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Enhanced Sialylation of Recombinant Erythropoietin in CHO Cells by Human Glycosyltransferase Expression

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Sialylation, the attachment of sialic acid residues to a protein, can affect the biological activity and *in vivo* circulatory half-life of glycoproteins. Human α2,3-sialyltransferase (α2,3-ST) and β1,4-galactosyltransferase (β1,4-GT) are responsible for terminal sialylation and galactosylation, respectively. Enhanced sialylation of human erythropoietin (EPO) by the expression of α2,3-ST and β1,4-GT was achieved using recombinant Chinese hamster ovary (CHO) cells (EC1). The sialic acid content and sialylation of N-glycans were evaluated by HPLC. When α2,3-ST was expressed in CHO cells (EC1-ST2), the sialic acid content (moles of sialic acid/mole of EPO) increased from 6.7 to 7.5. In addition, the amount of trisialylated glycans increased from 17.3% to 26.1%. When α2,3-ST and β1,4-GT were coexpressed in CHO cells (EC1-GTST15), the degree of sialylation was greater than that in EC1-ST2 cells. In the case of EC1-GTST15 cells, the sialic acid content increased to 8.2 and the proportion of trisialylated glycans was markedly increased from 17.3% to 35.5%. Interestingly, the amount of asialoglycans decreased only in the case of GTST15 cells (21.4% to 14.2%). These results show that coexpression of α2,3-ST and β1,4-GT is more effective than the expression of α2,3-ST alone. Coexpression of α2,3-ST and β1,4-GT did not affect CHO cell growth and metabolism or EPO production. Thus, coexpression of α2,3-ST and β1,4-GT may be beneficial for producing therapeutic glycoproteins with enhanced sialylation in CHO cells.

Keywords: Glycosylation, sialylation, recombinant erythropoietin, sialyltransferase, galactosyltransferase

N-Glycosylation, which is one of most common posttranslational modifications in eukaryotic cells, can affect the properties of glycoproteins, including their enzymatic activity, antigenicity, stability, solubility, cellular processing, secretion, and *in vivo* clearance [12, 28]. Many recombinant proteins manufactured for human therapy are glycoproteins derived from animal cells. Since the glycans found in the recombinant glycoproteins of Chinese hamster ovary (CHO) cells are similar in structure and composition to those found in human glycoproteins, CHO cells have been widely used for the production of recombinant therapeutic glycoproteins related to a number of diseases [7].

Sialic acid, the terminal sugar in N-linked complex glycans, is usually found in glycoproteins. Sialic acid residues play a major role in determining the circulatory lifespan of glycoproteins, because they occupy the terminal position on the oligosaccharide chain, thereby masking the penultimate sugar, galactose, from recognition and uptake by hepatocyte asialoglycoprotein receptor [32]. Therefore, it is often desirable to maximize the sialic acid content of glycoproteins used as therapeutic agents to ensure their quality and consistency. For example, sialylated recombinant human erythropoietin (EPO) has a longer serum half-life and greater *in vivo* potency than the nonsialylated form [8].

Sialylation is mediated by sialyltransferases, which catalyze the attachment of CMP-sialic acid to the terminal galactose of complex oligosaccharides in the *trans*-Golgi, and human glycoproteins usually contain a mixture of both α2,3- and α2,6-linked sialic acids [27]. The expression of human α2,6-sialyltransferase in CHO cells producing tissue plasminogen activator (tPA) enhances the α2,6-sialylation of tPA [35]. In addition, coexpression of α2,3-sialyltransferase and β1,4-galactosyltransferase (α2,3-ST and β1,4-GT, respectively) results in greater than 90% sialylation of TNK-tPA and TNFR-IgG [31].

To enhance glycoprotein sialylation, we introduced sialyltransferase (ST) and galactosyltransferase (GT) into recombinant CHO cells. GT transfers UDP-galactose to the N-acetylglucosamine residue of N-glycan chains as a donor molecule; however, a terminal sialic acid residue

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can only be attached in the presence of a terminal galactose [16]. Our expectation was that coexpression of ST and GT would result in the production of more galactose termini, leading to more terminal sialic acid residues.

In this study, recombinant human EPO was used as a model protein. Human EPO, which is produced in the kidneys, controls erythrocyte differentiation. As erythrocytes carry the oxygen in the blood, the plasma concentration of EPO is important in controlling the blood oxygen concentration [26]. With one O-linked and three N-linked glycosylation sites, human EPO is a heavily glycosylated protein with the carbohydrate portion accounting for more than 40% of its molecular mass [29]. We coexpressed human α2,3-ST and β1,4-GT in CHO EC1 cells, which produce recombinant human EPO, in order to improve sialylation. In our analysis, recombinant human EPO was produced and purified from the culture supernatant of CHO cells. The N-linked oligosaccharides were subsequently released from the purified EPO by glycoamidase-F, derivatized with 2-aminopyridine, and the relative amount of sialylation was evaluated by anion-exchange HPLC.

Materials and Methods

Construction of the α2,3-ST and β1,4-GT Expression Vectors

The gene encoding human α2,3-ST (1,128 bp) was cloned by reverse transcription-polymerase chain reaction (RT-PCR; AccuPower RT/PCR PreMix kit; Bioneer, Daejeon, Korea) from human fibroblast cells, based on a previously reported sequence [15]. The forward primer was 5'-ATGGGACTCTTGGTATTTGT-3' and the reverse primer was 5'-TCAGATGCCACTGCTTAGAT-3'. The amplified products were sequenced by dideoxy sequencing. Human β1,4-GT gene was provided by Dr. Fukuda of the Burnham Institute [19]. The genes encoding human α2,3-ST and β1,4-GT were then inserted into the mammalian expression vector pcDNA3.1/Zeo(+) (Invitrogen, Carlsbad, CA, U.S.A.) to generate pcSTz and pcGTz, which express α2,3-ST and β1,4-GT, respectively.

Cell Lines and Culture Maintenance

The recombinant human EPO-producing CHO cell line (EC1, provided by Dr. H. J. Hong of the Korea Research Institute of Bioscience and Biotechnology) was constructed by introducing the cDNA encoding human EPO under the control of the Cytomegalovirus (CMV) promoter.

EC1 cells were transfected with pcSTz and pcGTz using Lipofectamine (Gibco, Grand Island, NY, U.S.A.). Drug selection was performed for 2 weeks by seeding 10^4 cells/well in 96-well tissue culture plates (Nunc, Roskilde, Denmark) containing MEMα (Gibco) supplemented with 10% dialyzed fetal bovine serum (dFBS; JRH, Lenexa, KS), 3.5 g/l glucose, 500 μg/ml zeocin (Invitrogen), 20 nM methotrexate (MTX; Sigma, St. Louis, MO, U.S.A.), and 1% antibiotic-antimycotic solution (Gibco) in a humidified atmosphere containing 5% CO_2 at 37°C. EC1 cells were also cultured in the same medium without zeocin. All cells were maintained as monolayer cultures in 75-cm^2 T-flasks (Nunc, Roskilde, Denmark). Two different cell lines were produced: those that expressed α2,3-ST only (EC1-ST) and those that expressed both α2,3-ST and β1,4-GT (EC1-GTST).

Detection of α2,3-ST and β1,4-GT by RT-PCR

Total RNA was extracted from EC1, EC1-ST, and EC1-GTST cells with TRizol reagent (Invitrogen) as described by the manufacturer, and 1 μg from each sample was used as the template for RT-PCR (AccuPower RT/PCR PreMix kit) to confirm the mRNA expression of α2,3-ST and β1,4-GT. To amplify α2,3-ST, forward primer (ST-f, 106–130 bp) 5'-GAGGAGGACTCCAATTCAGTGGTTC-3' and reverse primer (ST-r, 973–949 bp) 5'-CATAGCCAAATCCTGCG-ACTGCCAC-3' were used. In the case of β1,4-GT, forward primer (GT-f, 94–118 bp) 5'-CACCTTGGCGTCACCCTCGTTTACT-3' and reverse primer (GT-r, 1004-980) 5'-GCATTTGGGCGAGATATAG-ACATGC-3' were used. The amplified products were electrophoresed in a 1% agarose gel and visualized by ethidium bromide staining.

Western Blot Assay of α2,3-ST and β1,4-GT Expressions

Total cell lysates were prepared and subjected to 10% SDS-PAGE (20 μg each). The samples were then transferred to a nitrocellulose membrane (Amersham Biosciences, Uppsala, Sweden) overnight at 40 V. The blots were soaked in 3% skim milk for 2 h at room temperature before probing with anti-human α2,3-ST rabbit polyclonal antibodies and anti-human β1,4-GT rabbit polyclonal antibodies. The blots were then treated with anti-rabbit IgG donkey polyclonal antibody-HRP conjugate (Amersham Biosciences). After washing the blots with TBS-T buffer (Tris-buffered saline containing 0.1% Tween-20), the α2,3-ST- and β1,4-GT-specific bands were visualized using the ECL Western blotting system (Amersham Biosciences).

Culture Analysis

The cells were counted using a hemacytometer after trypsinization. The viable cells were distinguished from the dead cells using the trypan blue dye exclusion method. The secreted EPO concentration was measured by sandwich ELISA as described previously [3]. The glucose and lactate concentrations were measured using a glucose/lactate analyzer (Model 2300 STAT; Yellow Springs Instruments, Yellow Springs, OH, U.S.A.).

Production and Purification of EPO

The cells were cultivated as monolayer cultures in 175-cm^2 T-flasks (Nunc) in MEMα supplemented with 10% dFBS, 3.5 g/l glucose, 1% antibiotic-antimycotic solution, and 20 nM MTX. Exponentially growing cells were seeded at a concentration of 6.0×10^6 cells. After 3 days, the culture medium was replaced with a commercially available serum-free medium (CHO-S-SFMII; Gibco). Twenty-four hours later, the culture supernatant containing EPO was harvested, filtered through a 0.45-μm membrane, and stored at −70°C.

To isolate EPO, the culture supernatant was concentrated and dialyzed with phosphate-buffered saline (PBS) by ultrafiltration (Amicon Ultra; Millipore, Bedford, MA, U.S.A.). Briefly, the supernatant was loaded onto an immunoaffinity column consisting of CNBr-activated Sepharose 4B (Amersham Biosciences) coupled with monoclonal anti-human EPO (R&D Systems, Minneapolis, MN, U.S.A.) that had previously been equilibrated with PBS. After sample application and washing, the bound EPO was eluted with 0.1 M glycine and 0.5 M NaCl (pH 2.8), and the fractions were immediately neutralized with 1.0 M Tris-Cl (pH 9.0). The purified EPO was further evaluated by SDS-PAGE. It was then dialyzed and

lyophilized in order to evaluate the sialylation profile. The purity of the preparation was analyzed by 12.5% SDS-PAGE with Coomassie blue staining.

Determination of the Sialic Acid Content

The sialic acid content of the purified EPO was quantified using the method described by Anumula [2]. Sialic acid was released from the purified EPO under mild acid conditions and derivatized with *o*-phenylenediamine-2HCl (OPD; Sigma). The derivatized sialic acid was then analyzed on a C18-reversed-phase column (Shim-pack CLC-ODS; Shimadzu, Kyoto, Japan). The monosaccharide derivatives were detected with a fluorescence detector (474; Waters, Milford, MA, U.S.A.) set at the following wavelengths: excitation, 230 nm; emission, 425 nm.

Isolation and Analysis of N-Glycans from Recombinant EPO

N-Glycans were prepared from the purified recombinant EPO as described previously [1, 4]. Briefly, a trypsin-chymotrypsin (1% each, w/w of the substrate protein; Sigma) digest of EPO (~250 μg) was treated with glycoamidase F (3 U; Roche, Mannheim, Germany) in 25 mM sodium phosphate, pH 6.5, at 37°C overnight, and the mixture was passed through a Dowex 50×2(H+) column (Dow Chemical, Midland, MI, U.S.A.). The purified glycans were subsequently lyophilized and derivatized by reductive amination with 2-aminopyridine (pyridylamino, PA) and sodium cyanoborohydride [21, 34], and the PA-derivatized glycans were purified by gel filtration on a Sephadex G-15 (Amersham Biosciences) column (1.0×40 cm) using 10 mM NH_4HCO_3.

The PA-labeled glycans were then separated using an anion-exchange column (TSKgel DEAE-5PW, 7.5×75 mm; Tosoh, Tokyo, Japan), and the degree of sialylation was determined from the elution position [18]. Elution was achieved using 10% CH_3CN, pH 9.5 (E1), and 3% acetic acid-triethylamine:CH_3CN=90:10, pH 7.3 (E2). The initial condition was 100% E1 at a flow rate of 0.8 ml/min at 30°C, followed by 0–20% E2 for 40 min. The PA-derivatized glycans were monitored by a fluorescence detector (474; Waters) set at the following wavelengths: excitation, 310 nm; emission, 370 nm.

RESULTS

Expression of α2,3-ST and β1,4-GT in CHO EC1 Cells

The vectors pcSTz and pcGTz were constructed to express α2,3-ST and β1,4-GT, respectively. α2,3-ST transfers CMP-sialic acid to a galactose residue for terminal sialylation, whereas β1,4-GT transfers UDP-galactose to the N-acetylglucosamine residue of N-glycans for galactosylation. pcSTz was transfected into CHO EC1 cells, which produce recombinant human EPO, and nine candidate transfectants (EC1-ST) were selected with zeocin. pcSTz and pcGTz were subsequently cotransfected into the same host, and 11 clones (EC1-GTST) were selected with zeocin. To evaluate the level of α2,3-ST and β1,4-GT expression in the clones, RNA was isolated from each transfectant, and RT-PCR was performed to detect α2,3-ST and β1,4-GT transcription. The expression of α2,3-ST and β1,4-GT was verified by Western blotting. No α2,3-ST and β1,4-GT transcripts were detected in the control cells (EC1). Among the EC1-ST clones, cells expressing α2,3-ST (EC1-ST2) were selected based on the amplification of a 0.9-kb band (Fig. 1). For the EC1-GTST clones, those cells that expressed both α2,3-ST and β1,4-GT (EC1-GTST15) were selected based on the amplification of two 0.9-kb bands (Fig. 1). To analyze the sialylation of EPO, EC1-ST2 and EC1-GTST15 cells were selected and cultured to produce recombinant EPO.

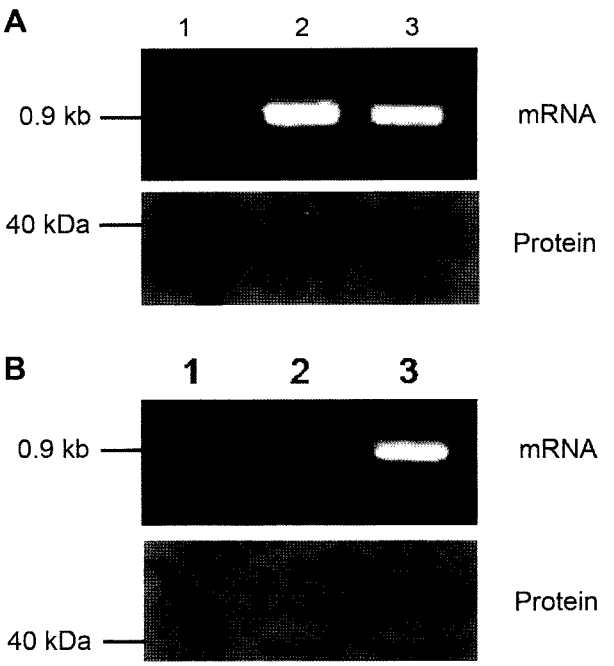

Fig. 1. RT-PCR and Western blot analysis of α2,3-ST (**A**) and β1,4-GT (**B**).
Total RNA isolated from the transfected cells was amplified by RT-PCR (α2,3-ST, 0.9 kb; β1,4-GT, 0.9 kb). The cell lysates were subjected to 10% SDS-PAGE and Western blot analysis. EC1 cells were used as a negative control. Lane 1, EC1; lane 2, EC1-ST2; lane 3, EC1-GTST15.

Cell Culture Analysis

The cells were cultivated as monolayer cultures in 7 ml of MEMα supplemented with 10% dFBS, 3.5 g/l glucose, 1% antibiotic-antimycotic solution, and 20 nM MTX in 25-cm^2 T-flasks (Nunc). After 3 days, the spent medium was replaced with an EPO production medium (CHO-S-SFMII).

Fig. 2 shows the amount of cell growth and EPO production in the EC1-ST2 and EC1-GTST15 cells. Both cell lines showed similar growth rates compared with the control (EC1). The cumulative EPO concentration in both the EC1-ST2 and EC1-GTST15 cells was similar to that of the EC1 cells in serum-free media. The glucose and lactate concentrations were measured in serum-free media (Fig. 2). Although the lactate formation rate of the EC1-ST2 cells was slightly lower than that of the other cell lines, the glucose and lactate concentrations in each culture were not

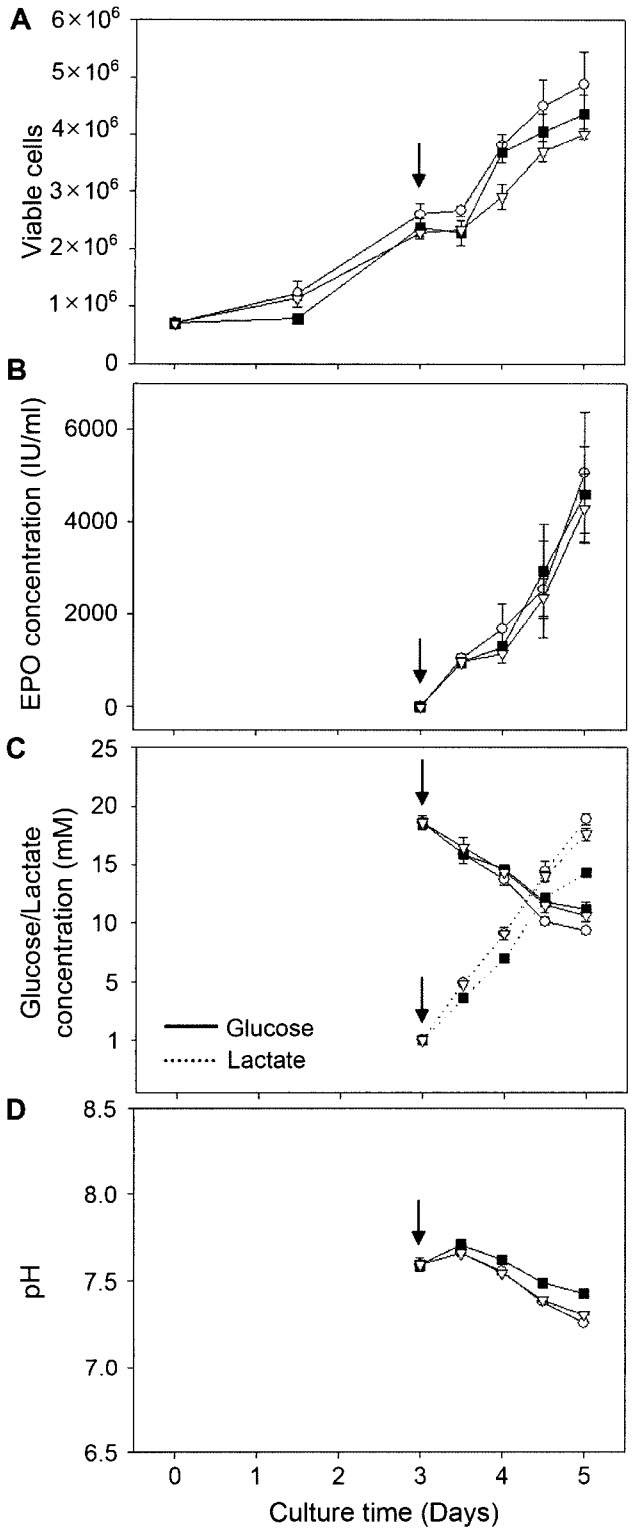

Fig. 2. Comparison of batch-cultured EC1 (○), EC1-ST2 (■), and EC1-GTST15 cells (▽).
Cell growth (**A**), EPO production (**B**), glucose consumption and lactate formation (**C**), and culture pH (**D**). When the cells were in the late-exponential growth phase (3 days after inoculation), the spent medium was replaced with serum-free medium (↓). Error bars represent the standard deviations calculated from the data obtained in triplicate experiments.

significantly different. The pH of the culture media over time was also similar. The specific metabolic rates were determined from a plot of the substrate and product concentrations against the time integral values of the growth curve [25]. The q_{EPO}, q_{Glc}, q_{Lac}, and $Y_{Lac/Glc}$ of the control and transfected cells are summarized in Table 1. The metabolic parameters of the EC1-ST2 and EC1-GTST15 cells were similar to those of the control (EC1). These data indicate that the expression of glycosyltransferases such as α2,3-ST and β1,4-GT in CHO cells under serum-free conditions does not significantly affect cell growth, EPO production, or other cellular metabolisms.

Sialylation Analysis of EPO in Recombinant CHO Cells (EC1-ST2/EC1-GTST15)

For EPO production, EC1, EC1-ST2, and EC1-GTST15 cells were cultivated in serum-free media for 24 h under a humidified atmosphere of 5% CO_2 at 37°C, and the supernatant was harvested from each culture. To analyze the sialylation of EPO, EPO was purified by immunoaffinity chromatography, yielding approximately 500 μg of purified protein. The purified EPO was then subjected to SDS-PAGE with Coomassie staining under reduced conditions (Fig. 3). No difference in molecular size was observed for EPO among the EC1 (control), EC1-ST2, and EC1-GTST15 cells (Fig. 3).

The sialic acid content of the purified EPO was also measured as the number of moles of sialic acid per mole of EPO. Human EPO has one O-linked and three N-linked glycosylation sites. Since each N-linked glycan has four sialic acid residues and each O-linked glycan has two sialic acid residues, there are 14 sialic acid residues possible per mole of EPO. Fig. 4 shows the sialic acid content of each CHO cell type. The sialic acid content was higher in EC1-ST2 than in EC1 (increased from 6.7 to 7.5); however, the increase was more pronounced in EC1-GTST15 (from 6.7 to 8.2).

To evaluate the sialylation pattern of EPO, the N-glycans from each purified EPO sample were isolated and labeled with 2-aminopyridine, as described above. The PA-glycans were then applied to an anion-exchange column (DEAE-5PW) and separated based on the number of attached sialic acid residues, which carry a negative charge. The PA-glycans were identified as neutral- (asialo-), mono-, di-, tri-, and tetrasialylated glycans. Our data indicate that the recombinant EPO produced by the CHO cells possessed asialo- and tetra-sialylated N-glycans, as does the EPO produced by human cells; thus, the glycosylation machinery of CHO cells is similar to that of human cells.

The elution profile of the PA-glycans from each sample is shown in Fig. 5. When α2,3-ST was expressed (EC1-ST2), relative sialylation increased; however, even more sialylated glycans were produced when both α2,3-ST and β1,4-GT were expressed (EC1-GTST15). The relative amounts of sialylated glycans in the control (EC1), EC1-

Table 1. Metabolic quotients of EC1, EC1-ST2, and EC1-GTST15 cells[a].

	q_{EPO} (IU/10^6 cells·day)	q_{Glc} (μmol /10^6 cells·day)	q_{Lac} (μmol/10^6 cells·day)	$Y_{Lac/Glc}$
EC1	4,750	7.9	16.1	2.0
EC1-ST2	4,500	6.3	14.1	1.9
EC1-GTST15	4,580	8.2	17.5	2.2

[a]Each value represents the mean of three independent cultures.

ST2, and EC1-GTST15 cells are shown in Table 2. In the case of EPO sialylation in EC1-ST2 and EC1-GTST15, total sialylation increased compared with the control. In particular, the pool of trisialylated glycans increased in both EC1-ST2 (17.3% to 26.1%) and EC1-GTST15 (17.3% to 35.5%). The proportion of monosialylated glycans in EC1-ST2 and EC1-GTST15 decreased from 31.2% to 21.2% and 18.1%, respectively. Interestingly, only in the case of the GTST15 cells did the amount of neutral glycans decrease (21.4% to 14.2%). These results indicate that expression of both α2,3-ST and β1,4-GT is more effective than expression of α2,3-ST alone in terms of sialylation.

DISCUSSION

To enhance EPO sialylation, we engineered CHO cells that overexpress the terminal glycosyltransferases α2,3-ST and β1,4-GT. α2,3-ST and β1,4-GT are responsible for the production of complex glycans. Based on our results, coexpression of α2,3-ST and β1,4-GT enhances the sialylation of EPO to a greater extent than does the expression of α2,3-ST alone.

In this study, the relative amount of monosialylated glycans was reduced compared with the other low-sialylated glycans. This suggests that monosialylated glycans are easily attached with sialic acid. Although the activity of α2,3-ST seemed to be more effective toward monosialylated glycans than other N-glycans, the proportion of tetrasialylated glycans remained relatively unchanged. Thus, there may be electrostatic hindrance when human α2,3-ST transfers CMP-sialic acid to trisialylated glycans, which inhibits the production of fully sialylated glycans in CHO cells. Previously, it was reported that the sialylation of human interferon-γ in CHO cells was improved by the transfection of sialyltransferases [10]. In that report, the amount of tetrasialylated glycans was slightly increased, whereas the amount of mono- and trisialylated glycans was significantly altered. Our results are in accordance with that report. Previous studies also reported that several sialyltransferases have branch specificity [13, 22]. Thus, to allow complete sialylation to be achieved, sialyltransferases that are less limited in terms of electrostatic hindrance and branch specificity must be identified, whether by screening in several species or by protein engineering.

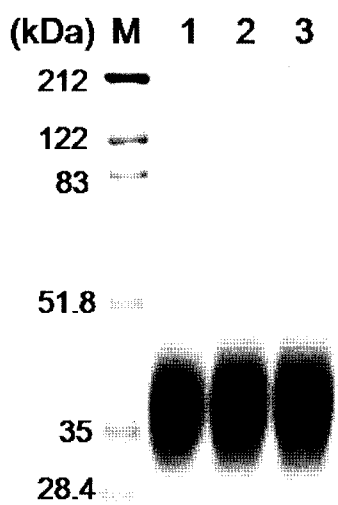

Fig. 3. SDS-PAGE of the purified EPO from each culture supernatant.
The supernatants were concentrated and dialyzed with PBS, and EPO was immediately isolated using an immunoaffinity column with 0.1 M glycine and 0.5 M NaCl (pH 2.8), and then neutralized with 1.0 M Tris-Cl (pH 9.0). The purified EPO was visualized by Coomassie staining. M, broad-range protein molecular size marker; Lane 1, EC1; lane 2, EC1-ST2; lane 3, EC1-GTST15.

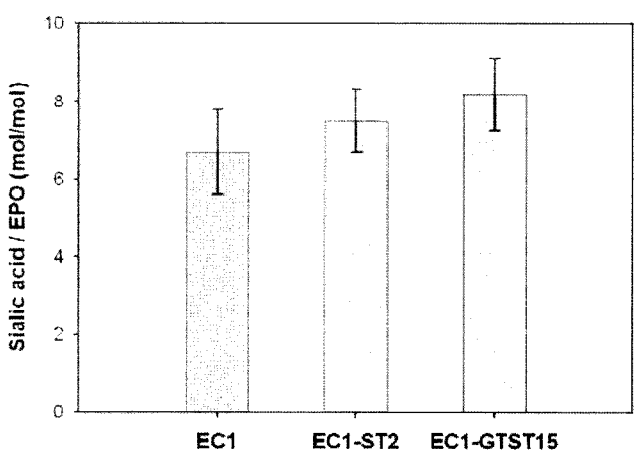

Fig. 4. EPO sialic acid contents of EC1, EC1-ST2, and EC1-GTST15 cells.
The sialic acid content was determined using the OPD-derivatization method. Error bars indicate the standard deviations calculated from data obtained in duplicate experiments.

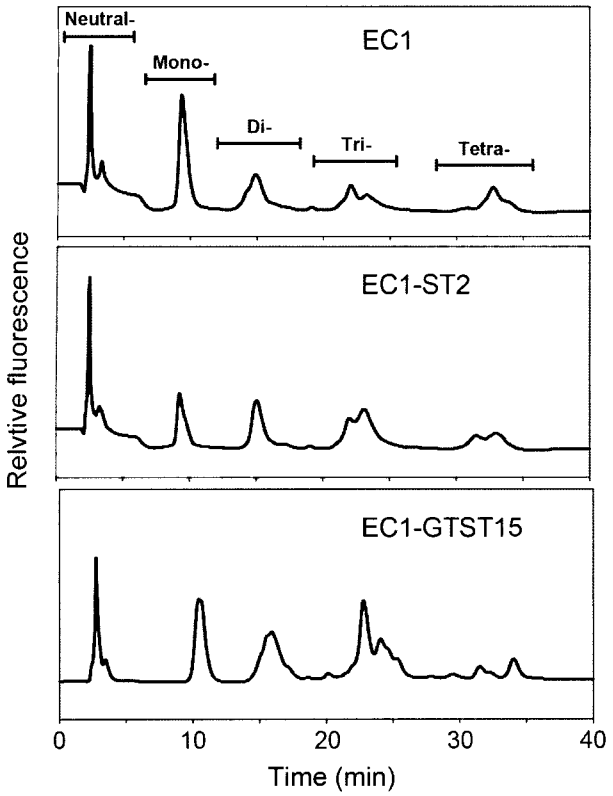

Fig. 5. Sialylation profile (DEAE-5PW column) of the PA-glycans from the EPO produced by EC1, ST2, and GTST15 cells cultured in serum-free medium.
The PA-glycans were separated based on the number of attached sialic acid residues. Asialo- (neutral) to tetrasialylated glycans were detected among the EPO glycans from the CHO cells.

The proportion of asialo-glycans was hardly changed by the overexpression of α2,3-ST alone, whereas it was reduced by the overexpression of α2,3-ST and β1,4-GT together. We speculate that α2,3-ST expression did not decrease the relative amount of asialo-glycans owing to the lack of galactose residues in asialo-glycans.

Many attempts have been made to increase the protein production rate and final titer in mammalian cells, including CHO cells, for industrial purposes. Most of these have involved altering the culture conditions or adding chemicals to the culture medium [3, 6, 17, 24], although genetic engineering has also been used to prolong CHO cell viability to increase protein production [14, 20]. However, these efforts have often resulted in reduced or incomplete sialylation. We previously reported that the addition of butyrate to CHO cells in order to increase EPO productivity resulted in decreased sialylation [5]. Moreover, the addition of N-acetylcysteine to cultured CHO cells for the same purpose caused decreased sialylation (unpublished results). Thus, our study is significant in that it offers the possibility of preventing decreased sialylation of EPO while still allowing increased EPO production.

To obtain enhanced or even complete sialylation of a glycoprotein, several approaches can be used to inhibit sialidase activity, which cleaves sialic acid residues from complex glycans [9, 23, 30]. For example, a greater than 30% increase in the sialic acid content of total glycoprotein was found by inhibiting sialidase activity with antisense RNA [9]. Moreover, removing limitations due to the size of the CMP-sialic acid pool or enhancing sugar-nucleotide transporter activity in the Golgi network may be required for complete sialylation. CMP-sialic acid, a precursor of the terminal sialic acid in glycans, is essential for terminal sialylation. Limiting the intracellular CMP-sialic acid pool can therefore hinder complete sialylation [11, 18]. The expression of CMP-sialic acid synthase elevates the level of CMP-sialic acid [18]. In addition, a sugar-nucleotide transporter is required for transporting CMP-sialic acid from the nucleus to the Golgi network. Sialylation involving CMP-sialic acid occurs in the Golgi network, but CMP-sialic acid is generated in the nucleus. The overexpression of a CMP-sialic acid transporter resulted in a 4–16% increase in interferon-γ sialylation [33]. Thus, if all of these methods are applied together, we may be able to maximize glycoprotein sialylation.

Table 2. Relative amount of sialylated glycans in the recombinant human EPO produced by EC1, EC1-ST2, and EC1-GTST15 cells[a].

Sialylated glycans (%)	EC1 (control)	EC1-ST2 (+α2,3-ST)	EC1-GTST15 (+α2,3-ST/β1,4-GT)
Neutral (asialo)	21.4±5.1	20.6±3.6	14.2±3.0
Mono-	31.2±4.2	21.2±2.9	18.1±0.8
Di-	19.1±1.3	19.6±1.1	21.8±1.3
Tri-	17.3±2.8	26.1±2.5	35.5±4.3
Tetra-	11.0±1.0	12.5±1.2	10.4±1.9

[a]The values are the means of duplicate cultures.

Acknowledgments

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