu5Gc content of CHO cell-derived epoetins is very low (0.08%) [25]. In contrast to other mammals, humans are genetically unable to produce Neu5Gc [26]. All normal humans have circulating antibodies against Neu5Gc, which are raised against Neu5Gc in nutrients [26,27]. After all, neutralizing anti-rhEPO antibodies are not usually directed

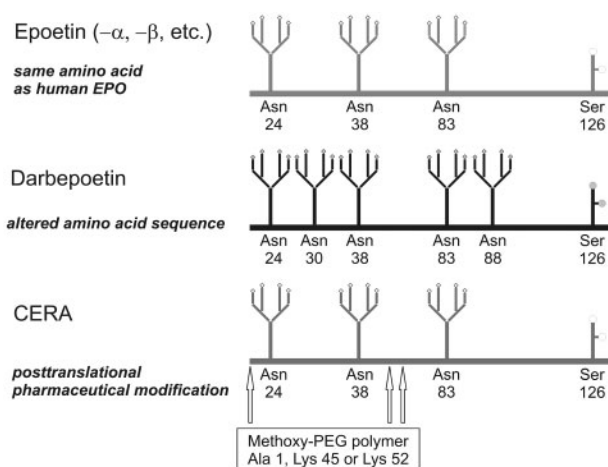


Fig. 2. Scheme of epoetin (EPO), darbepoetin and CERA (pegylated epoetin- β) and their glycosylation, respectively pegylation sites. While the amino acid sequence of all epoetins (α , β , ω , δ , etc.) is the same, they differ in the structure of their glycans and, thus, isoforms. Darbepoetin- α differs in five amino acids from epoetin and has two additional N-glycans.

against the carbohydrate portion of the molecules. Epoetin- δ is effective in increasing haemoglobin levels in predialysis [28] and dialysis [29] patients with CKD.

EPO congeners

The rhEPO mutein (a product with altered amino acid sequence) darbepoetin- α (Aranesp[®]) was approved as an anti-anemic drug 6 years ago. Darbepoetin- α has an increased molecular mass (37.1 kDa) and contains an increased proportion of carbohydrate (51%) compared with epoetins. The amino acid sequence of darbepoetin- α is modified through site-directed mutagenesis at 5 positions, resulting in 2 additional N-glycans at novel asparagine residues in positions 30 and 88 (Figure 2) [30,31]. Darbepoetin- α has a lower affinity for the EPO receptor (EPO-R) but a longer survival in circulation, because EPO-R mediated uptake by target cells, followed by intracellular proteolysis, is probably a major mechanism of the degradation of EPO [32]. The terminal half-life of i.v. administered darbepoetin- α is three to four times longer than that of epoetin- α or epoetin- β (25 vs 6–9 h) [33], which allows for less frequent application. The questions of dose requirements and costs of epoetin- α or epoetin- β vs darbepoetin- α have remained a matter of debate [34].

Pegylated epoetin- β (CERA, 'continuous erythropoiesis receptor activator'; Ro 50-3821), which is currently tested in clinical trials, has an even longer half-life (130–140 h) than darbepoetin- α on i.v. injection [35]. CERA contains a single methoxy-polyethylene glycol (PEG) polymer of ~30 kDa, integrated via amide bonds with the amino groups of either the alanine in position 1 or one of the

lysines in positions 45 or 52 of EPO. Details of the metabolic fate of CERA in humans have not yet been reported, but prolonged survival in the circulation is probably in part due to the large molecular mass (~60 kDa) and the low EPO-R binding affinity.

The use of conjugation with PEG has recently been extended beyond rhEPO. A phase 1 study has shown the erythropoietic stimulating potential of a novel agent ('Hematide'), which is composed of a synthetic (non-recombinant) dimeric EPO mimetic peptide linked to PEG [36]. Since Hematide has been proven to induce reticulocytosis and to increase haemoglobin levels in healthy volunteers, single and repeat dose phase 2 studies in patients suffering from CKD or cancer have been initiated [36]. The variety of compounds in pre-clinical studies or clinical trials is widened by the recent production of synthetic (SEP) and recombinant erythropoietic compounds, which are complexed with peptidic or non-peptidic polymers by chemical ligation or recombinant techniques [37].

Conclusion

Developments in the manufacture of erythropoietic biopharmaceuticals should aim at improving the efficacy and pharmacokinetics of the drugs and to reduce their costs. Technically, changes can relate to differences in the plasmids (site-directed mutagenesis of the target gene, type of promoter, marker genes, etc.), the transformed host cell (CHO, BHK, human lines), the production process (type of fermenter and culture medium), the purification steps, post-translational modifications (addition of PEG or other polymers) and the way of formulation. Unintended effects can occur on manufacturing changes, in particular increased immunogenicity. Critical issues include the absence of host cell DNA and proteins, the lack of aggregates in the products and integrity of the glycans. Eventually, the safety of novel biopharmaceuticals can be proven only by clinical experience.

Conflict of interest statement. The author has served on an Amgen advisory board and received honoraria from Amgen, BioPartners, Hoffmann-La Roche, Johnson and Johnson/Ortho Biotech and Shire for medical education lectures and consultations.

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Angiogenic factors in preeclampsia: so complex, so simple?

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Introduction

Preeclampsia is a common cause of fetal and maternal morbidity and mortality, that affects ~2–7% of all healthy nulliparous women [1]. Until recently, the

pathophysiology of preeclampsia was not well understood. Successive hypotheses have been proposed, each being challenged by subsequent publications. The current most plausible hypothesis involves abnormal placentation leading to placenta ischaemia [2]. Recently, ischaemic trophoblast cells [3] were shown to synthesize anti-angiogenic factors, notably the soluble form of fms-like tyrosine kinase-1 (sFlt-1), which is a receptor for vascular endothelial growth factor (VEGF). In addition, sFlt-1 mRNA is highly expressed in the placenta from preeclamptic patients, and administration of sFlt-1 induces a preeclampsia-like syndrome in pregnant rats [4]. Therefore, sFlt-1 could be the link between implantation disorders and maternal symptoms of preeclampsia.

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