

DYNEPO[®]: an erythropoiesis-stimulating agent with a difference?

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■ INTRODUCTION

In 2007 Shire Plc. launched epoetin delta (Dynepo[®]) in five European markets. It is approved for the treatment of symptomatic anaemia in adult patients with chronic renal failure. Epoetin delta is well tolerated and effective when given intravenously or subcutaneously to patients on dialysis or pre-dialysis, irrespective of dose-frequency; this has been documented in a series of clinical trials¹⁻⁶. Epoetin delta is the first and currently the only erythropoiesis-stimulating agent (ESA) that is produced in a human cell line. All other currently available ESAs including epoetin alfa, epoetin beta and darbepoetin alfa are derived from Chinese Hamster Ovary (CHO) cells.

■ HUMAN CELL PRODUCTION TECHNOLOGY

Human cell production of epoetin delta is accomplished through a proprietary technology called gene-activation. Hereby, a genetic switch that functions as a transcriptional activator is inserted in front of the endogenous EPO gene within the intact genome of the human producer cell. This switch overrides endogenous control of EPO gene transcription and enforces continuous activation of the EPO-gene, thus yielding continuous EPO protein expression (Fig. 1). In contrast, other ESAs are produced through recombinant gene technology, whereby the coding sequence for the 165 amino acids of the

human EPO gene (cDNA) is inserted into an expression vector which in turn permits random insertion of the human EPO cDNA into the genome of a CHO cell. This forces the hamster cell to produce a protein with the human EPO protein sequence^{7,8}.

■ SWEET TALK – THE IMPORTANCE OF GLYCOSYLATION

Human cell line derived epoetin delta and CHO-derived ESAs such as epoetin alfa or beta thus share the same human amino acid sequence of the human EPO protein. The amino acid sequence of darbepoetin alfa is slightly modified compared to endogenous human EPO cDNA at five amino acid sites, which creates two additional N-glycosylation sites and allows for the hyperglycosylation of this molecule⁹. However, these ESAs differ in that post-translational modifications are provided by human cells for epoetin delta but hamster cells for CHO-derived ESA. Post-translational modifications encompass all processing steps that the nascent protein is subjected to once the polypeptide chain synthesis is completed. They occur in a range of cytoplasmic compartments, including the endoplasmic reticulum or the Golgi apparatus and in the cytoplasm itself and include phosphorylation, acetylation, ubiquitination and glycosylation amongst others. More than 5% of the human genome encodes for enzymes dedicated to ensuring accurate and complete post-translational modifications, which are critical in influencing the functionality of a protein¹⁰.

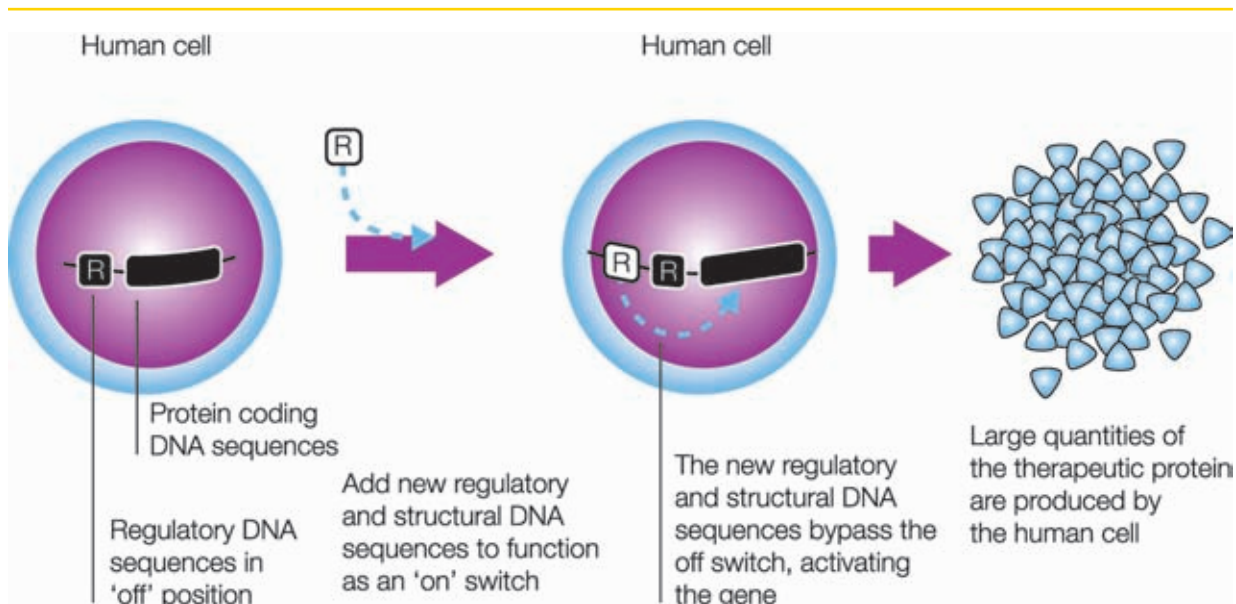


Figure 1

Gene-activation technology to facilitate production of Erythropoietin in a human cell. A transcriptional activator is inserted in front of the endogenous Epo-gene within the intact genome of the human cell. This activator overrides endogenous transcriptional regulation of the Epo-gene and causes continuous production of Epo by the cell.

For erythropoietin, glycosylation is of particular interest among post-translational modifications as the protein carries a total of four glycosylation sites, three N-glycosylation sites and one O-glycosylation site, and about 40% of both the natural as well as recombinant EPO molecular weight is made up of carbohydrate residues^{11,12}. In general, glycosylation of proteins is a complex and tightly regulated cell, site and protein specific process. Moreover, glycosylation is a species specific process, whereby human and non-human mammals differ in their repertoire of glycosylating enzymes and hence in the structure and design of carbohydrate residues^{13,14}. At the cellular level glycosylation influences cell differentiation, homing to specific tissues, cell adhesion and cell recognition. At the protein level glycosylation modifies functionality and provides orthogonal functions; it influences binding of proteins to their receptors as well as signaling cascades¹⁵. Glycosylation is also involved in pathological processes, it influences microbial pathogenesis and immunological recognition, congenital defects in proper glycosylation are associated with severe debilitating diseases¹⁶⁻¹⁹. Defined changes in the glycosylation pattern of self-proteins initiate

inflammatory and immune responses and faulty glycosylation processes are even implicated in the genesis of autoimmune diseases, such as rheumatoid arthritis^{16,20,21}. In relation to therapeutic proteins, glycosylation has been linked to changes in pharmacokinetics, clearance and bioavailability as well as immunogenicity of such products^{22,23}.

■ UNDERSTANDING IN VIVO IMPLICATIONS OF GLYCOSYLATION DIFFERENCES ACROSS ESA

Currently, our understanding as to how glycosylation might influence the properties of ESAs is very limited. The efficacy profile of epoetin alfa, beta and delta, as measured in their ability to promote erythropoiesis in patients with anaemia, appears similar. This, however, should be expected as these ESAs are standardized by the international unit (IU) definition, which is defined by the ability of each agent to promote erythropoiesis in a standardized experimental system. Any glycosylation-mediated differences in erythropoietic

activity that these ESAs may or may not have would be reflected in their respective specific activity but is neutralized through the IU definition. Known variations in the glycosylation profile lead to differences in half-life and bioavailability, with hyper-glycosylated darbepoetin alfa, which carries two additional N-linked carbohydrate residues offering the longest half-life²⁴. Any glycosylation-mediated differences in extra-erythropoietic activities across all available ESAs have not yet been systematically investigated.

At the molecular level, differences in half-life and bioavailability can be traced back to carbohydrate-mediated modifications of the binding affinity of these ESAs to the EPO-Receptor (R). For example, asialo-erythropoietin, which is deprived of all sialic acids, and normally glycosylated CHO-derived epoetin, share similar affinity to the EPO-R and similar potency in promoting proliferation of erythropoietic cells *in vitro*²⁵. In contrast, hyperglycosylated darbepoetin alfa has lower affinity to the EPO-R and is less potent *in vitro* in promoting erythropoietic activity such as the proliferation of erythropoietic cells²⁴. *In vivo*, on the other hand, asialo-erythropoietin has a very short half-life as it is rapidly cleared by hepatocytes through the galactose-binding protein and fails to promote erythropoiesis^{25,26}. Darbepoetin alfa in contrast, survives longer in serum because its hyperglycosylation interferes with its ability to bind to the EPO-R and therefore its intracellular EPO-receptor mediated uptake and degradation is reduced²⁷. Because of its very long half life it promotes erythropoiesis when given at lower frequency^{7,24,25}.

In the clinic, the glycosylation driven difference has thus far translated only to a range of choices of different frequency of ESA administration. Yet, given the broad ranging functions of carbohydrate residues, the fact that they constitute 40% of the molecular weight of EPO and the broad extra-erythropoietic activities of EPO that have been uncovered in recent years, it seems unlikely that these might be the only tangible differences. The arrival of the first human cell line derived epoetin now permits the investigation of how other qualitative and quantitative differences between human and hamster-cell derived glycosylation may affect ESA functionality. Areas yet to be explored are the relative immunogenicity of these agents, their inflammatory profile and their extra-erythropoietic functionalities.

■ NON-HUMAN CARBOHYDRATE RESIDUES

CHO cells harbour a different set of glycosylating enzymes than human cells, consequently, the carbohydrate profile between human cell line derived and hamster cell derived EPO differs²⁸. Specifically, hamster and all other mammalian cells express CMP-N-acetylneuraminic acid hydroxylase, which is the rate-limiting enzyme generating the N-glycolylneuraminic acid (Neu5Gc) carbohydrate residue. In contrast, human cells lack a functional copy of this enzyme and do not produce this carbohydrate residue²⁹. Epoetin delta has no detectable Neu5Gc residue while CHO-derived products such as epoetin alfa, epoetin beta and darbepoetin alfa contain approximately 1% to 1.4% of Neu5Gc residues relative to the total sialic acid content³⁰. This is consistent with prior publications describing between 1% and 3% Neu5Gc in hamster-cell derived ESA and lower than the 7% reported for recombinant GM-CSF³¹⁻³³. The genetic mutation incapacitating humans to generate Neu5Gc occurred about 2.5 to 3 million years ago, preceding the first evidence of meat-eating in the homo lineage³⁴. Consequently, the residue became variably immunogenic for humans²⁹. All humans investigated have circulating antibodies against Neu5Gc^{34,35}. Whether the Neu5Gc content of CHO derived ESAs may attract circulating Neu5Gc antibodies to such ESAs and reduce their bioavailability, pharmacokinetic or functionality in select patients, such as those with higher levels of circulating Neu5Gc antibodies, is currently unknown.

Natural anti-carbohydrate antibodies against alien carbohydrate residues may cause hypersensitivity reactions. This is well understood for plant derived allergens, where carbohydrate chains of glycosylated pollen bind IgE antibodies and elicit the release of inflammatory cytokines such as IL-4 from basophils following cross-linking of IgE receptors³⁶. More recently, circulating anti-carbohydrate antibodies against non-human carbohydrate residues have been linked to hypersensitivity reactions against therapeutic proteins carrying such carbohydrate residues. For example, circulating IgE antibodies against the non human carbohydrate residue galactose-alpha-1,3-galactose, which may evolve following a tick bite, are associated with hypersensitivity reactions to cetuximab, a chimeric mouse-

human IgG1 monoclonal antibody against the epidermal growth factor receptor that is approved for use in colorectal cancer and squamous-cell carcinoma of the head and neck³⁷. Galactose- α 1,3 galactose is present on the Fab portion of the cetuximab heavy chain. In a study, most patients with hypersensitivity reactions to cetuximab had circulating IgE antibodies against galactose- α 1,3-galactose before being exposed to this drug. Similarly, IgE antibodies causing hypersensitivity reactions and treatment withdrawal have been identified in Fabry disease patients receiving hamster-cell derived agalsidase beta for enzyme replacement therapy³⁸. No IgE antibodies emerged in Fabry disease patients under long-term treatment with human-cell derived agalsidase alfa³⁹. Whether or not the presence of an ESA carrying non-human carbohydrate structures may constitute an inflammatory challenge to select patients remains to be investigated.

Natural anti-carbohydrate antibodies against alien carbohydrate structures are capable of priming the immune system to antigens decorated with the antigenic carbohydrate residues and directing an immune-response against soluble serum proteins⁴⁰. In patients receiving ESAs, such an immune response against the protein structure itself is known as a very rare but severe side-effect called Pure Red Cell Aplasia (PRCA). It results from the evolution of neutralizing antibodies targeting the protein core common to ESA as well as endogenous EPO and neutralizing all erythropoietic activity of ESA and any remaining endogenous EPO⁴¹. Whether any specific carbohydrate residues such as Neu5GC may contribute to the emergence of PRCA is unknown.

■ POTENTIAL FOR FUNCTIONAL DIFFERENCES BETWEEN CHO-CELL DERIVED ESAS AND HUMAN CELL LINE DERIVED EPOETIN DELTA

While ESAs derived from CHO cells and human cells both control anaemia (6-8) nothing is known as to how they may compare in their extra-erythropoietic activities. The plethora of functions of EPO on a wide range of tissues and organs were discovered only over the past few years⁴². Notably, these

include the ability of EPO to promote endothelial cell proliferation and angiogenesis, to repair and regenerate the kidney and to protect heart and brain against acute toxic damage. From a mechanistic perspective therefore the therapeutic effects of ESAs may extend far beyond the correction of anemia. Little is known, however, how such pleiotropic effects of EPO play out in the renal anaemia patient receiving ESA and whether and how ESAs may differ in their extra-erythropoietic activities as a function of their carbohydrate profile.

Currently, there is controversy on how the extra-erythropoietic effects of EPO are mediated. Some argue that a different EPO-binding structure, consisting of one EPO-Receptor and two beta common chain receptors, is responsible for EPO's tissue-protective effects^{43,44}. This is consistent with the notion that the binding affinity of non-erythropoietic cells to EPO differs from that of erythropoietic cells⁴⁴. How differential glycosylation of ESAs influences binding to such tissue-protective receptors is currently unknown. If one extrapolates experience with the classical homodimeric EPO-Receptor, in that glycosylation affects receptor affinity and subsequent biological activity²⁴, glycosylation-driven differences are to be expected to influence tissue-protective activities of ESA.

More importantly and along these lines, preliminary data do suggest that ESAs differ in their extra-erythropoietic functionality. Human cell line derived epoetin delta for example is less angiogenic than hyperglycosylated CHO-derived darbepoetin alfa in human microvascular endothelial cells at equivalent and therapeutic relevant doses⁴⁵. Excess angiogenesis might be helpful for a patient with cardiovascular disease, where new vessel growth in the heart might be wanted but harmful for a patient with diabetic retinopathy, where additional new vessel growth in the retina would be detrimental.

Studies like these should help to further our understanding how different post-translational modifications such as glycosylation influence therapeutic properties of ESA for the renal anaemia patient and help to select the most appropriate compound to meet an individual patient's need.

Conflict of Interest statement. Marion A. Howard is a Consultant to Shire Pharmaceuticals, Ltd.

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