Trends in Analytical Chemistry 68 (2015) 18–27

Contents lists available at ScienceDirect

Trends in Analytical Chemistry

journal homepage: www.elsevier.com/locate/trac

Technologies for glycomic characterization of biopharmaceutical erythropoietins

Serenus Hua [a,b], Myung Jin Oh [a,b], Sureyya Ozcan [a], Young Suk Seo [a,b], Rudolf Grimm [b,c], Hyun Joo An [a,b,*]

[a] Asia Glycomics Reference Site, Daejeon, Republic of Korea
[b] Graduate School of Analytical Science and Technology, Chungnam National University, Daejeon, Republic of Korea
[c] Agilent Technologies, Santa Clara, CA, USA

ARTICLE INFO

Keywords:
Biopharmaceutical
Biosimilar
Darbepoetin alfa
Epoetin
Glycan
Glycomic characterization
Glycopeptide
Glycosylation
Mass spectrometry
Recombinant erythropoietin

ABSTRACT

Glycosylation is one of the most critical factors affecting the quality, the safety and the potency of recombinant erythropoietin. Small changes during production can significantly affect glycosylation, and so the potency, of recombinant erythropoietin. Due to patent expirations, we expect biosimilar erythropoietins to play an increasing role in healthcare in coming years. Governmental regulatory agencies and biopharmaceutical companies therefore have an urgent need for reliable methods that can accurately characterize and evaluate these biological products, particularly in terms of their glycosylation. In this review, we provide an overview of current analytical tools for qualitative and quantitative analysis of erythropoietin glycosylation.

Contents

1. Introduction

Erythropoietin (EPO) is a highly glycosylated protein hormone that stimulates erythropoiesis, the production of red blood cells. Human EPO, produced industrially by recombinant DNA technology, is used medically for the treatment of anemia and illicitly as a performance-enhancing drug. Since its introduction in 1989, recombinant EPO has made tens of billions of dollars for its original developers – Amgen in USA and Roche in Europe. In recent years, many of the patents on recombinant EPO began to expire, and more will expire within the next few years [1,2]. These patent expirations, combined with rising healthcare costs and an aging worldwide population, have created a veritable explosion of biosimilar manufacturers looking to create and to market their own, generic versions of EPO [3–5]. In this rapidly evolving landscape, government regulatory agencies and industry quality-control (QC) laboratories have

* Corresponding author. Tel.: +82 42 821 8547; fax: +82 42 821 8541.
E-mail address: hjan@cnu.ac.kr (H.J. An).

http://dx.doi.org/10.1016/j.trac.2015.02.004
0165-9936/

a responsibility to develop and to implement new analytical methodologies and protocols for evaluating the safety and efficacy of EPO.

1.1. A brief history of recombinant EPO

The first recombinant EPOs entered commercial production in 1989 with epoetin alfa, marketed by Amgen as Epogen. Soon afterwards, epoetin beta, marketed by Boehringer Mannheim (and later Roche) as Recormon, was launched in 1990. In contrast to earlier biologics, such as recombinant insulins, which are produced by *E. coli* cells, the first recombinant EPOs were produced by Chinese hamster ovary (CHO) cells in order to imitate the glycosylation patterns of endogenous human EPO and thereby preserve the biological activity of the drug.

A decade later, the blockbuster success of biotherapeutic EPO (combined with looming patent expirations) prompted Amgen to develop a second-generation "biobetter" EPO. Recognizing the role that glycosylation plays in determining EPO circulation times (and by extension, bioactivity), researchers modified the amino-acid sequence of first-generation EPO to add two additional N-glycosylation sites (Fig. 1), while simultaneously increasing the levels of glycan sialylation and O-acetylation through bioprocess engineering [6]. The resulting "novel erythropoiesis-stimulating protein" (NESP) exhibited a 3–4-fold increase in plasma half-life over first-generation EPO, allowing smaller doses and less-frequent administration [7–9].

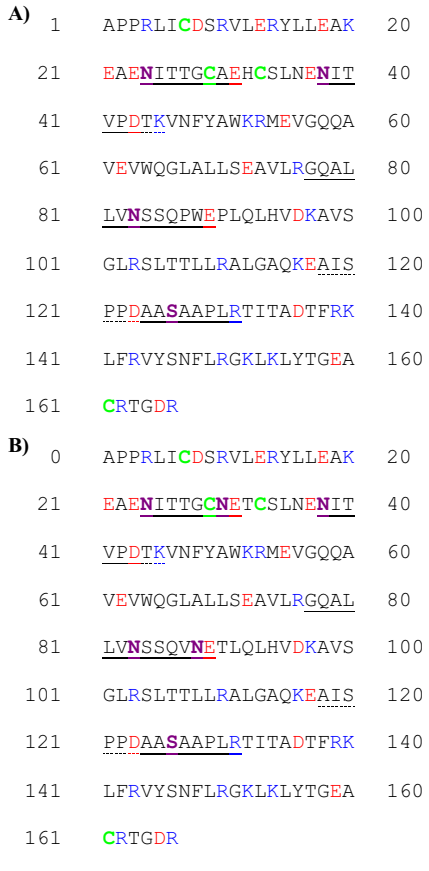

Fig. 1. Amino-acid sequences of A) first-generation epoetin, and B) second-generation darbepoetin alfa, with proteolytic cleavage sites annotated. Blue represents tryptic cleavage sites, red represents Glu-C cleavage sites, and bold green represents disulfide-bond locations. Glycopeptides resulting from trypsin/Glu-C cleavage are underlined. Solid underlines represent full digestions (with Glu-C cleavage after Asp) while dashed extensions represent incomplete digestions (without Glu-C cleavage after Asp).

From 2001 onwards, Amgen marketed its new product, darbepoetin alfa, under the trade name Aranesp.

Not long afterwards, patents for cloning and production of the original first-generation EPOs (epoetin alfa and epoetin beta) began expiring, enabling development of generic versions (biosimilars). In 2007, the first biosimilar epoetin alfa (marketed variously as Binocrit, Epoetin alfa Hexal, or Abseamed) was approved by the European Medicines Agency (EMA). Patent expirations also cleared the way for sales of novel first-generation EPOs, such as epoetin delta (Dynepo), which was advertised as the first EPO with fully human glycosylation due to the human fibrosarcoma cells used in its production. Since then, a multitude of first generation EPOs have been launched across Europe, Africa, Asia, and South America – though, notably, not in USA, where the Food and Drug Administration (FDA) has yet to develop a regulatory pathway for biosimilars.

Now, a quarter-of-a-century after the introduction of the first biopharmaceutical EPO, the next chapter in the history of EPO is unfolding. As patents for second-generation EPO (darbepoetin alfa) approach expiration in international markets, a flurry of biosimilar and biobetter development is taking place. A biosimilar darbepoetin alfa (marketed as Cresp) has already been released in India, and two more (Avdesp and Actorise) are currently in development. Biopharmaceutical companies are carrying out similar development for the Asian and European markets. However, demonstrating similarity (or dissimilarity) to this extraordinarily complex molecule is no easy task – and analytical scientists face a significant challenge in laying the analytical groundwork for upcoming generations of EPO biosimilars.

1.2. Pharmacokinetic considerations for analysis of EPO glycosylation

Endogenous EPO is a cytokine hormone with a compact globular form comprising four alpha-helical bundles connected by loops [10,11]. The amino-acid core structure represents about 60% (21 kDa) of its total molecular weight and is responsible for binding to the EPO receptor in order to stimulate erythropoiesis. The remainder (~40%) of the molecular weight of EPO is made up of various N- and O-glycan attachments that collectively mediate the circulation time of the protein [12–14]. Due to the non-template-driven nature of glycosylation, the distribution of EPO glycoforms is quite heterogeneous and varies significantly, depending on the specific growth conditions of the cells that produced them [15–17]. Fig. 2 and its caption summarize the types of glycans that might be found decorating first- or second-generation EPO. While the precise effects of these different glycans and glycosylation motifs are not all completely understood, *in-vivo* studies on glyco-engineered EPO variants have been able to pinpoint several glycosylation-associated critical quality attributes (CQAs) that significantly affect the safety and the efficacy of biopharmaceutical EPO.

Perhaps the most important CQA for EPO is its sialic-acid content. EPO molecules with exposed galactose residues are rapidly cleared from the bloodstream by galactose receptors in the liver. However, decoration of galactose residues with sialic acid blocks the action of the galactose receptors and extends EPO circulation time [18,19]. The protective effect of sialic acid can be further enhanced by O-acetyl modifications, which interfere with *in-vivo* enzymatic desialylation [20]. As a result, EPO manufacturers generally try to maximize the amounts of sialylation and O-acetylation on their products, adding as many as four sialic acids per N-glycan, two sialic acids per O-glycan, and two O-acetylations per sialic acid (Fig. 2). First-generation epoetin (with three N-glycosylation sites and one O-glycosylation site) can be decorated with up to 14 sialic acids and 28 O-acetylations per molecule, while second-generation darbepoetin alfa (with five N-glycosylation sites and one O-glycosylation site) can be decorated with up to 22 sialic acids and 44 O-acetylations