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Production platforms for biotherapeutic glycoproteins. Occurrence, impact, and challenges of non-human sialylation

DARIUS GHADERI[1], MAI ZHANG[1], NANCY HURTADO-ZIOLA[1], AND AJIT VARKI[2*].

[1]Sialix, Inc. 1396 Poinsettia Ave. Vista, CA 92081-8504 USA; [2]Glycobiology Research and Training Center, Departments of Medicine, and Cellular & Molecular Medicine, University of California, San Diego, La Jolla, CA, 92093, USA

Abstract

One of the fastest growing fields in the pharmaceutical industry is the market for therapeutic glycoproteins. Today, these molecules play a major role in the treatment of various diseases, and include several protein classes, i.e., clotting factors, hormones, cytokines, antisera, enzymes, enzyme inhibitors, Ig-Fc-Fusion proteins, and monoclonal antibodies. Optimal glycosylation is critical for therapeutic glycoproteins, as glycans can influence their yield, immunogenicity and efficacy, which impact the costs and success of such treatments. While several mammalian cell expression systems currently used can produce therapeutic glycoproteins that are mostly decorated with human-like glycans, they can differ from human glycans by presenting two structures at the terminal and therefore most exposed position. First, natural human N-glycans are lacking the terminal Galα1-3Gal (alpha-Gal) modification;

*To whom correspondence may be addressed (a1varki@ucsd.edu)

Abbreviations: ADA, anti-drug antibody; alpha-Gal, Galα1-3Galb1-(3)4GlcNAc; bp, base pair; BHK, baby hamster kidney; BSA, bovine serum albumin; CHO, Chinese hamster ovary; CMAH, CMP-N-acetylneuraminic acid hydroxylase; CMP, Cytidine monophosphate; DMB, fluorphore 1,2-diamino-4,5-methylenedioxybenzene; ELISA, Enzyme-linked immunosorbent assay; EPO, Erythropoietin; Fc, Fragment, crystallizable of Ig; FDA, Food and Drug Administration; Gal, galactose; GlcNAc, N-acetylglucosamine; hEScs, human embryonic stem cells; HD, Hanganutztiu-Deicher; HPLC, High-performance liquid chromatography; Ig, immunoglobulin; KO, knock-out; Mab, monoclonal antibody; Man, mannose; MEFs, mouse embryonic fibroblasts; Neu5Ac, N-acetylneuraminic acid; Neu5Gc, N-glycolylneuraminic acid; PEG, polyethylene glycol; Sia, sialic acid; and, TBA, thiobarbituric acid.

and second, they do not contain the non-human sialic acid N-glycolylneuraminic acid (Neu5Gc). All humans spontaneously express antibodies against both of these glycan structures, risking increased immunogenicity of biotherapeutics carrying such non-human glycan epitopes. However, in striking contrast to the alpha-Gal epitope, exogenous Neu5Gc can be metabolically incorporated into human cells and presented on expressed glycoproteins in several possible epitopes. Recent work has demonstrated that this non-human sialic acid is found in widely varying amounts on biotherapeutic glycoproteins approved for treatment of various medical conditions. Neu5Gc on glycans of these medical agents likely originates from the production process involving the non-human mammalian cell lines and/or the addition of animal-derived tissue culture supplements. Further studies are needed to fully understand the impact of Neu5Gc in biotherapeutic agents. Similar concerns apply to human cells prepared for allo- or auto-transplantation, that have been grown in animal-derived tissue culture supplements.

Introduction

Therapeutic proteins play an increasingly important role in the pharmaceutical industry, achieving annual total sales of ~$48 billion in 2009 (Aggarwal, 2010). Unlike in the past, therapeutic proteins are now given to patients with a whole variety of disease conditions, sometimes in high milligram quantities per dose. They represent an integrated part of treatment for various cancer types, autoimmune diseases, and replacement therapies such as enzyme and hormone substitutes. Among the biggest blockbusters in the biopharmaceutical industry are therapeutic proteins like Insulin (Lantus®; Sanofi Aventis), Erythropoietin (EPO, Epogen®; Amgen), and the chimeric IgG1• monoclonal antibody Infliximab (Remicade®; Centocor Ortho Biotech Inc.) with annual sale volumes of $2.6, $3.2, and $3.2 billion each in 2009, respectively (Aggarwal, 2010). Unlike insulin (which is not a glycoprotein), EPO, Infliximab and the vast majority of therapeutic proteins require posttranslational modification with N-glycans and less frequently, O-glycans.

Glycosylation is a very critical modification of therapeutic proteins, known to significantly modulate yield, bioactivity, solubility, stability against proteolysis, immunogenicity, and clearance rate from circulation (Arnold et al., 2007; Durocher and Butler, 2009; Higgins, 2009). This review begins with an overview about established and potentially new production platforms for such recombinant biotherapeutic glycoproteins, highlighting critical differences in the glycosylation patterns among available expression systems. Depending on the source, the glycosylation pattern of the recombinant protein product varies greatly: starting with bacterial systems that do not glycosylate, followed by yeast, plants and insect cell systems generating immunogenic glycan types that are absent in humans, to mammalian systems with human-like complex glycans (Figure 1). Significant progress has been made over the past decade to overcome the current limitations of non-mammalian expression systems by glycoengineering approaches to achieve expression of human-like glycosylation patterns (Durocher and Butler, 2009; Jacobs and Callewaert, 2009). Thus, non-mammalian expression systems might become more prevalent for therapeutic glycoprotein production in the future. But currently, production of therapeutic glycoproteins is dominated by mammalian production platforms with their natural ability to express human-like glycosylation.

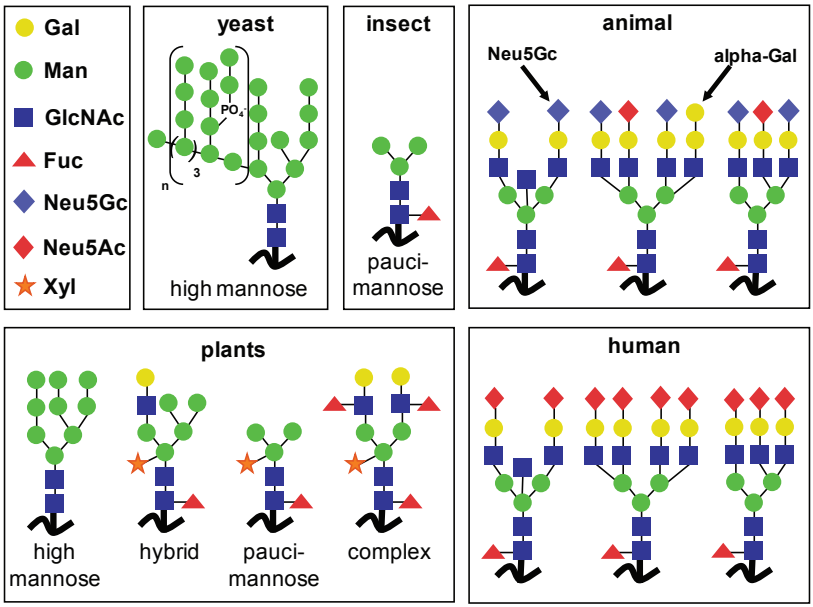

Figure 1. Non-human glycan structures in available glycoprotein expression systems. (Modified from Cummings and Doering, 2009). Eukaryotic cells share the ability to modify proteins by N-glycosylation. Shown are common examples of N-glycan structures occurring in different conceivable production platforms for biotherapeutic glycoproteins. Among eukaryotic cells, the first steps of N-glycosylation occur in the endoplasmatic reticulum (ER). The glycosylation machinery of the ER is highly conserved between all species and results in the biosynthesis of the common $Man_3GlcNAc_2$ core structure. All further modifications of the N-glycan core take place in the Golgi apparatus whereupon the glycosylation repertoire varies greatly among species. For example, yeast mainly expresses high-mannose glycan structures harboring up to 100 mannose residues in different linkages. By contrast, most N-glycans found on insect cell proteins belong to the paucimannosidic type which represents the core structure, but further modifications by additional mannose, fucose, and galactose residues have been found to a lesser extent. Higher plants even synthesize a significant portion of complex type glycans with two antennae. However, non-human highly immunogenic xylose residues occur with a high frequency. By contrast, animals mainly express multiantennary complex type N-glycans and carry sialic acids at outermost positions of glycan chains. But humans have lost the ability to synthesize two of the major mammalian glycan epitopes, Galα1-3Gal (alpha-Gal) and *N*-glycolylneuraminic acid (Neu5Gc). Normal humans have antibodies directed against these structures.

However, two critical differences have been identified between humans and most other mammals: humans have lost the ability to biosynthesize both the terminal Gal•1-3Galβ1-(3)4GlcNAc (alpha-Gal) epitope, and a major mammalian sialic acid, *N*-glycolylneuraminic acid (Neu5Gc), structures that are widely present on non-human mammalian cells (Padler-Karavani and Varki, 2011). These non-human glycan epitopes can potentially affect immunogenicity and/or efficacy of therapeutic glycoproteins in a disadvantageous manner for the patient, since all humans tested have circulating antibodies against them (Padler-Karavani and Varki, 2011). In contrast to the alpha-Gal epitope, Neu5Gc can even be taken up by human cells and metabolically incorporated into cell surface glycoconjugates, which occurs despite the presence of a circulating polyclonal anti-Neu5Gc antibody response (Tangvoranuntakul et al., 2003; Nguyen et al., 2005; Padler-Karavani et al., 2008; Padler-Karavani et al., 2011). This makes Neu5Gc the first example of a "xeno-auto-antigen" (Padler-Karavani and Varki, 2011; Varki et al., 2011).

Notably, pre-clinical trials in small animals to test for efficacy, potential side effects, and immunogenicity of a putative new drug cannot study consequences of the presence of immunoreactive Neu5Gc because organisms such as mice, rats, or rabbits are characterized by the natural occurrence of Neu5Gc. Thus, alternative modified animal models need to be developed to analyze Neu5Gc-dependent immunoreactivity. One available system is the *Cmah* knockout mouse, which is Neu5Gc-deficient (Hedlund et al., 2007) and can be induced to express anti-Neu5Gc antibodies at human-like levels (Padler-Karavani et al., 2011; Taylor et al., 2010). Recent work confirmed that immunoreactive Neu5Gc is not only found in biotherapeutic glycoproteins approved for treatment of various medical conditions, but also that its presence can reduce half-life and increase immunogenicity in the *Cmah* knockout mouse model (Ghaderi et al., 2010). In the following review, we will focus on the occurrence and impact of non-human sialylation patterns on recombinant biotherapeutic glycoproteins derived from mammalian cells. Table 1 summarizes current FDA-approved biotherapeutic glycoproteins and rates their chance of carrying critical immunoreactive Neu5Gc depending on the expression host (Table 1). Besides recombinant expression of biotherapeutic glycoproteins in mammalian cells, the issue of Neu5Gc-contamination also applies for recombinant glycoproteins derived from transgenic animals and animal plasma, as well as for human stem cells if maintained on non-human feeder cell layers and/or cultured in the presence of animal sera or animal-derived cell culture supplements. Finally, this review will discuss the currently available, highly sensitive methods to detect Neu5Gc-contamination and outline promising, cost-efficient approaches to reduce and/or eliminate Neu5Gc-contamination from recombinant biotherapeutic glycoproteins.

Glycosylation properties of available production platforms for biotherapeutic proteins

BACTERIAL EXPRESSION SYSTEMS. These have limitations in glycoprotein production due to the complete lack of the enzymatic machinery and compartmentalization required for mammalian-type glycosylation. However, several non-glycosylated biotherapeutic proteins are currently produced in prokaryotes including monoclonal antibodies, hormones, enzymes, and cytokines. Moreover, several FDA-approved biotherapeutics produced in bacteria are chemically modified with covalently linked polyethylene glycol (PEG) chains, such as Cimzia® Certolizumab pegol by UCB and Lucentis® Ranibizumab by Genentech Inc (DeFrees et al., 2006). This has proved to be successful in improving therapeutic capability, by prolonging drug half-life, enhancing stability, and reducing immunogenicity caused by the lack of glycosylation. Also, approaches involving the engineering and functional transfer of the required glycosylation machinery into prokaryotes to allow synthesis of mammalian-like N-glycans in bacterial systems has made significant progress over recent years (Wacker et al., 2002; Schwarz et al., 2010; Nothaft and Szymanski, 2010). However, additional improvements are needed to establish a cost-efficient, reliable system.

YEAST EXPRESSION SYSTEMS. This expression system is attractive as yeasts may be cultured in chemically defined media, have the ability to multiply rapidly to high densities, have well characterized glycosylation machineries, and the ability

Table 1. Overview of currently FDA-approved therapeutic glycoproteins derived from different mammalian sources and the likelihood for Neu5Gc contamination.

Agent	Marketing Company	Source	Chance of Neu5Gc
	Monoclonal Antibodies		
Actemra® Tocilizumab	Genentech Inc., Hoffmann-La Roche Ltd.	CHO	++
Avastin® Bevacizumab	Genentech Inc., Hoffmann-La Roche Ltd.	CHO	++
Campath®, Mabcampath® Alemtuzumab	Genzyme Corp.	CHO	++
Herceptin® Trastuzumab	Genentech Inc., Hoffmann-La Roche Ltd	CHO	++
Humira® Adalimumab	Abbott Laboratories	CHO	++
Prolia® Denosumab	Amgen	CHO	++
Rituxan® Rituximab	Genentech Inc, Biogen Idec	CHO	++
Simponi® Golimumab	Centocor Ortho Biotech Inc., Merck & Co	CHO	++
Stelara™ Ustekinumab	Centocor Ortho Biotech Inc.	CHO	++
Vectibix® Panitumumab	Amgen	CHO	n.d.
Xolair® Omalizumab	Genentech Inc., Novartis Pharmaceut. Corp. Tanox Inc.	CHO	++
Yervoy® Ipilimumab	BMS	CHO	++
Zevalin® Ibritumomab tiuxetan	Biogen Idec., Bayer Schering Pharma AG	CHO	++
Bexxar® Tositumomab-I131	GlaxoSmithKline	Hyb.	+++
Orthoclone Okt3® Muromonab-CD3	Centocor Ortho Biotech Inc.	Murine ascites	+++
Soliris® Eculizumab	Alexion Pharmaceuticals, Inc	Mye.	+++
Arzerra® Ofatumumab	GlaxoSmithKline	NS0	+++
Benlysta® Belimumab	Human Genome Sciences Inc.	NS0	+++
Mylotarg® Gemtuzumab ozogamicin	Wyeth Pharmaceuticals	NS0	+++
Synagis® Palivizumab	Abbott Labs, MedImmune Inc.	NS0	+++
Tysabri® Natalizumab	Élan Pharmaceut., Biogen Idec.	NS0	+++
Erbitux® Cetuximab	ImClone Systems, BMS	Sp2/0	+++
Ilaris® Canakinumab	Novartis Pharmaceuticals	Sp2/0	+++
Remicade® Infliximab	Centocor Ortho Biotech Inc.	Sp2/0	+++
Reopro® Abciximab	Centocor Ortho Biotech Inc., Eli Lilly & Co.	Sp2/0	+++
Simulect® Basiliximab	Novartis Pharmaceuticals Corp.	Sp2/0	+++

Table 1. Contd

Agent	Marketing Company	Source	Chance of Neu5Gc
Zenapax® Daclizumab	Hoffmann-La Roche Ltd., PDL BioPharma	Sp2/0	+++
	Fc-Fusion Proteins		
Amevive® Alefacept	Astellas Pharma Inc.	CHO	++
Arcalyst® Rilonacept	Regeneron Pharmaceut. Inc.	CHO	++
Enbrel® Etanercept	Amgen, Wyeth Pharmaceutical	CHO	++
Nulojix Belatacept	BMS	CHO	++
Orencia® Abatacept	BMS	CHO	++
	Hormones		
Follistim® Follitropin-β	Merck & Co	CHO	++
Gonal-F® Follitropin alfa	EMD Serono, Inc.	CHO	++
Luveris® LH	EMD Serono, Inc.	CHO	++
OP-1 Putty Osteogenic Protein-1	Stryker Biotech	CHO	++
Ovidrel® Choriogonadotropin α	EMD Serono, Inc.	CHO	++
Thyrogen® Thyrotropin α	Genzyme Corp	CHO	++
Serostim®, Saizen®, Zorbtive™ Somatropin	EMD Serono, Inc.	Murine C127	+++
Emdogain® tooth enamel proteins	Staumann USA	Pig	+++
	Cytokines		
Aranesp® Darbepoetin α	Amgen	CHO	++
Avonex® Interferon β-1a	Biogen Idec, Inc.	CHO	++
NeoRecormon Epoetin β	Hoffmann-La Roche Ltd.	CHO	++
Procrit®, **Epogen®** Epoetin α	Amgen, Centocor Ortho Biotech Inc.	CHO	++
Rebif® Interferon β-1a	Pfizer, Inc., EMD Serono, Inc.	CHO	++
	Clotting Factors		
Xigris® Drotrecogin α	Eli Lilly & Co.	Hek293	+
Helixate FS Factor VIII	ZLB Behring	BHK	++
Kogenate FS Factor VIII	Bayer Schering Corp.	BHK	++
NovoSeven® Factor VIIa	Novo Nordisk	BHK	++
Advate® Antihemophilic Factor	Baxter International Inc.	CHO	++

Table 1. Contd

Agent	Marketing Company	Source	Chance of Neu5Gc
BeneFIX® Factor IX	Wyeth Pharmaceuticals	CHO	++
ReFacto® Antihemophilic Factor	Wyeth Pharmaceuticals	CHO	++
Xyntha® Factor VIII	Wyeth Pharmaceuticals	CHO	++
	Enzyme Inhibitor		
ATryn® Antithrombin/ ATIII	GTC Biotherapeutics	Goat milk	+++
	Enzymes		
Elaprase® Idursulfase	Shire Pharmaceuticals	HT-1080	+
Activase®, Cathflo® **Activase®, Actilyse®** Alteplase	Genentech Inc, Boehringer Ingelheim Pharma KG	CHO	++
Aldurazyme® Laronidase	Genzyme Corp	CHO	++
Cerezyme® Imiglucerase	Genzyme Corp.	CHO	++
Fabrazyme® agalsidase-β	Genzyme Corp	CHO	++
Hylenex®, Cumulase® Hyaluronidase	MediCult A/S, MidAtlantic Diagnostics, Inc., Halozyme Baxter Healthcare	CHO	++
Myozyme®, Lumizyme® Alglucosidase alfa	Genzyme Corp	CHO	++
Naglazyme® GalNAc 4-sulfatase	BioMarin Pharmaceutical Inc.	CHO	++
Pulmozyme® Human DNase	Genentech Inc, Hoffmann-La Roche Ltd.	CHO	++
TNKase® Tenecteplase	Genentech Inc	CHO	++
Amphadase® Hyaluronidase	Amphastar Pharmaceuticals	Bovine	+++
Creon® Pancrelipase	Abbott Products, Inc.	Pig	+++
Pancreaze® Pancrelipase	Ortho McNeil Janssen	Pig	+++
Vitrase® Hyaluronidase	ISTA Pharmaceuticals	Sheep	+++
	Antisera		
Atgam® Anti-thymocyte globulin	Pfizer, Inc.	Equine Serum	+++
Thymoglobulin® Anti-thymocyte globulin	Genzyme Corp.	Rabbit Serum	+++

Table 1. Contd

Agent	Marketing Company	Source	Chance of Neu5Gc
CroFab® Crotalidae Polyvalent Immune Fab	Savage Laboratories	Sheep Serum	+++
DigiFab® Digoxin Immune Fab	Savage Laboratories	Sheep Serum	+++

Details of information used to generate Table 1, with more information regarding common FDA-approved biotherapeutic agents. Information and sources obtained by searching publicly accessible web sites only, as of July 2011. The list is not meant to be comprehensive nor can its accuracy be guaranteed. (updated from Ghaderi et al., 2010).

Although there are no published studies of the sialic acid types of most of the products listed, it is reasonable to predict the following ascending order of *relative* Neu5Gc content, based on cell-type or source: HEK293/HT-1080 (+) < CHO (++)< BHK (++) < Animal sources (+++)< Myeloma/Hybridoma (+++). Due to an inactivated *CMAH* gene in humans potential Neu5Gc contamination could only be from exogenous sources in human cell lines (+), whereas in mammalian cell lines and animal sources potential Neu5Gc contamination depends on *Cmah* expression, which is lower in CHO and BHK cell lines (++), and higher in Myeloma/Hybridoma (+++). The *absolute* Neu5Gc content will also depend on the extent of glycosylation and sialylation of a given biotherapeutic agent.

Abbreviations used in the table: BHK, Baby Hamster Kidney cells; BMS, Bristol-Myers-Squibb; CHO, Chinese Hamster Ovary cells; Hek293, Human embryonic kidney cells; HT-1080, human HT-1080 cells; Hyb., hybridoma cells; Mye., murine myeloma; n.d., not detectable.

to scale up fermentation to industrial levels is well established. Although humans and yeasts share the same N-glycan core structure ($Man_3GlcNAc_2$; Figure 1), the hypermannosylated yeast N-glycans with up to 100 additional mannose residues is highly immunogenic for humans. Among other drawbacks of yeasts are the low to absent amounts of fucose and complete lack of terminal sialic acids (Cummings and Doering, 2009). Similar to bacteria, yeasts thus represent a well-established platform for the production of non-glycosylated biotherapeutic proteins. However, great strides have been made over the last decade to genetically modify yeasts with the intent of ultimately producing human-like N-glycosylated therapeutic glycoproteins (Chiba and Akeboshi, 2009; De Pourcq et al., 2010). Recently, a process to purify recombinant monoclonal antibodies with human-like N-glycans produced in glycoengineered yeast (Hamilton and Gerngross, 2007) has been reported to be successful (Jiang et al., 2011). However, the commercialization of a recombinant N-linked glycoprotein with human-like sialylation produced in yeasts has yet to be reported.

PLANT-BASED EXPRESSION SYSTEMS. These have been established as an alternative production platform for bioactive therapeutic proteins as they are have several advantages such as the ease of scale-up for industrial production at comparatively low cost (Gomord et al., 2010). However, the N-glycosylation

machinery of plants and humans is distinctive and results in significant structural differences of the N-glycan repertoire (Stanley et al., 2009) (Figure 1). Plants lack the bisecting GlcNAc residue, β1-4Galactose residues, sialic acids, and core α1-6-Fucose residues. Instead, plant N-glycans may carry immunogenic β1-2Xylose and α1-3Fucose residues that are absent in humans. Furthermore, while complex-type human N-glycans frequently consist of multiantennary structures with more than two terminal branches, plant N-glycans typically carry only two antennae (Gomord et al., 2010; Varki et al., 2009) (Figure 1). Plant-derived glycosylated biotherapeutics that are currently in clinical trials such as human transferrin or human lysozyme still contain plant glycosylation. However, products like lysozyme are designed for oral delivery suggesting that the plant glycan structures are not always immunogenic and will not hinder FDA-approval and commercialization (Boothe et al., 2010). Significant progress was achieved over the past years towards glycoengineered transgenic plants equipped with different components of the mammalian glycosylation machinery even allowing multiantennary and α2-6-sialylated N-glycosylation to some extent (Castilho et al., 2010; Gomord et al., 2010; Castilho et al., 2011; Nagels et al., 2011). However, additional improvements are needed to establish glycoengineered transgenic plants as a reliable production platform for glycoproteins carrying homogenous humanized glycosylation (Faye and Gomord, 2010).

INSECT CELLS. These represent an alternative system for efficient expression of processed recombinant proteins (Durocher and Butler, 2009; Kost et al., 2005; Mena and Kamen, 2011). However, recombinant proteins derived from insect cells also carry glycan structures that differ significantly from those present in mature human glycoproteins, including paucimannose type glycans where 3-7 mannose residues are predominant (Durocher and Butler, 2009; Kim et al., 2005) (Figure 1). Insect cell N-glycans may also contain immunogenic α1-3Fucose attached to the innermost GlcNAc residue, in contrast to mammals, which exclusively synthesize Core α1-6fucose residues (Staudacher et al., 1999). A serious issue is the lack of complex-type glycans with terminal sialic acids in insect cells (Hillar and Jarvis, 2010). For example, while human serotransferrin was found to have mostly α2-6sialyl-diantennary and trisialyl-triantennary N-glycan structures (Fu and van Halbeek, 1992), recombinant human serotransferrin expressed by insect cells contains lower molecular weight glycoforms mainly of the paucimannosidic type, with a complete lack of galactose and sialic acids (Ailor et al., 2000). Consequently, recombinant human glycoproteins expressed in insect cell systems are currently not suitable for therapeutic purposes. These major limitations may be overcome by glycoengineering approaches to allow improved N-glycan processing and generate human-type complex glycosylation (Jacobs and Callewaert, 2009). For example, Schneider-2 insect cells that produce fully complex type N-glycans, lacking only terminal sialic acids have been reported very recently (Kim et al., 2011).

EXPRESSION OF GLYCOPROTEINS IN MAMMALIAN SYSTEMS. This is to date the only platform applicable to routinely produce biopharmaceutical proteins harboring human-like complex glycosylation at an industrial scale. Consequently, most FDA-approved drugs of this category are currently either purified directly from mammalian plasma or milk, orderived from mammalian cell lines such as Chinese

Hamster Ovary (CHO) cells, baby hamster kidney (BHK-21) cells, murine myeloma and hybridoma cell lines (NS0 and Sp2/0) and to a lesser extent, human cells (Table 1). However, as mammalian cell lines, they can express terminal alpha-Gal structures, and Neu5Gc (Figure 1), the two non-human epitopes against which all humans tested thus far have an antibody repertoire. As mentioned earlier, human cells are capable of incorporation of exogenous Neu5Gc into glycan structures (Bardor et al., 2005). Therefore, even human cell lines used to produce biotherapeutic glycoproteins may express Neu5Gc-containing glycoproteins if animal sera and/or animal-derived media additives were used in the production process. In addition to cell lines, this problem extends to transgenic animals, as the farm animals currently used (cow, pig, sheep, and goat) can have large amounts of Neu5Gc (Tangvoranuntakul et al., 2003).

In the rest of this review we focus on the non-human sialic acid Neu5Gc, its occurrence and impact on biotherapeutic glycoproteins. Sources of Neu5Gc contamination in established production platforms will be discussed and methods to detect this non-human contaminant are outlined. Finally, applicable approaches to reduce and/or eliminate Neu5Gc from mammalian cell culture will be suggested.

Diversity of sialic acids

Sialic acids (Sias) are nine-carbon backbone monosaccharides with a carboxylic function in the C-1 position and are usually present at the outermost (non-reducing) end of glycan chains in the deuterostome lineage of animals (Angata and Varki, 2002; Varki, 2007; Schauer, 2000; Chen and Varki, 2010). Several possible substituents at positions 4, 5, 7, 8, and 9 of the neuraminic acid backbone and the formation of different α-ketosidic linkages between the C-2 position of Sias and the underlying sugar chain generate their natural diversity (Angata and Varki, 2002; Varki and Varki, 2007). More than 20 sialyltransferases have been described to date, which attach different Sias onto various acceptor structures in highly specific linkages (Harduin-Lepers et al., 2005). The most common Sia is *N*-acetylneuraminic acid (Neu5Ac), which serves as the biosynthetic precursor for most others. The predominant sialic acids found on mammalian cell surfaces are Neu5Ac and Neu5Gc. Biosynthesis of Neu5Gc occurs exclusively by hydroxylation of the *N*-acetyl group of CMP-Neu5Ac to yield CMP-Neu5Gc (Figure 2). This reaction is catalyzed by the CMP-*N*-acetylneuraminic acid hydroxylase (Cmah) (Shaw and Schauer, 1988; Kozutsumi et al., 1990; Muchmore et al., 1989). The activated Sias CMP-Neu5Ac and CMP-Neu5Gc serve as donors for >20 Golgi-resident sialyltransferases that link sialic acids via the C-2 position onto underlying glycan chains (Berninsone and Hirschberg, 2000; Varki, 1998; Takashima, 2008).

Human specific loss of *de novo* Neu5Gc biosynthesis.

Being positioned at the outermost end of glycan chains Sias represent the receptors most frequently targeted by pathogens that use Sia-binding proteins to access host cells, and specificity towards either Neu5Ac or Neu5Gc may be observed (Lewis et al., 2009). They are also known to serve as ligands for intrinsic sialic acid-binding

lectins such as Siglecs (Cao and Crocker, 2011). Some Siglecs even discriminate between Neu5Ac and Neu5Gc, although the two Sias differ only by the presence or absence of a single oxygen atom (Figure 2) (Kelm et al., 1994; Sonnenburg et al., 2004; Brinkman-Van der Linden et al., 2000; Crocker et al., 2007). The expression of a functional *CMAH* gene thus impacts the regulation of cell-cell interactions mediated by Siglecs. Whereas most mammals express Neu5Gc-containing glycans on their cell surfaces, glycans on human cells primarily express the precursor molecule Neu5Ac, and Neu5Gc was thought to be completely absent in healthy humans (Muchmore et al., 1998). Indeed, humans generate immune responses against molecules carrying Neu5Gc, e.g. the "serum sickness" reaction to equine anti-thymocyte globulin therapy (Higashi et al., 1977; Merrick et al., 1978). In 1998, the single copy *CMAH* gene was found inactivated in all humans by an *Alu*-mediated replacement of a 92-bp exon resulting in a frameshift mutation (Chou et al., 1998; Hayakawa et al., 2001). The identical genomic mutation was reported by another group (Irie et al., 1998), albeit defining an incorrect reading frame. This loss of Neu5Gc occurred ~3 million years ago in hominid ancestors and represented the first known genetic difference between humans and chimpanzees that could be directly linked to an altered phenotype (Varki, 2009). The *Cmah* gene is highly conserved from sea urchins to primates and the only known homologs are distantly related hydroxylases in bacteria and plants (Schmidt and Shaw, 2001).

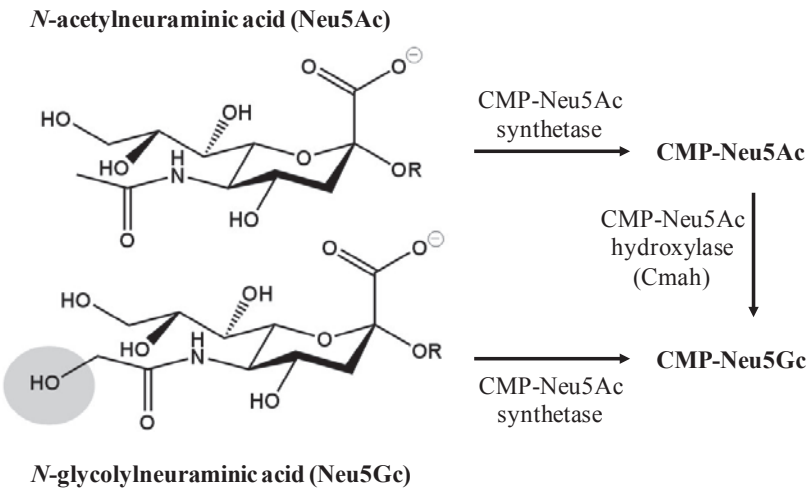

Figure 2. Biosynthesis of Neu5Gc. The two major mammalian sialic acids Neu5Ac and Neu5Gc, differ only in the presence or absence of a single oxygen atom (highlighted with a grey circle). The only known biosynthetic pathway yielding Neu5Gc is hydroxylation of CMP-Neu5Ac to form CMP-Neu5Gc, a reaction catalyzed by the highly conserved CMAH enzyme. Due to a genetic mutation, humans lack a functional CMAH enzyme and therefore express an excess of the precursor Neu5Ac on their cell surfaces (Varki, 2007).

Accumulation of exogenous Neu5Gc in human tissues

Despite the human-specific loss of Neu5Gc *de novo* biosynthesis, Neu5Gc has been unambiguously detected in several malignant human tumors by various immunological and chemical techniques (Malykh et al., 2001; Hedlund et al., 2008)

and in much smaller amounts even in normal human epithelial and endothelial cells (Tangvoranuntakul et al., 2003; Hedlund et al., 2008; Diaz et al., 2009). In contrast, *Cmah* knockout mice were found to lack detectable amounts of Neu5Gc, effectively ruling out an alternate mammalian pathway for endogenous Neu5Gc biosynthesis in mammals (Hedlund et al., 2007). Meanwhile, human biosynthetic pathways could theoretically allow exogenous Neu5Gc to be metabolically incorporated (Varki, 2001; Oetke et al., 2001). Indeed, human cells fed with free Neu5Gc incorporated it into endogenous glycoproteins, achieving levels comparable to those found in chimpanzee cells (Tangvoranuntakul et al., 2003; Nguyen et al., 2005). The pathway for uptake and incorporation of Neu5Gc into human cells involves pinocytosis, followed by delivery to the lysosome and release by lysosomal sialidase, and finally export into the cytosol by the Sialin transporter. Thereafter, free Neu5Gc is available for activation and subsequent transfer to glycoproteins in the Golgi apparatus, in a manner similar to endogenously synthesized Neu5Ac (Bardor et al., 2005). Since Neu5Gc biosynthesis was eliminated during human evolution only ~3 million years ago (Chou et al., 2002), the enzymes involved in activation, transport, and transfer of Sias do not yet consider it as a foreign molecule. Similar accumulation takes place in the human body due to exogenous Neu5Gc derived from foods of mammalian origin (in particular, red meats) (Tangvoranuntakul et al., 2003; Bardor et al., 2005). This is in line with the well-known epidemiological association of human cancers with the excessive consumption of red meat (Willett, 2000; Norat et al., 2002; Zhang and Kesteloot, 2005; Wiseman, 2008). The observed increased accumulation of Neu5Gc in cancers likely originates from increased metabolism of such cells, enhanced pinocytosis (Dharmawardhane et al., 2000), and upregulation of the lysosomal sialic acid transporter gene Sialin due to hypoxia (Yin et al., 2006). In addition to dietary sources, exogenous Neu5Gc is present on multiple therapeutic glycoproteins that are currently approved for treatment of various medical conditions (Ghaderi et al., 2010).

Naturally occurring anti-Neu5Gc antibodies in healthy humans

What we now now know as anti-Neu5Gc antibodies were already described in the 1920's by Hanganutztiu and Deicher (Hanganutziu, 1924; Deicher, 1926). They identified heterophile antibodies causing agglutination of red cells in human patients injected with animal serum - that were later referred to as HD antibodies (Higashi et al., 1977). Subsequently, HD antibodies were also found in patients who never received any injections of animal sera. Interestingly, HD antibodies were mainly identified in patients suffering from leprosy, rheumatoid arthritis or cancer. Decades later, the unknown epitope of HD-antibodies could finally be demonstrated to be Neu5Gc (Morito et al., 1986; Nishimaki et al., 1979; Takiguchi et al., 1984; Morito et al., 1982). As HD-antibodies were mostly found in cancer patients in the early days, the search for tumor-specific Neu5Gc-containing structures began. Neu5Gc-containing glycosphingolipids were found in various human tumors shortly thereafter (Higashi et al., 1985; Kawachi et al., 1988). During these classic studies, a very low frequency of such HD antibodies was reported in healthy human individuals (Morito et al., 1986; Morito et al., 1982; Higashihara et al., 1991). However, the detection systems used in the past were either dependent on animal erythrocyte agglutination, or assumed a very high arbitrary cut-off value as background in ELISA assays. In

some studies healthy humans were even just assumed to lack anti-Neu5Gc antibodies. Recently, a more sensitive assay system was made available to detect anti-Neu5Gc antibodies. This method is based on two chemically designed targets, which carry either Neu5Ac or Neu5Gc. Using this new approach, all normal humans studied were found to have circulating antibodies against Neu5Gc (Tangvoranuntakul et al., 2003; Nguyen et al., 2005).

In addition, all humans are known to have antibodies against the alpha-Gal epitope. The titers against the alpha-Gal epitope were shown to reach as much as 1% of total human circulating antibodies (Galili, 1993; Joziasse and Oriol, 1999), and IgE antibodies were shown to explain acute reactions to infusions of antibodies bearing the epitope (Chung et al., 2008). The highest levels of xenoreactive anti-Neu5Gc antibodies found in normal human individuals reach comparable titers (Padler-Karavani et al., 2011). Another research group reached the same conclusion using different methods (Zhu and Hurst, 2002). As the Neu5Gc monosaccharide alone is too small to fill the complete binding site (paratope) of an antibody, the human antibody response against xenoreactive Neu5Gc is diverse and polyclonal. The variety of glycan structures carrying Neu5Gc was indeed found to result in an abundant and diverse spectrum of anti-Neu5Gc antibodies in humans. This was investigated using paired sets of various glycans either carrying Neu5Gc or, as a background control, bearing Neu5Ac. Variable and complex patterns mostly of IgG, IgM, and IgA antibody reactivities were detected and some individuals were found to have remarkably high titers of anti-Neu5Gc antibodies, even surpassing levels of some antibodies directed against blood groups, which are also anti-glycan antibodies (Padler-Karavani et al., 2008; Padler-Karavani et al., 2011).

Xenosialitis: Chronic inflammation due to the non-human glycan epitope Neu5Gc

Naturally present human anti-Neu5Gc antibodies can induce complement-mediated cytotoxicity upon contact to cells expressing the xenoreactive Neu5Gc epitope (Nguyen et al., 2005). As mentioned, the major sources of exogenous Neu5Gc appear to be foods of mammalian origin i.e., red meat (beef, pork, and lamb) in the standard Western Diet (Tangvoranuntakul et al., 2003). Interestingly, consumption of red meat is associated with increased cancer risk (Rose et al., 1986; Fraser, 1999; Key et al., 1999; Phillips et al., 1980; Kolonel, 2001; Willett, 2000), whereas vegetarians predominantly consume products with very low Neu5Gc amounts and are associated with lower cancer rates (Fraser, 1999; Phillips et al., 1980). While Neu5Gc is likely not the only cause for the epidemiological associations of red meat and various diseases, it may very well be an important component that was overlooked in the past. In normal humans, accumulation of Neu5Gc is predominantly found on the endothelia of blood vessels and the epithelial cells lining hollow organs (Tangvoranuntakul et al., 2003), which also represent the location where common human pathological alterations preferentially occur, i.e. large-vessel occluding atherosclerosis and carcinomas of epithelial origin (Varki, 2009).

It was demonstrated that tumors expressing human-like levels of Neu5Gc show accelerated growth in syngeneic Neu5Gc-deficient mice, coincident with the induction of anti-Neu5Gc antibodies as well as increased infiltration of inflammatory cells

(Hedlund et al., 2008). In addition, transfer of polyclonal monospecific syngeneic mouse anti-Neu5Gc serum enhanced growth rates of transplanted syngeneic tumors bearing human-like levels of Neu5Gc, with evidence for antibody deposition, enhanced angiogenesis and chronic inflammation (Hedlund et al., 2008). These results suggest a human-specific mechanism for increased occurrence of chronic inflammation as a result of diet-related Neu5Gc tissue accumulation in the face of an anti-Neu5Gc immune response. This observation may help explain the predisposition to various diseases involving chronic inflammation, which prominently occur in humans (Varki et al., 2011).

Neu5Gc-contamination in biotherapeutic glycoproteins produced in mammalian cell lines

Due to potential viral contamination, protein purity and consistency issues, biotherapeutic glycoproteins directly isolated from human plasma are now becoming less popular comparing to mammalian cell-derived recombinant proteins. As mentioned previously, current biotherapeutic glycoproteins are mainly derived from cultured mammalian cell lines.

CHINESE HAMSTER OVARY (CHO) CELLS. These are a preferred host for production of biotherapeutic glycoproteins, and the great majority of FDA-approved therapeutic glycoproteins are currently produced in CHO cells (Table 1). CHO cells are a traditional host expression system to fulfill the requirements of the biopharmaceutical industry due to its low risk for the transmission of the major human viruses, high protein yield, and robustness towards pH, temperature, oxygen level, and pressure variations (Wurm, 2007). Also, CHO cells have the ability to carry out post-translational modifications and decorate therapeutic glycoproteins with human-like N-glycans, leading to more compatible, stable, and bioactive therapeutic glycoproteins (Jacobs and Callewaert, 2009). However, CHO cells can express the non-human Sia Neu5Gc as well as the non-human alpha-Gal epitope (Bosques et al., 2010). For example, EPO produced in CHO cells contains both Neu5Ac and Neu5Gc (Yuen et al., 2003) and Abatacept (Orencia®) derived from CHO cells contains the alpha-Gal epitope (Bosques et al., 2010). The presence of anti-Neu5Gc (Higashi et al., 1977; Hokke et al., 1990) and anti-Gal antibodies in healthy humans (Hamadeh et al., 1992) leads to an immune response against compounds carrying these two glycan epitopes. However, it is likely that other clinical factors also determine the immune reaction in individual patients.

Several glycoengineering attempts have been made to reduce Neu5Gc, including an anti-sense RNA strategy (Chenu et al., 2003) and changing of culture conditions (Borys et al., 2009). But the fastest, easiest and most cost-efficient strategy to eliminate Neu5Gc from currently FDA-approved CHO cell products and secreted glycoproteins is based on metabolic competition by feeding CHO cells with the human Sia Neu5Ac (Ghaderi et al., 2010).

BABY HAMSTER KIDNEY CELLS. Besides CHO cells, baby hamster kidney (BHK-21) cells are the most commonly used cell lines for the expression of biopharmaceuticals (Brooks, 2004). Although these cells can synthesize mammalian N-linked and O-linked glycosylation, only a fraction of the expressed protein is suitably glycosylated for use

in humans. For example, only 20-25% of recombinant human erythropoietin (EPO) from BHK-21 or CHO cells is sufficiently sialylated, whereas the remaining product partially lacks terminal sialic acids (Grabenhorst et al., 1999). In analogy to CHO cells described above, BHK-21 cells also have the biosynthetic machinery to synthesize the Neu5Gc *de novo* and may incorporate additional Neu5Gc from the media depending on the culture conditions. Thus, this immunogenic non-human sialic acid is likely present in the glycans of biotherapeutic products expressed in BHK cells.

MURINE MYELOMA CELL LINES. These are currently used mostly in the production of monoclonal antibodies (Mabs), with NS0 and Sp2/0 myeloma cells being the most popular (Table 1). Murine myeloma cell lines are often selected due to the excellent protein yield, higher sialylation compared to CHO cells and the ability to present Sias mostly in α2-6 linkage, a common linkage in humans (Byrne et al., 2007; Yoo et al., 2002). In terms of non-human glycosylation, murine cell lines express both Neu5Gc and alpha-Gal, similar to CHO and other mammalian cell lines, but at considerably higher levels (Muchmore et al., 1989). Thus, therapeutic glycoproteins produced in murine cell line are more likely to be immunogenic. For example, Cetuximab (Erbitux®), a chimeric Mab expressed in Sp2/0 myeloma cells, both Neu5Gc and alpha-Gal have been described as part of an additional N-glycan in the Fab fragment of the Mab (Qian et al., 2007). As mentioned earlier the alpha-Gal epitope on Cetuximab has been shown to induce anaphylaxis in patients triggered by pre-existing anti-Gal IgE antibodies (Chung et al., 2008). Similarly, anti-Neu5Gc antibodies from normal humans interact with Cetuximab in a Neu5Gc-specific manner and generate immune complexes *in vitro* (Ghaderi et al., 2010). Moreover, Neu5Gc present on Cetuximab can induce an immune reaction upon injection into Neu5Gc-deficient *Cmah* knockout mice (Ghaderi et al., 2010; Hedlund et al., 2007). In contrast, Panitumumab (Vectibix®, Amgen), a fully human antibody produced in CHO cells, does not contain detectable levels of Neu5Gc and did not react to anti-Neu5Gc antibodies *in vivo* and *in vitro*. Also, Panitumumab did not trigger a Neu5Gc-specific immune response in the *Cmah* deficient mice described above (Saadeh and Lee, 2007; Ghaderi et al., 2010). The presence of anti-Neu5Gc antibodies also enhanced clearance of the tested Neu5Gc-presenting therapeutic glycoprotein from circulation (Ghaderi et al., 2010). Notably, the interaction between Neu5Gc presenting therapeutic glycoproteins and anti-Neu5Gc containing human serum can result in formation of immune complexes *in vitro* (Ghaderi et al., 2010) and likely explains reduced half-life in *Cmah* null mice with circulating anti-Neu5Gc antibodies (Ghaderi et al., 2010). A similar interaction *in vivo* could potentially fix complement and cause untoward reactions in some patients depending on the anti-Neu5Gc antibody titer, possibly explaining some reported clinical differences (Chung et al., 2008). The variable anti-Neu5Gc antibody titers found in humans might also be an explanation for the known variability in dosage of biotherapeutics between human individuals: Individuals with high antibody titers would show increased clearance rates compared to patients with low anti-Neu5Gc antibodies potentially requiring differential dosing.

HUMAN CELL LINES. Production of therapeutic antibodies in human cell lines has been avoided for a long time due to the theoretical risk of human pathogen contamination. Thus, they still play a relatively minor role in the pharmaceutical

industry. However, human cells have potential in the future as they obviously express homogenous human glycosylation and are *per se* devoid of immunoreactive Neu5Gc and alpha-Gal epitopes. Nonetheless, culture conditions for cells have to be chosen with care as Neu5Gc can still be incorporated from exogenous sources. Therefore, even human cells may produce Neu5Gc-contaminated biotherapeutic proteins if animal sera, animal-derived culture supplements or stabilizers were used in the process. Recently, the first therapeutic glycoproteins expressed in human cell lines were approved by the FDA e.g., Xigris® (Eli Lilly) and Elaprase® (Shire Pharmaceuticals) produced in human embryonic kidney Hek293 and HT-1080 cells, respectively (table 1). PER.C6®, another human cell line was specifically designed for large-scale industrial production purposes and grows at much higher densities than other continuous cell lines. PER.C6®-based therapeutic glycoproteins i.e. mostly vaccines against tuberculosis, malaria and HIV, are also currently in clinical trials.

Stem cells and other cellular therapies may be contaminated with Neu5Gc

Standard tissue culture methods for human embryonic stem cells (hESCs) include murine embryonic fibroblast (MEF) feeder cell co-culture as well as "serum-free" media, which are not free of animal-derived products carrying Neu5Gc. Exogenous Neu5Gc from these sources is metabolically incorporated into and expressed on the surface of hESCs as if it had been internally produced (Martin et al., 2005). Because standard culturing techniques are used in most stem cell laboratories, it can be assumed that most hESCs are contaminated to some degree in this manner (Bardor et al., 2005; Hampton, 2005; Heiskanen et al., 2007). Similar to hESCs, other cellular therapies are confronted with the same Neu5Gc contamination problem when cultured in an environment with Neu5Gc. The presence of the immunoreactive contaminant Neu5Gc on cultured stem cells has been shown to result in Ab-mediated complement deposition when the cells are exposed to human sera *in vitro* (Martin et al., 2005). If this was to happen *in vivo* on hESCs during therapy, it could easily lead to early immune rejection or serious immune responses against these Neu5Gc-contaminated therapeutic stem cells. Other cellular therapies can be affected as well, if the cells are cultured with Neu5Gc containing animal-derived supplements, e.g. there is a cellular therapy already in clinical trials using allogeneic natural killer cells that were cultured in 20% fetal bovine serum before adaptive transfer into patients (Iliopoulou et al., 2010). Another example is the *ex vivo* preparation of vascular grafts (Dahl et al., 2011). Although the authors indicate that "cellular material was removed with detergents to render the grafts non-immunogenic", they actually used 20% serum in the culture stages and then only tested in animals that would not have circulating anti-Neu5Gc antibodies. Meanwhile, stem cell clinics (e.g. Regenerative Sciences in Broomfield/Colorado) are even arguing that cellular therapies using cells from the same patient do not need the usual FDA cellular therapy regulations, downplaying the potentially disadvantageous effects of Neu5Gc contamination due to the cell culture conditions.

It is currently possible to culture small amounts of hESCs in "animal-free" media using human Neu5Gc-free sera or other "serum-free" approaches with defined media (Martin et al., 2005). However, for scale-up to produce large amounts of differentiated cells for therapeutic purposes, it is unlikely that animal materials can

be avoided completely. Neu5Gc contamination of hEScs and their differentiated products is thus likely to be a roadblock that must be overcome before their routine use in biotherapeutic applications. Recently published data show that incorporation of immunoreactive Neu5Gc into mammalian cell lines can be efficiently reduced by non-toxic metabolic competition using Neu5Ac (Ghaderi et al., 2010). These findings might also be applicable to hEScs and therewith provide a cost-efficient and non-invasive method to reduce and/or remove the non-human contaminant Neu5Gc from hESc cultures used in biopharmaceutical industry.

Transgenic animals and animal organ transplants.

The use of transgenic animals has been estimated to be more cost-efficient than mammalian cell culture. For example antibody production in transgenic goats would cost approximately $100/g compared to $300–$3000/g in standard mammalian cell culture (Farid, 2007). After several hurdles towards generation of transgenic farm animals were passed in recent years, multiple biotherapeutic glycoproteins produced in transgenic animals are currently in development (Wells, 2010). The first product derived from transgenic animals, recombinant human antithrombin III (ATryn; Table 1) was FDA-approved in 2009. Comparable with the recombinant biotherapeutic glycoproteins produced in mammalian cell lines, products derived from transgenic animals likely contain the non-human epitopes alpha-Gal and Neu5Gc. Interestingly, ATryn is produced in goat milk, which is known to be extremely rich in Neu5Gc (Tangvoranuntakul et al., 2003). Farm animals such as cow, pig, goat, and sheep which are currently being established as production platforms for biotherapeutic glycoproteins, were all found to synthesize comparably high levels of Neu5Gc (Tangvoranuntakul et al., 2003). However, expression of Neu5Gc varies between tissues depending on the regulation of the *Cmah* gene (Malykh et al., 1998). Thus, direct analysis of each product is needed.

Additionally, the generation of transgenic pigs that might allow xenotransplantation into humans has been investigated, to address the shortage of donated human organs for transplant (Wells, 2010). Transplanting discordant xenografts from pig to primates result in immediate severe and destructive hyperacute rejection due to binding of xenoreactive antibodies to the xenograft endothelium, and this cannot be prevented with immunosuppressive agents (Petersen et al., 2009). As the non-human immunogenic alpha-Gal epitope is present in pigs and about 1% of total human antibodies are directed against alpha-Gal, removal of the corresponding α1-3-galactosyltransferase gene in pigs indeed reduced hyperacute reaction (Phelps et al., 2003). While a small amount of alpha-Gal remained present in the pig-KO, presumably due to action of a second galactosyltransferase, a significant increase in organ survival was achieved. However, there was still medium term rejection (Kuwaki et al., 2005). Besides the remaining low amount of alpha-Gal, the second known non-human immunogenic glycan-epitope Neu5Gc is also present in pigs. As mentioned above, Neu5Gc content was found high especially in red meat such as pork (Tangvoranuntakul et al., 2003). Recently, Neu5Gc-deficient *Cmah* knockout mice induced to have anti-Neu5Gc antibodies to mirror the human situation were found to reject allotransplanted islets from syngeneic Neu5Gc-positive wildtype mice (Tahara et al., 2010), demonstrating

the potential impact of the non-human sialic acid Neu5Gc on xenotransplant rejection risk (Padler-Karavani and Varki, 2011). Notably, Neu5Gc is also naturally present in primates such as baboons, which have been considered as an alternatives to pig-to-human xenotransplantation.

Pre-existing antibodies against an immunogenic glycan epitope on a glycoprotein can result in increased antibody formation against the underlying protein backbone

The pre-existing xenoreactive antibodies against alpha-Gal and Neu5Gc may also be able to induce production of additional antibodies that are directed against the protein moiety of an immunoreactive glycoprotein. This has already been conclusively demonstrated for the alpha-Gal epitope using an alpha-1-3galactosyltransferase deficient mouse model (*GGTA1*-/-) capable of producing alpha-Gal antibodies (Benatuil et al., 2005). Immunization of such mice with alpha-Gal conjugated BSA (alpha-Gal-BSA) resulted in a significant generation of new anti-BSA IgG antibodies without the need for adjuvant. This immune response was shown to depend on the presence of alpha-Gal xenoreactive antibodies in the mice. By contrast, immunization of wild-type mice with alpha-Gal-BSA failed to induce any anti-BSA immune response. Moreover, *GGTA1*-/- mice modulated to generate pre-existing anti-Gal antibodies before exposed to the alpha-Gal-BSA showed a significantly increased T-cell response towards the protein moiety of BSA itself. Thus, antibodies that bind alpha-Gal can increase the efficiency of priming to protein antigens decorated with such glycan epitopes. This is presumably because the immune complexes formed are cleared via Fc-receptors into antigen-presenting dendritic cells. In like manner, it is quite possible that the presence of Neu5Gc on glycoprotein biotherapeutic agents can help explain the development of "anti-drug" antibodies (ADAs) against these agents seen in various patients (De Groot and Scott, 2007; Shankar et al., 2007; Geng et al., 2005; Koren et al., 2008).

Highly sensitive and specific methods to detect Neu5Gc-contamination

Given the structural similarities of Neu5Ac and Neu5Gc (Neu5Gc has one additional oxygen atom; Figure 2), detection and quantification of Neu5Gc is critical to confirm contamination. General methods to measure Sia content, e.g. thiobarbituric acid (TBA) assay cannot distinguish between Neu5Gc and Neu5Ac. Traditional Neu5Gc analysis is based on mild acid treatment of glycan-containing samples to release Sias, followed by derivatization of the released Sias with the fluorophore 1,2-diamino-4,5-methylene-dioxybenzene (DMB), with final analysis using reverse-phase liquid chromatography and fluorescence detection (Hara et al., 1986). This method is well established and accurate. Thus, it is ideal for quantification of Neu5Ac and Neu5Gc contents side by side. However, the method itself is time-consuming, requires expensive equipment, and is therefore not cost-efficient, especially for screening of large sample sizes typical in pharmaceutical industry. An alternative approach for Neu5Gc detection was established by using specific IgY antibodies directed against

Neu5Gc. Due to the lack of Neu5Gc in birds, these anti-Neu5Gc IgY antibodies are raised in chickens (chicken egg yolk). Along with a control IgY, anti-Neu5Gc IgY antibodies allow a whole spectrum of lab techniques including ELISA, Western blotting, flow cytometry, and Immunohistochemistry for Neu5Gc detection (Diaz et al., 2009). Moreover, detection of Neu5Gc appears to be more sensitive than HPLC analysis with fluorescent detection (Diaz et al., 2009).

Approaches to reduce and/or eliminate Neu5Gc contamination

Neu5Gc contamination of therapeutic cells and therapeutic glycoproteins is caused by endogenous sources - when the chosen cell line is of non-human, mammalian origin - and/or by exogenous sources when the cells are cultured in tissue culture media that contains additives of mammalian origin, e.g. Fetal Calf Serum (FCS). Thus, reduction of Neu5Gc contamination can be achieved by eliminating both endogenous and exogenous sources. In most cases, substitution of the culture conditions is likely more feasible than substitution of the entire cell line.

As a consequence of the potential presence of Neu5Gc on therapeutic glycoproteins and its immunogenic character in humans, several glycoengineering attempts have been made to reduce its presence, including an anti-sense RNA strategy (Chenu et al., 2003) and changing of culture conditions (Borys et al., 2009). For instance, sodium butyrate, a cell culture additive in the pharmaceutical industry is capable of decreasing Neu5Gc levels in CHO cells by 50-62% (Borys et al., 2009). Furthermore, substituting sodium carbonate with sodium hydroxide, increasing the pCO_2 level, and decreasing the cell culture temperature near the stationary phase of a cell culture were described to lower Neu5Gc levels by 33%, 46% and 59%, respectively (Borys et al., 2009).

Probably the fastest, easiest and most cost-efficient strategy to eliminate Neu5Gc from currently FDA-approved animal cell products and secreted glycoproteins is based on metabolic competition by feeding cells in culture with the human Sia, Neu5Ac (Ghaderi et al., 2010). Recently, it has been shown that the addition of Neu5Ac to the culture media can dramatically reduce Neu5Gc levels of secreted recombinant proteins and membrane glycoproteins of Hek293 and CHO cells in as little as 3 days, even in the presence of Neu5Gc-containing FCS in the culture media (Ghaderi et al., 2010). The Neu5Gc reduction could be verified with Western blotting and HPLC. Addition of Neu5Ac to the culture media not only impacts incorporation of exogenous Neu5Gc, but also seems to affect the endogenous expression of Neu5Gc, although the mechanism remains unknown (Ghaderi et al., 2010). The power of this approach is greatest when host cell or major product changes are not feasible options and/or when the therapeutic glycoprotein is still in a preclinical stage, so the potential switch of culture conditions is not yet determined by FDA regulations. An earlier concern was the relatively large amounts of Neu5Ac needed and the resulting additional costs. However, the price of Neu5Ac has dropped substantially in recent years making the "Neu5Ac flushing" approach more feasible as a mid-term improvement of products from already established processes.

Changing the culture conditions as described above decreases Neu5Gc levels dramatically, but it may not eliminate Neu5Gc from therapeutic glycoprotein completely. To ensure complete elimination of endogenous Neu5Gc, cell systems may

have to be chosen that lack an active *Cmah* gene and consequently are not capable of Neu5Gc *de novo* biosynthesis. Non-mammalian cell systems like transgenic bacteria, yeast, plants, and insect cell systems could become reliable, cost-efficient alternatives but are far away from practical application (Gomord et al., 2010; Nothaft and Szymanski, 2010; De Pourcq et al., 2010; Jacobs and Callewaert, 2009). Given these hurdles for non-mammalian cell lines in term of proper human glycosylation (Figure 1), the *Cmah* gene could be knocked out from the standard mammalian cell lines used in the pharmaceutical industry, i.e. CHO, BHK, and myeloma cell lines (Sp2/0 and NS0) as well as from murine feeder cells for hEScs. The *CMAH* gene in transgenic animals used for the production of therapeutic glycoproteins could be deleted as well. As a first attempt in this direction, generation of *Cmah* deficient murine embryonic fibroblasts (MEFs) are currently in development that could serve as feeder cells for hEScs (unpublished data). Ideally, both genes responsible for alpha-Gal and Neu5Gc expression on recombinant therapeutic glycoproteins should be deleted from the genome of cells used in the biopharmaceutical industry. Human cell expression systems such as PER.C6® would represent an alternative and currently available production platform for industrial use.

Conclusions and future perspectives

Among all conceivable expression platforms, only mammalian systems are currently established in pharmaceutical industry to produce biotherapeutic glycoproteins with homogenous human-like complex-type N-glycosylation. However, humans lost the ability to synthesize two common mammalian glycan epitopes, alpha-Gal and Neu5Gc, and normal humans were found to have circulating antibodies against both these non-human structures. In the face of this human antibody repertoire, the use of biotherapeutic glycoproteins carrying such immunoreactive epitopes needs to be revisited. Humanized transgenic animals, which lack the genes to synthesize critical alpha-Gal and Neu5Gc epitopes, as well as cell lines derived from these animals represent conceivable future possible. As bacteria-, yeast-, and plant-based expression systems represent the preferred industrial production platforms for non-glycosylated proteins today, they could be additional interesting alternatives for the future, once successful glycoengineering approaches establish systems capable of expressing non-immunogenic human-like glycosylation patterns. Currently approved therapeutic glycoproteins are based on already optimized production platforms and could at first be tested for potential Neu5Gc contamination. If contamination is indeed present, addition of the precursor molecule Neu5Ac to the feeding media may contribute to reduce and/or eliminate Neu5Gc contamination by metabolic competition. This simple, non-invasive, and cost-efficient method was recently demonstrated to work in cell culture. However, further studies are required to evaluate the efficiency and feasibility of this “Neu5Ac-flushing” method, especially with regard to industrial scale production platforms.

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