Journal of Bioscience and Bioengineering
VOL. xx No. xx, , 2018

www.elsevier.com/locate/jbiosc

Enhancement of glycosylation by stable co-expression of two sialylation-related enzymes on Chinese hamster ovary cells

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Received 20 October 2017; accepted 12 January 2018
Available online xxx

Sialic acid plays important roles in stabilization and modulation of the interaction of molecules and membranes in organisms. Due to its high electronegativity, sialic acid can promote binding effects of molecules and support the transportation of drugs and ions in cells. This also strengthens cells against degradation from glycosidases and proteases. Hence sialic acid helps glycoproteins extend their half-lives and bioactivity. On the other hand, Chinese hamster ovary (CHO) cells have been widely used as a workhorse in biopharmaceutical fields in part due to the similarity between their glycan properties and those in humans. Thus, a high sialylation produced by CHO host cell line is strongly desired. In this study, we simultaneously overexpressed two key sialylated-based enzymes human β-galactoside α(2,6) sialyltransferase I and UDP-GlcNAc 2-epimerase/ManNAc kinase to achieve greater sialylation pattern produced host cells. The single-cell line thus-generated produced an approximately 41.6% higher level of total free sialic acid, and the glycan profiles showed a significant increase of more than 7-fold in the relative amount of total sialylated *N*-glycan as compared to the wild-type. These results demonstrated that co-expression of these two sialylated-based key enzymes yielded a cell line that effectively produced glycoproteins with superior sialylation and achievable human-like glycoforms.

[**Key words:** Chinese hamster ovary cell; Sialylation; Glycosylation; Sialyltransferase; UDP-GlcNAc 2-epimerase/ManNAc kinase; Sialic acid]

Glycosylation is an important post-translational modification during translation process of proteins in organism (1), and the glycosylated proteins are known as “glycoproteins”. The oligosaccharides linked to glycoproteins are of two types: (i) *N*-linked oligosaccharides are attached to the side chain of asparagine only when the consensus sequence is Asn-X-Ser/Thr, where X can be any amino acid except proline. (ii) *O*-linked oligosaccharides are linked to a serine or threonine residue (1), with the former predominating (2). Thus, there are a large number of bioactive molecules occurring in nature, including glycoconjugates, which can significantly affect the biological functions and stability of glycoproteins on an organism. Therefore, a significance of human disease therapies obtained by changing in glycoforms can be used as diagnostic and/or therapeutic products (1).

Because the glycan structures resulting from some mammalian cells are close to human glycoforms (3), they have been prominently used for biopharmaceutical production. Chinese hamster ovary (CHO), 3T3, HEK293, BHK, HeLa and HepG2, rodent- or human-derived cells, are often used in biopharmaceutical research for numerous protein expressions (4). Today, nearly 70% of recombinant protein therapeutics is produced in CHO cells (4). The use of biopharmaceuticals generated from CHO cells has escalated in the last twenty years, and these agents are now among the ten best-selling biopharmaceutical products (5).

Because numerous biopharmaceutical products produced in CHO cells have been recognized for their potential clinical use, CHO cells have become a biopharmaceutical workhorse for the safe production of glycoprotein products (4). Moreover, CHO cells exhibit resistance to many human pathogenic viruses, including HIV, influenza, polio, and herpes (4,6–8). On the other hand, CHO cells can be readily scaled-up because of their adaptability and their ease of genetic modification (4). Thus, CHO cells have been used on an industrial scale with a high accretion in cell cultures. With so many benefits, CHO cells may well remain a premier host cell line for biopharmaceutical production, at least into the near future.

The terminal sialylation of *N*-glycans is one of the most important considerations for the quality of biopharmaceuticals, which should be studied in recombinant protein production (9). *N*-Acetylneuraminic acid (NeuAc/Neu5Ac), commonly known as sialic acid, is well-known as an important moiety because of its biological functions. Due to its strong electronegativity, Neu5Ac has the ability to enhance the stabilization and modulation of the interaction between molecules and membranes in various organisms. Sialic acid helps to enhance the stability of protein conformations and to protect cells from digestion by proteases and glycosidase. Neu5Ac is also believed to be able to modulate signaling in the transmembrane, as well as the differentiation and growth of cells. Moreover, Neu5Ac is reported to have antioxidant effects and to confer protection against apoptosis (1). With so many important effects on cells, glycans with terminal sialic acid are highly desired in biopharmaceutical glycoprotein production.

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This research was conducted by Nguyen Thi Sam in partial fulfillment of the requirements for a Ph.D.

1389-1723/$ – see front matter
https://doi.org/10.1016/j.jbiosc.2018.01.010

Please cite this article in press as: Thi Sam, N., et al., Enhancement of glycosylation by stable co-expression of two sialylation-related enzymes on Chinese hamster ovary cells, J. Biosci. Bioeng., (2018), https://doi.org/10.1016/j.jbiosc.2018.01.010

Sialylation can occur through α(2,3), α(2,6) or α(2,8) linkage. Neu5Ac residues can be attached to galactose by glycosyltransferases β-galactoside α(2,3) sialyltransferases (ST3GAL-I – ST3GAL-VI) and β-galactoside α(2,6) sialyltransferases (ST6GAL-I and II) (10). In human proteins, all these linkages have been observed, but the α(2,6) linkages are predominant. However, CHO glycans have primarily only α(2,3) linkages (3,11,12). Thus, glycoproteins that contain α(2,6) linked sialic acids would be more appropriate for the development of biopharmaceuticals for human use. Hence the recombinant proteins obtained by sialylation of terminal galactose are believed to be less immunogenic due to more human-like glycoproteins. The α(2,6) linked sialic acid residues are important for the anti-inflammation activity of human intravenous immunoglobulin (13,14). This may be an important factor contributing to the quality of the biopharmaceuticals. Accordingly, there is high demand in the biopharmaceutical field for a CHO host cell line containing α(2,6) linkage.

The gene encoding cytidine monophosphate (CMP)-N-acetylneuraminic acid hydroxylase in humans does not have an N-terminal domain required for its activity. Hence, NeuGc does not occur in humans, but it does exist in CHO-derived glycoproteins (15). UDP-GlcNAc 2-epimerase/ManNAc kinase (GNE) is known as a key enzyme for Neu5Ac synthesis. Humans can sometimes produce high level of circulating anti-NeuGc antibodies (16) suggesting that glycoproteins with NeuGc residues are not desirable products in the biopharmaceutical field.

Glycoforms are critical to the quality of many pharmaceutical products. There are many research approaches to controlling the glycosylation of CHO cells, such as changing the process, supplying media or engineering glycosylation genes. Adjusting bioprocess parameters such as pH, dissolved oxygen (DO), temperature and time; or cell culture parameters such as optimization and media components supplementation are common strategies (17–22). These methods are considered as attractive due to their simplicity and time efficiency. Nonetheless, these are expensive and low performance strategies with varying results depending on the cell types and targeted proteins. This suggests that there may be a better way to modulate glycoforms of targeted proteins, such as platform engineering, in specific CHO cells. Hence, genetic modification to modify the glycosylation of recombinant proteins is the most common strategy. As mentioned above, the production of drugs and/or therapeutics is most often accomplished using CHO cells, and there is thus a high demand for a better host cell line.

In consideration of the above, we attempted the glycoengineering of CHO *N*-glycans by introducing human ST6GAL-I and mouse GNE genes. The effects of the stable expression of these two Neu5Ac-based genes on the glycosylation profiles of host cell lines were elucidated. Specifically, the enzyme activity and free sialic acid content were elucidated using reverse phase-high-performance liquid chromatography (RP-HPLC). In addition, the *N*-linked glycosylation profiling was revealed in detail using liquid chromatography–mass spectrometry (LC–MS). This reveals an increase of sialylation in the total *N*-glycans of total proteins with an increase of free sialic acid content. We therefore conducted the present study to obtain proof of concept that the resulting glycosylation mutant would consistently exhibit superior sialylation.

MATERIALS AND METHODS

Cells and culture media Cell lines CHO-DG44 were cultured in a Minimum Essential Medium Eagle – Alpha Modification medium (Alpha MEM) (Invitrogen, MA, USA) with nucleosides and L-glutamine supplemented with 10% fetal bovine serum (FBS). Transfected CHO cell lines were cultured in a medium supplemented with 100 μg/mL neomycin. Selected transfectants were cultured in an (−) alpha MEM medium (Invitrogen) without nucleosides, supplemented with 10% dialyzed FBS as described above. All cells were cultured in a cell culture incubator maintained at 37°C and 5% CO_2.

Expression construct An attenuated internal ribosome entry site (IRESmt) was obtained by altering the IRES wild-type (IRESwt) from the Encephalomyocarditis virus (Invitrogen) at the ATG 10th, 12th with point mutation. By using primers, IRESwt was point mutated from A to G as described previously (23). The plasmid pcDNA-SG2 was formed by the insertion of open reading frame sequences for glycosylation genes ST6GAL-I, GNE and DHFR linked by IRESwt and IRESmt, respectively, into a pcDNA 3.1 (+) expression plasmid (Invitrogen) that contained a neomycin resistance coding sequence. All of the DNA fragments were prepared by PCR in a 96-well thermal cycler (Applied Biosystems, MA, USA) with the following primers:

5′ GTTGGTGTGAATCATCAAGCTTAAGTTTAAACGCTAGCCAG 3′ (sense for pcDNA 3.1 (+)), 5′ GTTGGTGTGAATCATCAAGCTTAAGTTTAAACGCTAGCCAG 3′ (anti-sense for pcDNA 3.1 (+)); 5′ TTAAACTTAAGCTTGATGATTCACACCAACCTGAAGAAAAAGTTCAGCTG 3′ (sense for ST6GAL-I), 5′ GGGGAGGGAGAGGGGT TAGCAGTGAATGGTCCGGAAGCC 3′ (anti-sense for ST6GAL-I); 5′ ACCATTCACTGCTAACCCCTCTCCCTCCCCCCC 3′ (sense for IRESwt), 5′ CCCATTCTTCTCCATAACATGGTTGTGGCAACATATTATAACATCGTGTTTTTCAAAG 3′ (anti-sense for IRESwt); 5′ AGCCACAACCATGTAATGGAGAAGAATGGGAATAACCGAAAGCTTC 3′ (sense for GNE), 5′ GGGGAGGGAGAGGGGCTAGTGGATCCTGCGGGTTGTG 3′ (anti-sense for GNE); 5′ CGCAGGATCCACTAGCCCCTCTCCCTCCCCCCC 3′ (sense for IRESmt), 5′ CAATGGTCGAACCATCACGGTTGTGGCCATATTATCACCGTG 3′ (anti-sense for IRESmt); 5′ ATGGCCACAACCGTGATGGTTCGACCATTGAACTGCATCG 3′ (sense for DHFR) and 5′TCAGCGGGTTTAAACTTAGTCTTTCTTCTCGTAGACTTCAAACTTATACTTGATGCC 3′ (anti-sense for DHFR).

The obtained DNA fragments were ligated using an In-fusion cloning kit (Takara, Shiga, Japan). Then, this plasmid was transfected into CHO-DG44 cells using Lipofectamine 2000 (Thermo Fisher Scientific, MA, USA) according to the manufacturer's instructions. The plasmid of pcDNA-SG2 was not linearized. Next, the plasmid was transfected into 80–90% frequency of CHO wild-type cells using Lipofectamin 2000 with the final concentration of neomycin sulfate G418 (Wako, Tokyo, Japan) 800 μg/mL in (−) alpha MEM medium (Invitrogen) without nucleosides. Then, the selected transfectants were maintained in the concentration of 100 μg/mL G418 in (−) alpha MEM medium.

Quantitative real-time PCR Cultured cells (1×10^7) were washed well with phosphate buffered saline (PBS) and harvested. An RNeasy Mini Kit (Qiagen, Hilden, Germany) was employed to extract total RNA from collected cells according to the manufacturer's instructions. The RNA was then used to synthesize complementary DNA (cDNA) using a SuperScript VILO cDNA Synthesis Kit (Invitrogen) following the manufacturer's instructions. To examine the expression of the investigated genes in transfected cells, quantitative real-time PCR (qRT-PCR) using FastStart Universal SYBR Green Master Mix (Rox) (Roche, Basel, Switzerland) was performed using a StepOnePlus real-time PCR system (Applied Biosystems) with the following primers: 5′ TGTGTGGAAGGAAAAGAAGAAAGG 3′ (sense primer for ST6GAL-I), 5′ GGGTGCTGCTTGAGGATACA 3′ (antisense primer for ST6GAL-I), 5′ GTGGAAGGG ATGTCAGTACCAAA 3′ (sense primer for GNE) and 5′ CCAAGTCCCAAAGCAGTTCC 3′ (antisense primer for GNE).

Preparation of cell extracts CHO cell lines were homogenized by ultrasonication with 25 mM Tris–HCl (pH 7.4), 0.25 M sucrose, 1 mM $MgCl_2$, 50 mM KCl, and protease inhibitor (Roche). Then, this mixture was ultra-centrifuged at 4°C and 112,000 ×*g* for 1 h. The resulting pellet was used as a microsomal fraction for the ST6GAL-1 enzymatic assay and the supernatant was used for sialic acid quantitation assays.

ST6GAL-I enzymatic assays The microsomes were re-suspended in 20 mM cacodylic acid (pH 6.0) with 0.1% Triton X-100. The suspension was then incubated at 4°C for 1 h and centrifuged at 10,000 ×*g* for 10 min to collect the supernatant. The collected supernatant was added to an assay mixture consisting of 20 mM cacodylic acid (pH 6.0), 20 mM $MgCl_2$, 5 mM CMP-Neu5Ac, 10 pmol Gal2-Gn2-M3-PA (Gal, galactose; Gn, *N*-acetylglucosamine; M3, 3 mannose and 2 Gn; PA, pyridylamine) and protease inhibitor. Thus, the mixture was then incubated at 37°C. After incubation, the mixture was heated at 100°C for 3 min and centrifuged at 10,000 ×*g* for 5 min. The supernatant was then subjected to a RP-HPLC. Neu5Ac(B)α(2,6)-Gal2-Gn2-M3- PA, Neu5Ac(A)α(2,6)Gal2-Gn2-M3-PA, Neu5Ac2α(2,6)-Gal2-Gn2-M3-PA, Gal2-Gn2-M3-PA, M8C (Takara) and Neu5Ac(B) α(2,3)-Gal2-Gn2-M3-PA, Neu5Ac(A)α(2,3)-Gal2-Gn2-M3-PA, and Neu5Ac2α(2,3)-Gal2-Gn2-M3-PA (Masuda, Japan) were used as glycan standards. PA-sugar chains were monitored on an RP-HPLC apparatus (Hitachi 7000 HPLC system, Hitachi, Tokyo, Japan) using a Cosmosil C18 column (4.6 × 250 mm; Nacalai Tesque, Kyoto, Japan) at the excitation and emission wavelengths of 310 nm and 380 nm, respectively, and eluted by linearly increasing the acetonitrile concentration from 0 to 35% in 0.02% trifluoroacetic acid (TFA) at a flow rate of 1.2 mL/min at 30°C for 50 min.

Free sialic acid quantitation Free Neu5Ac and NeuGc from the supernatant of cell extracts was labeled with 1,2-diamino-4,5-methylenedioxybenzene (DMB) using a sialic acid fluorescence labeling kit (Takara) according to the manufacturer's instructions. The DMB-sialic acids level were then monitored on an RP-HPLC apparatus (Hitachi 7000 HPLC system) using a Cosmosil C18 column (4.6 × 250 mm; Nacalai Tesque) at the excitation and emission wavelengths of

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