Recombinant glycoprotein drugs require proper glycosylation for optimal therapeutic efficacy. Glycoprotein therapeutics are rapidly removed from circulation and have reduced efficacy if they are poorly sialylated. *Ricinus communis* agglutinin-I (RCA-I) was found highly toxic to wild-type CHO-K1 cells and all the mutants that survived RCA-I treatment contained a dysfunctional N-acetylglucosaminyltransferase I (GnT I) gene. These mutants are named CHO-gmt4 cells. Interestingly, upon restoration of GnT I, the sialylation of a model glycoprotein, erythropoietin, produced in CHO-gmt4 cells was shown to be superior to that produced in wild-type CHO-K1 cells. This addendum summarizes the applicability of this cell line, from transient to stable expression of the recombinant protein, and from a lab scale to an industrial scale perfusion bioreactor. In addition, CHO-gmt4 cells can be used to produce glycoproteins with mannose-terminated N-glycans. Recombinant glucocerebrosidase produced by CHO-gmt4 cells will not require glycan remodeling and may be directly used to treat patients with Gaucher disease. CHO-gmt4 cells can also be used to produce other glycoprotein therapeutics which target cells expressing mannose receptors.

**Introduction**

Sialylation of glycoprotein therapeutics plays an important role in regulating the circulatory half-life of recombinant drugs. Poorly-sialylated glycoproteins with exposed galactose residues bind to asialoglycoprotein receptors expressed on liver cells which leads to its removal from circulation, resulting in a shorter in vivo half-life of the therapeutic protein. This has been demonstrated in several recombinant therapeutic glycoproteins such as erythropoietin (EPO), Factor VIII, and chorionic gonadotrophin. Commercially available recombinant EPO used to treat patients with anemia contains only highly sialylated isoforms as shown in the left lane of Figure 1. However, transiently expressed recombinant EPO produced in CHO-K1 cells also contains many isoforms that are less sialylated as shown in the right lane in Figure 1. The less sialylated isoforms, sometimes constituting up to 80% of the total product, has to be discarded during purification. Hence, the biotechnology industry has focused on improving the sialylation of recombinant glycoproteins through host cell line engineering, process control and optimization. We have developed a novel technology that is able to produce superiorly sialylated glycoprotein therapeutics with the use of novel glycosylation mutants, CHO-gmt4 cells.

**The Isolation of CHO-gmt4 Cells that Possess Dysfunctional GnT I**

CHO-gmt4 cells belong to a panel of CHO glycosylation mutant (CHO-gmt) lines that have been established in our group. All CHO-gmt lines are able to grow in suspension culture using...
CHO-gmt4 mutants we have analyzed so far, the GnT I loss-of-function mutation was due to a single point mutation in the GnT I coding region that resulted either in missense and nonsense mutations.\textsuperscript{8} Theoretically, mammalian cells that are unable to transport UDP-GlcNAc into the Golgi or with a dysfunctional GnT I should both produce truncated N-glycans with the structure Man5-GlcNAc2. However, more than 100 RCA-I-resistant CHO mutants that we analyzed, all had lost GnT I activity (data not shown). None of the mutants was due to a mutated UDP-GlcNAc transporter. This may be due to the existence of several functionally redundant UDP-GlcNAc transporters in the mammalian cells.\textsuperscript{13} No other glycosylation mutations were obtained using this lectin, demonstrating the specific use of RCA-I in isolating naturally occurring CHO mutants with dysfunctional GnT I.

The study of CHO glycosylation mutants was first pioneered by Stanley and coworkers.\textsuperscript{14} Lec1 cells, which belong to this group of CHO mutants, do not possess GnT I activity. They were isolated using the lectin, Phaseolus vulgaris (L-PHA) from a Pro5 CHO cell line and was used to determine the coding sequence of human GnT I.\textsuperscript{15,16} Other cell lines with dysfunctional GnT I have also been isolated. CHO-DUXK cells were also isolated using L-PHA lectin-resistant selection after undergoing ethyl methysulphonate (EMS) chemical mutagenesis. However, the authors found that these cells could not be adapted to protein-free suspension culture.\textsuperscript{17} Another group utilized zinc-finger technology to inactivate the GnT I gene in CHO cells, leading to the generation of a panel of GnT I deficient cell lines that could be adapted to suspension culture and used for the production of recombinant proteins.\textsuperscript{18} The authors also relied on the use of RCA-I to identify the mutant cells after the expression of the zinc-finger nuclease. GnT I deficient HEK293S cells have been isolated using EMS followed by lectin-resistant selection using ricin. The mutant cell line was used to produce a recombinant glycoprotein for crystal structure studies because the mutant produced highly homologous Man5GlcNAc2 N-glycans.\textsuperscript{19} In another report, baby hamster kidney cells (BHK-21) that are deficient in GnT I due to single point mutations was isolated following treatment with chemical mutagenesis and lectin-resistant selection using ricin.\textsuperscript{20}

CHO-gmt4 cells were found to produce glycoproteins bearing glycans terminating in mannose residues. A fusion protein, EPO linked to IgG1 Fc (EPO-Fc), was transiently expressed in CHO-gmt4 and wild-type CHO-K1 cells. The N-glycans were cleaved from the respective EPO-Fc samples using PNGase F, purified, and permethylated before analysis using matrix-assisted laser desorption and/or ionization time-of-flight mass spectrometry (MALDI-TOF MS). The results are shown in Figure 2. The N-glycans found on EPO-Fc produced in CHO-K1 are mostly of the complex type, with di-, tri- and tetra-antennary structures with incomplete sialylation. EPO-Fc produced by CHO-gmt4 cells contains afucosylated and fucosylated glycans with five mannose as the two major glycan species (Man5 and Man5F), followed by afucosylated and fucosylated glycans with four terminal mannose residues (Man4 and Man4F). The afucosylated glycans were in greater proportion to fucosylated glycans for both Man4 and Man5 glycans. Other pauci- and oligo-mannose N-glycans (Man4, Man6, Man7, Man8, and Glc1Man9) were also detected although at much lower relative abundance.

**Superior Sialylation Observed in Transient and Stable Expression of EPO in CHO-gmt4**

Interestingly, the sialylation of transiently expressed EPO produced in CHO-gmt4 with functionally restored GnT I was more superior to that produced in wild-type CHO-K1 cells. The better sialylation was not due to the overexpression of GnT I as the overexpression of GnT I in wild-type CHO-K1 did not show an improvement in sialylation.\textsuperscript{8,21} All CHO-gmt4 lines analyzed so far produced better sialylated EPO when functional GnT I was restored.\textsuperscript{8} In addition, the restoration of GnT I function also led to the improved sialylation of recombinant EPO produced...
in Lec1 cells. Only one kind of mutation in the GnT I coding sequence was found in each CHO-gmt4 line and this also holds true for the Lec1 cells. This may suggest that only one GnT I allele exists in CHO cells. The molecular mechanism for the enhanced sialylation remains to be discovered.

The overexpression of GnT I not only improved the sialylation of transiently expressed EPO in CHO-gmt4 cells, the enhanced sialylation was also observed for stably transfected CHO-gmt4 cells. The detailed analysis of the glycan structures using high pH anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) revealed that this was due to a higher proportion of tri- and tetra-antennary branched glycans. GnT I deficient CHO cell lines engineered with zinc-finger nuclease technology were also shown to have improved the sialylation of IgG1 molecules.

To extend the applicability of this cell line to industrial use, the mutant cell line was further engineered using zinc-finger nuclease technology targeting the dihydrofolate reductase gene (DHFR), resulting in a CHO-gmt4 cell line that can undergo methotrexate amplification. A panel of stable cell lines co-expressing EPO and GnT I showed that the superior EPO sialylation was maintained after gene amplification with methotrexate. Furthermore, the sialylation profile of EPO was also maintained when one of the stable cell lines was cultured in an industrial perfusion bioreactor. Purified EPO from this bioreactor run was analyzed using MALDI-TOF MS analysis, which showed a greater proportion of tri- and tetra-antennary sialylated glycans. This result is in agreement with the earlier HPAEC-PAD analysis of transiently expressed EPO-Fc in CHO-gmt4 cells.

Figure 2. MALDI-TOF mass spectrometry analysis of N-glycan profile from purified EPO-Fc fusion protein transiently produced in CHO-K1 cells (top) and CHO-gmt4 cells (bottom). Red diamonds, sialic acid; yellow circles, galactose; blue squares, mannose; red triangles, fucose.

CHO-gmt4 cells Can Be Used to Produce Recombinant Proteins Targeting Cells with Mannose Receptors for Better Efficacy

Besides improving the efficacy of recombinant glycoproteins through better sialylation, the efficacy of other biologics can also be improved through the production of glycoproteins with more specific glycosylation for targeted cells. In the treatment of Gaucher disease, recombinant glucocerebrosidase is produced to target macrophages that have been engorged due to the inability to metabolize glycolipids. Following production in CHO cells, purified glucocerebrosidase has to be enzymatically modified by glycosidases resulting in glycans containing terminal mannose residues for better efficacy as mannose-specific receptors are expressed on the surface of macrophages.
produced in a human cell line using gene activation is called velaglucerase alfa (VPRIV). Its glycosylation is tailored to target macrophage cells by the addition of kifunensin, an inhibitor of α-mannosidase-I, resulting in the recombinant protein containing oligomannoses.25 Taliglucerase alfa, glucocerebrosidase produced in carrot cells with terminal mannose glycans26 has also been recently approved.

CHO-gmt4 is a good candidate host cell line for the production of glucocerebrosidase. The glycosylation mutant cells express recombinant glycoproteins with mannose-terminated N-glycans (Fig. 2), therefore bypassing the requirement for enzymatic deglycosylation to produce the mannose-terminated N-glycans. The use of kifunensine to alter the glycosylation pattern may affect cell growth and, in addition, some complex N-glycan structures may still persist due to incomplete inhibition of the mannosidase enzyme.27

Terminal-mannosylated proteins have been shown to elicit better immunogenicity as these mannosylated proteins are much more efficiently targeted to the mannose receptors of antigen-presenting cells such as dendritic cells.28,29 Hence CHO-gmt4 cells may potentially be a good host for producing cancer vaccines due to their ability to produce proteins containing terminal five-mannose sugars.

Conclusion

CHO-gmt4 cells are able to produce recombinant glycoproteins with two different glycosylation characteristics that cater to different applications. It can be used to produce therapeutic glycosylation with superior sialylation when functional GnT I is restored. CHO-gmt4 cells can also be used to produce glycoproteins with mannose-terminated N-glycans, such as glucocerebrosidase for Gaucher disease and protein-based cancer vaccines targeting the mannose receptors of antigen-presenting cells.

We propose that the CHO-gmt4 glycosylation mutants represent a significant step toward the improvement in the quality of glycoprotein therapeutic production.

Disclosure of Potential Conflict of Interest

A’STAR has filed a patent application to cover the findings.

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