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Assessment of the coordinated role of ST3GAL3, ST3GAL4 and ST3GAL6 on the α2,3 sialylation linkage of mammalian glycoproteins

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ABSTRACT

In this research, we examined which genes are involved in N-linked sialylation in Chinese Hamster Ovary (CHO) cells using siRNA knockdown approaches. Three genes from the sialyltransferase family (ST3GAL3, ST3GAL4 and ST3GAL6) were chosen as knockdown targets with siRNA applied to reduce their expression. Single, double and triple gene knockdowns were investigated, and the reduction levels of sialylation on the total cell lysate were monitored by enzyme-linked lectin absorption assays (ELLA) and sialic acid quantification with high performance liquid chromatography (HPLC). All transfection groups showed effective reduction in 2,3-linked sialylation whereas the trend of reduction levels of triple siRNA transfection outweighed both the dual siRNA groups and single siRNA transfection groups. Next, this transfection approach was applied to CHO cells producing erythropoietin (EPO). Quantification of EPO sialylation showed similar result to total cell lysate except that the ST3GAL4 siRNA transfection exhibited the largest reduction according to the HPLC analysis as compared with other single siRNA transfections. Finally, the N-glycan released from the EPO transfected with ST3GAL4 siRNA showed a prominent reduction in sialyation level among the single siRNA transfections. From these experiments, we concluded that each of these three genes were involved in N-linked sialylation and ST3GAL4 may play the critical role in glycoprotein sialylation of recombinant proteins such as EPO.

1. Introduction

Glycosylation is one of the critical post translational modifications occurring in eukaryotic cells. Glycan structures affect numerous biological properties including development, growth, and survival by serving protective, stabilizing and signaling functions [1]. Sialylation represents the terminal step for many glycans with sialic acid playing an influential role in the biological function due to its size, hydrophilic characteristic and electronegative negative charge [2].

Another feature is that glycoprotein bearing sialic acid can hide the galactose residues from being recognized by asialoglycoprotein receptors (ASPR), a lectin in hepatocytes which captures the non-sialylated glycoproteins from blood circulation [3]. As a result, asialylated proteins will be cleared more rapidly than their sialylated counterparts. Consequently, researchers have observed that glycoprotein therapeutics bearing sialic acid often exhibit prolonged circulatory half-lives in vivo and improved efficacies [4].

On the other hand, limiting sialylation of glycoproteins can have other important in vivo implications for biomedicine and diagnostics. It has been reported that antibodies containing sialic acid exhibit reduced antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) [5]. In addition, radiolabeled diagnostic proteins such as radiolabeled EPO represent potential diagnosis candidates in order to evaluate the lung cancer prognosis [6]. However, radioactive diagnostic proteins having long circulatory half-lives may lead to overexposure of patients to radiation and can be detrimental to patient health. Therefore, modulating the sialic acid content on antibodies or radioactive diagnostic proteins may alter the application of these proteins' in biomedical contexts.

Sialylation of N-glycans is accomplished by the sialyltransferases which transfer the sialic acid from CMP-sialic acid onto a glycoprotein terminating in galactose. HEK 293 and NS0, the common protein production vehicles derived from human and murine, contain glycoprotein bearing both α2–3 and α2–6

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sialylation linkages. However, CHO cells produce glycoproteins having only α2−3 sialylation linkages because CHO cells contain α2−3 but no α2−6 sialyltransferases [7]. Six α2- 3 sialyltransferase family members, ST3GAL1 through ST3GAL6, have been identified and generally differentiated based on each sialyltransferase's substrate [8]. ST3GAL1 and ST3GAL2 have been shown to mask the galactose residues with sialic acids in O-glycans and glycolipids [8]. ST3GAL3 preferentially utilizes type I glycans (Galβ1-3GlcNAc) over the type II glycan (Galβ1-4GlcNAc), whereas ST3GAL4 has a preference of acting on type II glycan from the N-glycan [9,10]. ST3GAL5 is believed to act solely on lactosyceramide substrates [8]. ST3GAL6 has been shown to exhibit weak substrate specificity toward glycoproteins and has acted preferentially on glycolipids with substrates containing Galβ1-4GlcNAc-R [11]. Thus, ST3GAL3, ST3GAL4 and ST3GAL6 are believed to act on glycoproteins.

Previous studies of ST3GAL3, ST3GAL4 and ST3GAL6 have focused primarily on overexpression of these genes to enhance the sialylation and examine the biological consequences such as its effect on glycoproteins' circulatory half-lives and cancer metastasis [4,12,13]. Much less work has been performed to knockdown or knockout these genes individually or together. Mo et al. used siRNA to down-regulate the ST3GAL3 and/or ST3GAL4 expression on the endolyn produced in Madin-Darby canine kidney(MDCK) cells and addressed the effect of reducing sialylation on apical sorting of endolyn [14]. Yang et al. created ST3GAL4 and ST3GAL6 knockout mice and demonstrated that both genes are involved in the generation of functional selectin ligands in vivo [15]. However, previous research has not investigated the relevance of ST3GAL3, ST3GAL4 and ST3GAL6 simultaneously and especially their role on glycosylation of biotherapeutic molecules. Thus, the present study is to transiently knockdown the ST3GAL3, ST3GAL4 and ST3GAL6 genes through siRNA interference and perform a comprehensive discussion of the individual and coordinated roles of ST3GAL3, ST3GAL4 and ST3GAL6 sialylatransferases involved in erythropotein (EPO) expressing CHO cells, a heterologous and highly glycosylated biotherapeutic protein used in the treatment of anemia andradiolabelling imaging studies.

2. Materials & methods

2.1. Cell lines

A CHO-K1 cell line was purchased from Sigma−Aldrich (European Collection of Cell Culture, ECACC), and cultured with Ham's F-12K media with 10% FBS and 2 mM L-glutamine (Life technologies). An EPO-producing cell line was created by transfecting a mammalian cell expression plasmid containing a codon-optimized human EPO cDNA sequence linked with a six-histidine tag at the 3′ end. The CHO-EPO cell line was cultured in the same media as the CHO-K1 cell line except for the addition of 300ug/mL of Zeocin to maintain stable EPO expression.

2.2. siRNA transfection

The siRNAs targeting the ST3GAL genes were designed and purchased from Sigma. A negative control siRNA from Sigma was used as a negative control. The nucleotide sequences for ST3GAL3 siRNA were 5′ GUCACGAAUUGACGACUAUTT 3′ (sense) and 5′ AUAGUCGUCAAUUCGUGACTT 3′ (antisense); for ST3GAL4 siRNA were 5′ CCAUCACUAGCUAUUCUAUTT 3′ (sense) and 5′ AUAGAAUAGCUAGUGAUGGTT 3′ (antisense); and for ST3GAL6 siRNA were 5′ CCUUAAACUUGAUCUAUAATT 3′ (sense) and 5′ UUAUAGAUCAAGUUUAAGGTT 3′ (antisense). The siRNA transfection procedure was adapted from the Lipofectamine RNAiMax transfection protocol following the manufacturer's protocol. Briefly, 5×10^5 cells were seeded to each well in a 6-well plate. 24 h after seeding, dilute 9 ul of lipofectamine RNAiMax transfection reagent with 150 ul Opti-MEM media (Life technologies). Meanwhile, dilute 3 ul of 10 uM siRNA with 150 ul Opti-MEM media. Mix the diluted siRNA solution with the diluted lipofectamine RNAiMax solution and incubate for 20 min. Then the mixture was added to each well in the 6-well plate.

2.3. RT-PCR

RNA was isolated from the siRNA transfected CHO cells using an RNAeasy kit from Qiagen. 2 ug of the isolated RNA were used as the template for the first strand cDNA synthesis. A 15 ul mixture was created containing 2ug of isolated RNA, 1 ul of OligodT, and deionized water. This mixture was incubated at 70 °C for 5 min, and then put on ice immediately. A second mixture, containing 5 μl of 5 × MMLV buffer, 1.25 μl of 10 mM dNTP, 1 μl of RNase inhibitor, and l μl of Moloney murine leukaemia virus (MMLV) reverse transcriptase were added subsequently into the first mixture and incubated at 42 °C for 1 h to synthesize the cDNA.

The gene sequences of the sialyltransferase genes were obtained from CHOgenome.org. Specific PCR primer pairs of sialyltransferase genes were designed by the Primer3 website. The sequences of primers were as follows: ST3GAL3 (forward primer: 5′ CTGCTTGGAAGTTGCACTTG 3′ reverse primer: 5′ GTTTCTACGGAAGCTGGTGA 3′); ST3GAL4 (forward primer: 5′ AATCCCGCTGTAAGTTCCTG 3′ reverse primer: 5′ CACAATGTCTCCCAAGAGGC 3′); ST3GAL6 (forward primer: 5′ TGGCCTTAGTCTTGTGTTCC 3′ reverse primer: 5′ CTACTATGGAAACGCCACCA 3′); The gene fragments were amplified using a Veriti® thermocycler. For each reaction, 12.5 ul of 2x PCR Mastermix (Promega), 1ul each of 10 uM forward and reverse primer, 50 ng of cDNA, and nuclease-free water were added subsequently to make the final volume of 25 ul. Gel electrophoresis was performed to detect the gene expression levels.

2.4. SDS-page and western blotting analysis

CHOK1 cells transfected with siRNAs were lysed using RIPA buffer followed by brief sonication. The total cell lysate was subjected to SDS-PAGE and the protein samples were transferred to a PVDF membrane using Biorad wet blotting system under 100 V for an hour. For western blot, the membrane was blocked with 5% milk in PBST (Phosphate-buffered saline with 0.5% Tween 20) for an hour, followed by another hour of incubation in either rabbit anti-ST3GAL3 or rabbit anti-ST3GAL4 antibody. The membrane was then washed multiple times by 1X PBST and incubated with HRP-conjugated goat-anti-Rabbit IgG antibody for an extra hour. Finally, the membrane was visualized by an ECL detection substrate.

2.5. Maackia amurensis lectin II enzyme-linked lectin absorption assay (MAL II ELLA assay)

The protein concentration of total cell lysate or purified EPO from the siRNA transfected CHO cells was quantified by BCA assay (Pierce Biotechnology). 100 ul of Bicarbonate buffer(pH 9.6) was first added to each well in the 96-well microtiter plate. Add 2 ug of total cell lysate or purified EPO to each well subsequently and allow the protein samples to be coated at 4 °C overnight. Then remove the coating buffer and wash the plate three times with PBST (phosphate buffered saline with 0.5% (v/v) Tween 20). The wells were then blocked by 1X Carbo-Free blocking solution (Vector Lab) at 37 °C for 1 h. Remove the blocking buffer and wash the plate with PBST three times. 100 ul of 2 ug/mL of biotinylated Maackia amurensis lectin II (Vector Lab) in PBST was added to each well,