Pharmaceutical BIOPROCESSING

ics

Improving sialylation of recombinant biologics for enhanced therapeutic efficacy

"CHO-gmt4 cells represent an attractive cell line to produce highly sialylated recombinant therapeutics."

Keywords: CHO • EPO • glycosylation mutant • GnT I • sialylation

In 1971, Anatol Morell and Gilbert Ashwell discovered that the removal of sialic acids from circulating glycoproteins significantly reduced their serum half-life [1]. The asialoglycoprotein receptor responsible for this phenomenon was identified in the liver 3 years later [2]. The interaction between the asialoglycoproteins and the receptor was then believed to be the physiological mechanism for the removal and degradation of serum glycoproteins. However, in knockout mice that lack the receptor, no phenotypic abnormalities was observed and no accumulated plasma glycoproteins was detected [3], suggesting that the biological purpose of asialoglycoprotein receptor remains to be elucidated. A recent study suggested that the actual endogenous ligands of the asialoglycoprotein receptor might be the glycoprotein components involved in blood coagulation and thrombosis [4]. Nonetheless, it is clear that the asialoglycoprotein receptor mediates the capture and endocytosis of a variety of exogenously administered glycoprotein therapeutics. Removal of sialic acid from some glycoprotein drugs can reduce their *in vivo* half-life from a few hours to minutes (see references in [5]). In order to maintain optimal efficacy, less sialylated glycoforms of the recombinant drugs have to be discarded during purification. Therefore, substantial research has been devoted to the improvement of protein sialylation in mammalian cells, especially Chinese hamster ovary (CHO) cells as they are the main workhorse of the biopharmaceutical industry.

N-glycosylation of recombinant therapeutics produced by CHO cells presents two major challenges to the biotech industry: heterogeneity and batch-to-batch consistency of the glycans. Many factors such as the genetic background of the cell line and culture conditions can affect the glycosylation patterns of biologics produced by CHO cells [6]. It has been estimated that at least 600 genes are involved in the complex process of protein glycosylation [7]. Factors that directly or indirectly affect these genes will subsequently affect the glycosylation of the recombinant products. Therefore, it is not an easy task to identify a single method to improve sialylation in CHO cells.

Many approaches have been introduced to improve sialylation in CHO cells. They include a feeding strategy which involves feeding the cells with nucleotide sugar precursors such as N-acetylmannosamine (ManNAc) to increase intracellular sialic acid pools [8]. However, this method would be too costly and therefore impractical for large-scale productions. The use of antisense RNA targeting cytosolic sialidase Neu2 was reported to produce recombinant DNase with increased sialic acid content [9]. Stable expression of siRNA targeting Neu2 was shown to improve sialic acid content of glycoproteins [5]. Research into improving the sialylation in CHO cells stably expressing different glycoproteins by the overexpression of glycogenes has resulted in fairly convincing results [10–13]. It is important to note that the so-called 'wild-type' CHO cells are genetically heterogeneous and individual cells may exhibit variations in many attributes including glycosylation [14]. We previously showed that randomly picked single clones from sta-

Kah Fai Chan
Bioprocessing Technology Institute, Agency for Science, Technology & Research (A*STAR), 20 Biopolis Way, #06–01 Centros, Singapore 138668, Singapore
and
Department of Biochemistry, National University of Singapore, Singapore 119077, Singapore

John SY Goh
Bioprocessing Technology Institute, Agency for Science, Technology & Research (A*STAR), 20 Biopolis Way, #06–01 Centros, Singapore 138668, Singapore

Zhiwei Song
Author for correspondence:
Bioprocessing Technology Institute, Agency for Science, Technology & Research (A*STAR), 20 Biopolis Way, #06–01 Centros, Singapore 138668, Singapore
Tel.: +65 64078844
Fax: +65 64789561
song_zhiwei@bti.a-star.edu.sg

FUTURE SCIENCE part of fsg

bly transfected 'wild-type' CHO-K1 cells expressed human EPO with varying extents of sialylation [15]. Therefore, the glycosylation capability of each stably transfected clone can be different from other clones and the results observed in the overexpression studies [10–13] may not be generalized to other CHO lines.

We used a transient transfection approach to study the impact of 31 *N*-glycosylation-related genes on sialylation. We showed that none of the glycosyltransferases or the nucleotide sugar transporters tested was able to improve EPO sialylation in CHO-K1 cells [16]. The results suggested that under our experimental conditions the endogenous expression levels of all 31 genes were sufficient for CHO-K1 cells to glycosylate recombinant EPO. However, it should be pointed out that human EPO contains only three *N*-glycans and one *O*-glycan. It would be interesting to determine if any of the 31 genes would become a 'bottle neck' if the cells were producing a protein with additional *N*-glycans sites, such as the novel erythropoiesis stimulating protein. It remains to be determined whether a similar transient transfection study might yield a different outcome if novel erythropoiesis stimulating protein is used as the model glycoprotein.

> "...under our experimental conditions the endogenous expression levels of all 31 genes were sufficient for CHO-K1 cells to glycosylate recombinant EPO."

The accurate assessment of the degree of sialylation of a recombinant protein represents another challenging task. Different methods have been used to analyze the content of sialic acid of glycoproteins. The thiobarbituric acid assay has been traditionally used to quantify sialic acid [17]. This method however requires purified glycoprotein samples. A high-throughput method proposed recently offers rapid quantification of glycoprotein sialic acid content in crude supernatant samples [18]. This process offers 10-fold higher sensitivity than other sialic acid quantification kits and can be carried out in microplate format within 15 min. Purification of glycoprotein is a time-consuming process and certain glycoforms of the protein can be partially lost during the procedure. Therefore, it raises the concern of the accuracy of the sialylation assays. To establish a rapid platform to analyze the glycosylation patterns of a recombinant protein without the need for purification, we have adapted the EPO/isoelectric focusing (IEF) method to study sialylation patterns of EPO. Recombinant human EPO expressed in CHO cells is first separated on an IEF gel into different bands/glycoforms based on their charge difference. EPO molecules with different number of sialic acid residues are separated by the gel and then detected by an anti-EPO antibody using a typical immunoblotting assay. This assay offers a semi-quantitative estimation of all the recombinant EPO in the conditioned medium [15]. The IEF strategy can also be used to screen for better-sialylated clones in the early stages of clone selection. To obtain detailed structural information on glycans, EPO fused to the Fc region of human IgG1 (EPO-Fc) has been produced in CHO cells. The Protein A-based purification procedure ensures the full recovery of the all the glycoforms of EPO-Fc for sialylation assessment. The *N*-glycans attached to the EPO-Fc were analyzed using more powerful analytical methods such as high pH anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD), MALDI-TOF MS and LC–MS.

The CHO Lec mutants isolated for resistance to plant lectins by Stanley and colleagues have brought us in-depth knowledge of the glycosylation pathway in mammalian cells (reviewed in [19]). However, the potential use of these glycosylation mutants in the production of recombinant therapeutics with desired glycoforms is hampered by difficulties in adapting the Lec mutants to serum-free suspension culture. In order to isolate CHO glycosylation mutants with the potential to be host cells in biomanufacturing, we have isolated a panel of glycosylation mutants from CHO-K1 cells. Cytotoxic lectin RCA-I was one of the lectins used to isolate CHO mutants. RCA-I was selected because it was reported to be specific for terminal β1,4-linked galactose and thus would allow us to isolate a panel of CHO glycosylation mutants with genetic defects in the *N*-glycosylation pathway upstream of galactose addition. Unexpectedly, genetic analysis of more than 100 RCA-I-resistant CHO mutants showed that they are all the same type of mutants with different genetic mutations in the *Mgat1* gene [15], similar to Stanley's Lec1 mutant. This gene codes for GnT I. Without functional GnT I, the cells fail to transfer N-acetylglucosamine to $Man_5GlcNac_2$ glycan. A plausible explanation for having only GnT I-deficient mutants surviving RCA-I selection is that the lectin is not specific for terminal β1,4-linked galactose but possibly binds many glycan structures except for $Man_5GlcNac_2$ [20]. All these CHO mutants with dysfunctional GnT I have been named CHO-gmt4 cells. Different from Lec1 cells, CHO-gmt4 cells can be easily adapted to serum-free suspension cultures.

An important finding in this work was that the restoration of functional GnT I in these CHO-gmt4 cells led to an increase in the sialylation of recombinant EPO both in transient expression as well as in stably transfected clones [15]. The overexpression of GnT I

in wild-type CHO cells did not yield an equivalent increase in sialylation of EPO. Quantitative analysis of the *N*-glycans released from recombinant EPO-Fc produced by GnT I-rescued CHO-gmt4 revealed a ~20% increase in sialic acid content compared with that produced by wild-type CHO-K1 cells. This increased in sialylation was attributed to increased *N*-glycan branching in CHO-gmt4. The molecular mechanism for this phenomenon remains unknown [15,21].

To further enhance the industrial applicability of CHO-gmt4 cells, the dihydrofolate reductase gene in CHO-gmt4 cells was inactivated using zinc-finger nucleases to allow for the amplification of the EPO transgene with methotrexate. We found that several methotrexate-amplified clones maintained the ability to produce highly sialylated EPO. One of these clones was cultured in a perfusion bioreactor that was used in an existing industrial EPO-production bioprocess. The EPO produced by the mutant line again maintained superior sialylation compared with the commercially used EPO producing CHO clone cultured under the same conditions [21]. HPAEC-PAD and MALDI-TOF MS analyses showed that the EPO produced by the GnT I-rescued CHO-gmt4 cells contained higher amount of tri- and tetra-antennary glycans [21]. These results demonstrated the industrial potential of CHO-gmt4 mutant as a production cell line for producing highly sialylated glycoprotein therapeutics.

Less sialylated glycoforms of recombinant protein drugs are required to be removed during the purification steps by the regulatory authorities to ensure therapeutic efficacy. Enhancing sialylation for the recombinant biologics will significantly simplify the purification process and reduce the cost of drug production. Different strategies have been previously employed to improve sialylation of recombinant proteins in CHO cells. Through the use of CHO-gmt4 cells we offer a novel strategy to improve the sialylation of recombinant EPO. We aim to analyze the sialylation profiles of other glycoproteins using the same cells. CHO-gmt4 cells represent an attractive cell line to produce highly sialylated recombinant therapeutics.

Financial & competing interests disclosure

This work was funded by the Agency for Science, Technology and Research (A*STAR) of Singapore. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

References

1 Morell AG, Gregoriadis G, Scheinberg IH, Hickman J, Ashwell G. The role of sialic acid in determining the survival of glycoproteins in the circulation. *J. Bio. Chem.* 246(5), 1461–1467 (1971).

2 Hudgin RL, Pricer WE, Jr, Ashwell G, Stockert RJ, Morell AG. The isolation and properties of a rabbit liver binding protein specific for asialoglycoproteins. *J. Bio. Chem.* 249(17), 5536–5543 (1974).

3 Tozawa R, Ishibashi S, Osuga J *et al.* Asialoglycoprotein receptor deficiency in mice lacking the major receptor subunit. Its obligate requirement for the stable expression of oligomeric receptor. *J. Bio. Chem.* 276(16), 12624–12628 (2001).

4 Grewal PK, Uchiyama S, Ditto D *et al.* The Ashwell receptor mitigates the lethal coagulopathy of sepsis. *Nat. Med.* 14(6), 648–655 (2008).

5 Ngantung FA, Miller PG, Brushett FR, Tang GL, Wang DI. RNA interference of sialidase improves glycoprotein sialic acid content consistency. *Biotechnol. Bioeng.* 95(1), 106–119 (2006).

6 Hossler P, Khattak SF, Li ZJ. Optimal and consistent protein glycosylation in mammalian cell culture. *Glycobiology* 19(9), 936–949 (2009).

7 Lauc G, Essafi A, Huffman JE *et al.* Genomics meets glycomics-the first GWAS study of human N-glycome identifies HNF1alpha as a master regulator of plasma protein fucosylation. *PLoS Genet.* 6(12), e1001256 (2010).

8 Gu X, Wang DI. Improvement of interferon-gamma sialylation in Chinese hamster ovary cell culture by feeding of N-acetylmannosamine. *Biotechnol. Bioeng.* 58(6), 642–648 (1998).

9 Ferrari J, Gunson J, Lofgren J, Krummen L, Warner TG. Chinese hamster ovary cells with constitutively expressed sialidase antisense RNA produce recombinant DNase in batch culture with increased sialic acid. *Biotechnol. Bioeng.* 60(5), 589–595 (1998).

10 Weikert S, Papac D, Briggs J *et al.* Engineering Chinese hamster ovary cells to maximize sialic acid content of recombinant glycoproteins. *Nat. Biotechnol.* 17(11), 1116–1121 (1999).

11 Fukuta K, Yokomatsu T, Abe R, Asanagi M, Makino T. Genetic engineering of CHO cells producing human interferon-gamma by transfection of sialyltransferases. *Glycoconj. J.* 17(12), 895–904 (2000).

12 Bork K, Reutter W, Weidemann W, Horstkorte R. Enhanced sialylation of EPO by overexpression of UDP-GlcNAc 2-epimerase/ManAc kinase containing a sialuria mutation in CHO cells. *FEBS Lett.* 581(22), 4195–4198 (2007).

13 Son YD, Jeong YT, Park SY, Kim JH. Enhanced sialylation of recombinant human erythropoietin in Chinese hamster ovary cells by combinatorial engineering of selected genes. *Glycobiology* 21(8), 1019–1028 (2011).

14 Davies SL, Lovelady CS, Grainger RK, Racher AJ, Young RJ, James DC. Functional heterogeneity and heritability in CHO cell populations. *Biotechnol. Bioeng.* 110(1), 260–274 (2013).

15 Goh JS, Zhang P, Chan KF, Lee MM, Lim SF, Song Z. RCA-I-resistant CHO mutant cells have dysfunctional GnT I and expression of normal GnT I in these mutants enhances sialylation of recombinant erythropoietin. *Metab. Eng.* 12(4), 360–368 (2010).

16 Zhang P, Tan DL, Heng D, Wang T, Mariati, , Yang Y, Song Z. A functional analysis of N-glycosylation-related genes on sialylation of recombinant erythropoietin in six commonly used mammalian cell lines. *Metab. Eng.* 12(6), 526–536 (2010).

17 Warren L. The thiobarbituric acid assay of sialic acids. *J. Bio. Chem.* 234(8), 1971–1975 (1959).

18 Markely LR, Ong BT, Hoi KM, Teo G, Lu MY, Wang DI. A high-throughput method for quantification of glycoprotein sialylation. *Anal. Biochem.* 407(1), 128–133 (2010).

19 Patnaik SK, Stanley P. Lectin-resistant CHO glycosylation mutants. *Meth. Enzymol.* 416, 159–182 (2006).

20 Iskratsch T, Braun A, Paschinger K, Wilson IB. Specificity analysis of lectins and antibodies using remodeled glycoproteins. *Anal. Biochem.* 386(2), 133–146 (2009).

21 Goh JS, Liu Y, Liu H *et al.* Highly sialylated recombinant human erythropoietin production in large-scale perfusion bioreactor utilizing CHO-gmt4 (JW152) with restored GnT I function. *Biotechnol. J.* 9(1), 100–109 (2014).