Current Topics

New Era of Glycoscience: Intrinsic and Extrinsic Functions Performed by Glycans

The Significance of Glycosylation Analysis in Development of Biopharmaceuticals

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Received October 24, 2008

Many glycoproteins and glycosaminoglycans are approved for clinical use. Carbohydrate moieties in biopharmaceuticals affect not only their physicochemical properties and thermal stability, but also their reactivity with their receptors and circulating half-life. Modification of glycans is one target of drug design for enhancement of efficacy. Meanwhile, there have been reports of serious adverse events caused by some carbohydrates. It is crucial to maintain the constancy of carbohydrate moieties for the efficient and safe use of glycosylated biopharmaceuticals. On the other hand, for scientific, safety-related, and economic reasons, changes in the manufacturing process are frequently made either during the development or after the approval of new biopharmaceuticals. Furthermore, the development of biosimilar glycoprotein products has been attempted by different manufacturers. Changes in pharmaceutical manufacturing processes possibly cause alteration of glycosylation and raise concerns about alteration of their quality, safety, and efficacy. In this review we provide some current topics of glycosylated biopharmaceuticals from the viewpoints of efficacy, safety, and the manufacturing process and discuss the significance of glycosylation analysis for development of biopharmaceuticals.

Key words glycoprotein; glycosaminoglycan; biopharmaceutical; efficacy; safety; manufacturing process

1. INTRODUCTION

Many biological molecules containing carbohydrates are approved for clinical use in Japan (Table 1). In the beginning, most therapeutic glycoconjugates were naturally occurring glycoproteins and glycosaminoglycans (GAGs) derived from human and healthy animal tissues, such as gonadotropins from human urine, and heparins from the porcine intestine. Recombinant glycoproteins, including erythropoietin and tissue plasminogen activator (tPA), have been developed as copies of native human glycoproteins since the early 1990s in Japan. In many cases their carbohydrate moieties were different from the original human ones. Carbohydrate moieties in glycoproteins affects not only their physicochemical properties and thermal stability but also their reactivity with their receptors and circulating half-life.¹⁾ Modification of carbohydrate moieties is one target of drug design to enhance the efficacy of the original ones. Meanwhile, there have been reports from around the world of serious adverse events caused by carbohydrate-related drugs. For the efficient and safe use of glycoprotein/GAG products, it is necessary to maintain the structures and heterogeneity of carbohydrate moieties in glycosylated biopharmaceuticals.

On the other hand, carbohydrate moieties in biotechnology-derived drugs are variable when the manufacturing process is changed. For scientific, safety-related and economic reasons, it has become common for companies to change the manufacturing process for their approved products. Furthermore, biosimilar glycoprotein products, which are manufactured by different companies, have been developing in many regions.^{2,3)} One of the main issues for the devel-

Table 1. Glycosylated Biopharmaceuticals in Japan

Origin	Japanese accepted name
Granulocyte-colony stimulating factor	Lenograstim
Granulocyte macrophage colony-stimulating factor	Mirimostim
Interferon	Interferon Alfa (NAMALWA), Interferon Alfa (BALL-1), Interferon Beta, Interferon Beta-1a, Interferon Gamma-n1
Erythropoietin	Epoetin Alfa, Epoetin Beta, Darbepoetin Alfa
Monoclonal antibody	Ibritumomab Tiuxetan, Basiliximab, Infliximab, Rituximab, Cetuximab, Gemtuzumab, Ozogamicin, Palivizumab, Tocilizumab, Trastuzumab, Bevacizumab, Adalimumab
Receptor	Etanercept
Follicle stimulating hormone	Follitropin Alfa, Follitropin Beta
Gonadotropin	Human Menopausal Gonadotropin, Human Chorionic Gonadotropin, Serum Gonadotropin
Factor VII	Eptacog Alfa (Activated)
Factor VIII	Octocog Alfa, Rurioctocog Alfa
Thrombomodulin	Thrombomodulin Alfa
Urokinase	Urokinase
Pro-urokinase	Nasaruplase
Tissue-plasminogen activator	Alteplase, Monteplase, Pamiteplase
Enzymes	Kallidinogenase, Agalsidase Alfa, Agalsidase Beta, Alglucosidase Alfa, Alglucerase, Idursulfase, Imiglucerase, Laronidase, Galsulfase
Heparins	Heparin Sodium, Heparin Calcium, Parnaparin Sodium, Dalteparin Sodium, Enoxaparin Sodium, Reviparin Sodium
Hyaluronate	Sodium Hyaluronate
Chondroitin sulfate	Chondroitin sulfate

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opment of biosimilar products is how to assure the similarity of carbohydrate moieties between the biosimilar products and the reference products.

In this review we provide some current topics of glycosylated biopharmaceuticals in terms of efficacy, safety, and manufacturing process and discuss the significance of glycosylation analysis in the development of biopharmaceuticals.

2. ROLE OF CARBOHYDRATES ON EFFICACY

Glycosylation in some biopharmaceuticals is crucial for their biological activity. Lysosomal storage diseases are characterized by deficiencies of lysosomal enzymes that degrade the glycoconjugates, such as mucopolysaccharides and glycolipids, and consequent cellular damages by their accumulated metabolites.⁴⁾ For use in enzyme-replacement therapy against lysosomal storage diseases, several recombinant glycoprotein products have been approved, namely *agalsidase alfa* and *agalsidase beta* for Fabry's disease, *alglucosidase alfa* for Pompe's disease, and *laronidase*, *idursulfase*, and *galsulfase* for mucopolysaccharidosis I, II, and VI, respectively.^{4–8)} These drugs contain *N*-linked oligosaccharides attached to mannose 6-phosphate (M-6-P), and the secreted enzymes are transported to an acidified prelysosomal compartment through the M-6-P receptor.^{9,10)} The carbohydrate residue is essential for lysosomal targeting and to exhibit complete enzyme activity in lysosomes. *Imiglucerase* is an analog of human β -glucocerebrosidase, which is used for the treatment of Gaucher's disease. This glycoprotein is produced by recombinant DNA technology and exoglycosidase treatment to expose mannose residues in *N*-linked oligosaccharides. The carbohydrate modification facilitates incorporation of this drug into macrophages through mannose-binding receptors.¹¹⁾ These recombinant lysosomal enzymes have achieved dramatic therapeutic effects against the lysosomal storage diseases.

Several human glycoprotein analogs whose carbohydrates are modified to enhance their efficacy have been developed in recent years. Erythropoietin is a glycoprotein containing three *N*-glycans and one *O*-glycan, and sialylation on its non-reducing ends is closely associated with its circulating half-life.¹²⁾ *Darbepoetin alfa* is an erythropoietin analog to which two additional *N*-glycans are attached by replacement of five amino acid residues.¹³⁾ The modification of glycosylation prolongs the half-time of this analog compared to its native form. Similar genetic engineering for improvement of half lives has been successfully attempted in antithrombotic drugs. T-PA consists of finger, epidermal growth factor (EGF), kringle1, kringle2 and catalytic domains, and three *N*-glycans. High-mannose type oligosaccharides at the kringle1 domain and EGF domain are related to the short circulation half-life of t-PA.¹⁴⁾ Extension of half-life in blood has been achieved by eliminating the kringle1 domain from human t-PA and replacing one amino acid residue (*pamiteplase*).¹⁵⁾ These improvements have contributed to reducing the frequency and dose of administration.

Recombinant monoclonal antibodies, which have been successfully used in the treatment of cancers and immune diseases, might be the next target of drug design by glyco-engineering. Several anti-tumor monoclonal antibodies that contain a constant region derived from immunoglobulin

(Ig) G have antibody-dependent cellular cytotoxicity (ADCC), and removal of a fucose (Fuc) residue from *N*-linked oligosaccharides at the constant region causes the enhancement of ADCC.^{16,17)} A non-fucosylated IgG-derived antibody is expected to improve the therapeutic effects of these anti-tumor pharmaceuticals.

Glycosylation has also been used for the site-selective modification of proteins with polyethylene glycol (PEGylation).¹⁸⁾ In the GlycoPEGylation method, sialic acid covalently substituted with polyethylene glycol (PEG) can be enzymatically transferred to *O*-glycans at serine or threonine positions in proteins produced in *Escherichia coli*. This strategy has overcome the problems of the previous PEGylation, which provided a heterogeneous mixture of PEG positional isomers. There is increasing interest in utilization and modification of glycans in the development of biopharmaceuticals.

3. IMPACT OF CARBOHYDRATES ON SAFETY

Some glycans have caused serious adverse events in clinical stages. Heparin is a highly sulfated GAG composed of a disaccharide unit, β 1-4 linked α -D-glucosamine and α -D-iduronic acid or β -D-glucuronic acid (averaging 2.5 sulfate groups per disaccharide). In 2007–2008, a serious adverse event associated with *heparin sodium*, including over eighty deaths, occurred in the United States (US).¹⁹⁾ Over-sulfated chondroitin sulfate (OSCS), which consists of fully sulfated β 1-3 linked α -D-*N*-acetylgalactosamine (GalNAc) and a β -D-glucuronic acid unit, was identified as the contaminant in the heparin sodium that had caused hypersensitivity reactions.^{20,21)} It has been reported that OSCS activated the kinin-kallikrein system and induced generation of C3a and C5a, potent anaphylatoxins derived from complement proteins. The contaminated heparin sodium was distributed to at least twelve countries and raised concern about a shortage of heparin products. For ensuring the safety of heparin products, pharmacopoeias in Japan, the US and EU immediately amended their heparin sodium monograph to confirm the absence of OSCS by ¹H-NMR and/or capillary electrophoresis (Fig. 1A). This adverse event has left concerns about the safety of GAGs and motivated the development of a sensitive and selective analytical method for GAGs products all over the globe (Fig. 1B).

An alternative concern for a carbohydrate-related adverse event is immunogenicity of nonhuman glycan motifs. The galactose- α 1,3-galactose (Gal(α 1-3)Gal) motif is known as one of the major problems in the transplantation of organs from pigs to humans.²²⁾ Mouse myeloma cell lines, which are often used for production of recombinant glycoproteins, also expresses the Gal(α 1-3)Gal motif in *N*-linked oligosaccharides. Recently, a high prevalence of hypersensitivity reactions was reported in patients who had been injected with *cetuximab*, which is produced in mouse myeloma cells.²³⁾ This chimeric mouse-human antibody is attached to *N*-linked oligosaccharide containing the Gal(α 1-3)Gal motif at the Fab region.²⁴⁾ It is reported that IgE antibodies against the Gal(α 1-3)Gal motif had been present in serum before therapy, and these antibodies caused hypersensitive reactions after cetuximab treatment. Another immunogenic nonhuman glycan is *N*-glycolylneuraminic acid (NeuGc), a sialic acid.²⁵⁾

Fig. 1. ^1H -NMR Spectra (A) and Anion-Exchange Chromatogram (B) of Heparin Sodium Containing 10% OSCS

^1H -NMR spectrum was obtained at 298 K using a 500 MHz JEOL JNM-ECA500 instrument equipped with a 5-mm filed gradient tunable probe with standard JEOL software. Chemical shifts were referenced to the signal of 3-(trimethylsilyl)propionic-2,2,3,3- d_4 acid sodium salt (TSP) as internal standard (0.00 ppm). Anion-exchange chromatogram was obtained using TSKgel DEAE-5PW (21.5 mm I.D. $\times$ 15 cm, 10 μm) at a flow rate of 1.0 ml/min at 40 $^\circ\text{C}$. The UV detector was set at 230 nm. GAGs were eluted by a linear gradient of 0–100% B buffer (buffer: A, 20 mM Tris-HCl (pH 8.0); B, 20 mM Tris-HCl (pH 8.0) containing 2 M NaCl) within 40 min.

Since Varki *et al.* reported the incorporation of NeuGc into human embryonic stem (ES) cells through the animal serum and the feeder layer, contamination of cell therapy products with NeuGc has become a serious issue in clinics.²⁶⁾ These reports imply the significance of glycan analysis in recombinant glycoprotein products and risk assessment of nonhuman glycans.

4. ALTERATION OF CARBOHYDRATES BY CHANGES IN EXPRESSION SYSTEM AND MANUFACTURING PROCESS

In addition to recombinant technology using mammalian cells, a transgenic technique has been also focusing on large-scale production of therapeutic glycoproteins.^{27,28)} Antithrombin III is an anticoagulant factor, and a freeze-dried preparation of human antithrombin III has been approved for treatment of disseminated intravascular coagulation and thrombotic tendencies. In 2006, the European Medicine Agency (EMA) authorized the marketing of *antithrombin alfa*, a recombinant human antithrombin III produced from the milk of transgenic goats. Detailed structural analysis revealed that these two glycoproteins were structurally identical except for a difference in glycosylation.²⁷⁾ An alternative approach for the large-scale and low-cost production of biopharmaceuticals is the use of transgenic plants.²⁹⁾ Plant cells express nonhuman glycans, such as Fuc (α 1-3) *N*-acetylglucosamine (GlcNAc), xylose (Xyl) (β 1-2) GlcNAc and Gal (β 1-3) GlcNAc at the reducing-end of complex-type *N*-linked oligosaccharides. Currently, plant glyco-engineering, including modification of glycosyltransferases, is developing to overcome limitations in the production of glycoprotein products.³⁰⁾

For various scientific, safety-related and economic reasons, changes in manufacturing processes for biopharmaceuticals are often attempted during the development phase and after marketing authorization. Meanwhile recombinant glycoprotein products that have been claimed to be similar to a reference medical product already authorized (biogeneric/biosimilar/follow-on biologics) have been approved by different manufacturers. The changes in the manufacturing process possibly cause the alteration of glycosylation in the glycoprotein products and consequent changes in quality, efficacy and safety. The first biosimilar glycosylated pharmaceutical, *epo-*

etin alfa, was approved in the EU recently. According to the Japanese guidelines for the biosimilar products, the applicants have to submit some efficacy and safety study data but not all if they can show the data on structural properties and physicochemical/biological similarity between the authorized and biosimilar products. One of the challenging issues in the development of biosimilar glycoprotein products is a comparison of glycosylation between the authorized products and the new entry biosimilar products.

Using some *epoetin* products, we have studied the possibility of several analytical methods for comparison of the glycosylation between closely related biopharmaceuticals. Commercially available *epoetin* products (products A–D) were electrophoresed, and *N*-linked oligosaccharides were released from bands at 30 kDa by an in-gel glycopeptidase F digestion (Fig. 2A). The resulting oligosaccharides were reduced with NaBH_4 and subjected to LC/MS. In Fig. 2, *International nonproprietary names (INN)* of *epoetins* contained in the products A–C and D are tentatively named as *epoetin α* and *β* , respectively. Products A and B are marketed in country X, while products C and D are manufactured and distributed in country Y. Figure 2B shows the total ion chromatograms of *N*-linked oligosaccharides released from four *epoetin* products, and the mass spectra acquired from the most intense peaks (peak z_{1-4}) are shown in Fig. 2C. The m/z values of the most intense ions (m/z 1226.8) and a series of triply charged ions with m/z 14 spacing pattern reveal that the most abundant glycan in *epoetin* products are a tetrasialyl fucosylated-tetraantennary oligosaccharide in common but there are tangible differences in acetylation of sialic acids between products A–C (*epoetin α*) and product D (*epoetin β*) (Fig. 2C). Even among *epoetin α* products there were some significant differences, such as the non-fucosylated oligosaccharides that were found in *epoetin α* products in country X (products A and B) but not in country Y (product C). The latest analytical technology allows us to evaluate the similarity of the glycosylation of closely related biopharmaceuticals.^{31,32)}

5. CONCLUSION

As described above, glycosylation in most biopharmaceuticals affects their efficacy and safety, and the glycosylation is dependent on the manufacturing process and the expres-

Fig. 2. Glycosylation Analysis of Epoetin Products

(A) SDS-PAGE images. Sample: lane 1, product A; lane 2, product B; lane 3, product C; lane 4, product D. (B) *N*-glycan profiles of products A—D acquired by LC/MS in the negative ion mode. (C) Mass spectra of peaks z_1 — z_4 . Product: A, an epoetin α product manufactured/distributed by company a in country X; B, an epoetin α product manufactured by company a and distributed by company b in country Y; C, an epoetin α product manufactured/distributed by company c in country Y; D, an epoetin β product manufactured/distributed by company d in country Y. INN of products A—C, and D are tentatively named as epoetin α and epoetin β , respectively. Symbols: ●, Man; ○, Gal; ■, GlcNAc; ▲, Fuc; ◆, NeuAc. * Bands of epoetins. LC: instrument, nanoFrontier nLC system (Hitachi High-Technologies Corporation); column, graphitized carbon (0.075×150 mm, ThermoFisher Scientific); flow rate, 200 nL/min; buffer A, 5 mM ammonium acetate with 2% acetonitrile (pH 9.6); buffer B, 5 mM ammonium acetate with 80% acetonitrile (pH 9.6); gradient condition, 5—35% B (110 min). MS: instrument, LTQ-TF (ThermoFisher Scientific); electron voltage, 2.0 kV (negative ion mode).

sion system. Physicochemical and biological characterization of such glycosylation is crucial at various stages, namely the development of new biotherapeutic glycoproteins, the establishment of changes in the manufacturing process, and the development of biosimilar products. Appropriate glycan testing must be adopted if the carbohydrate moiety influences safety and efficacy of the pharmaceutical. Furthermore, an *in vivo* assay could be replaced by the glycan test if the glycan profile is strongly associated with *in vivo* activity. Advances in analytical techniques for carbohydrate moieties are expected to facilitate the development of biopharmaceuticals.

Acknowledgments The work was supported in part by a Grant-in-Aid from the Japanese Ministry of Health, Labor, and Welfare.

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