Enhanced sialylation of recombinant erythropoietin in genetically engineered Chinese-hamster ovary cells

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Sialic acid, the terminal sugar in N-linked complex glycans, is usually found in glycoproteins and plays a major role in determining the circulatory lifespan of glycoproteins. In the present study we attempted to enhance the sialylation of recombinant EPO (erythropoietin) in CHO (Chinese-hamster ovary) cells. To enhance EPO sialylation, we introduced human α2,3-ST (α2,3-sialyltransferase) and CMP-SAS (CMP-sialic acid synthase) into recombinant human EPO-producing CHO cells. The sialylation of EPO was increased by the expression of α2,3-ST alone. Although the co-expression of α2,3-ST and CMP-SAS did not further increase sialylation, an increase in the intracellular pool of CMP-sialic acid was noted. On the basis of these observations, it was postulated that the transport capacity of CMP-sialic acid into the Golgi lumen was limited, thereby causing the reduced availability of CMP-sialic acid substrate for sialylation. Therefore, we co-expressed human α2,3-ST and CMP-SAS, as well as overexpress Chinese hamster CMP-sialic acid transporter (CMP-SAT) in CHO cells, which produced recombinant human EPO. When α2,3-ST, CMP-SAS, and CMP-SAT were overexpressed in CHO cells, there was a corresponding increase in sialylation compared with the co-expression of α2,3-ST and CMP-SAS. The present study provides a useful strategy for enhancing the sialylation of therapeutic glycoproteins produced in CHO cells.

Introduction

The development and production of recombinant proteins for human therapeutic use has increased dramatically, and many of these products are glycoproteins expressed in mammalian cells. The physiological and pharmacological properties of glycoproteins are known to be affected by their covalently bound oligosaccharide moieties [1]. Sialic acid, the terminal sugar in N-linked complex glycans, is commonly 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 the hepatocyte asialoglycoprotein receptor [2]. Therefore, it is often desirable to maximize the sialic acid content of glycoproteins used as therapeutic agents in an attempt to ensure therapeutic effectiveness, quality and consistency.

Sialylation, the last event of the post-translational glycosylation modification process inside the Golgi, requires the metabolic generation of the nucleotide sugar CMP-sialic acid, followed by the transfer of the sialic acid to an acceptor oligosaccharide in the Golgi apparatus by sialyltransferases. Previous results suggested that an insufficient concentration of CMP-sialic acid within the Golgi may have been a potential cause for incomplete sialylation [3,4]. For this reason, several research groups have attempted to increase sialylation of recombinant proteins by the incorporation of nucleotide sugar precursors [5–7]. Although the intracellular pool of CMP-sialic acid has been shown to increase significantly due to nucleotide sugar precursors, the net result has been low or no change in the sialylation of the recombinant proteins. On the basis of these results it has been suggested that the transport capacity of CMP-sialic acid into the Golgi lumen is intrinsically limited, thereby reducing the availability of CMP-sialic acid substrate for sialylation [8,9]. It was also noted that an insufficient activity of sialyltransferase could cause incomplete sialylation [10].

To enhance sialylation, we overexpressed sialyltransferase, CMP-SAS (CMP-sialic acid synthase) and CMP-SAT (CMP-sialic acid transporter) in recombinant CHO (Chinese-hamster ovary) cells. In the case of mammalian cells, CMP-sialic acid is generated from sialic acid by CMP-SAS in the nucleus [11] and translocated across the Golgi membrane by CMP-SAT [12]. Human CMP-SAS [13] and

Key words: Chinese-hamster ovary cells (CHO cells), CMP-sialic acid synthase, CMP-sialic acid transporter, erythropoietin, sialylation, sialyltransferase.

Abbreviations used: 2-AP, 2-aminopyridine; CHO, Chinese-hamster ovary; CMP-SAS, CMP-sialic acid synthase; CMP-SAT, CMP-sialic acid transporter; dFBS, dialysed fetal bovine serum; EK, UDP-N-acetylglucosamine 2-epimerase/N-acetylmannosamine kinase; EPO, erythropoietin; GlcNAc, N-acetylglucosamine; ManNAc, N-acetylmannosamine; MEM, Eagle’s minimum essential medium; α modification; MTX, methotrexate; OPD, o-phenylenediamine; PA, pyridylamine; RT-PCR, reverse transcription PCR; α2,3-ST, α2,3-sialyltransferase; α2,6-ST, α2,6-sialyltransferase.

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hamster CMP-SAT [14] genes have been cloned. Recently, Wong et al. [15] reported that hamster CMP-SAT, expressed from vector functions in CHO cells, as well as the overexpression of CMP-SAT, resulted in an increase in interferon-γ sialylation. We postulated that the overexpression of CMP-SAS would elevate the level of CMP-sialic acid, and overexpression of CMP-SAT would facilitate the translocation of increased CMP-sialic acid into the Golgi apparatus. This would therefore lead to the improvement of sialylation through the enhanced transfer of the CMP-sialic acid to oligosaccharides by overexpression of sialyltransferase.

In the present study, recombinant human EPO (erythropoietin) was used as a model protein. Human EPO, which is produced in the kidneys, controls erythrocyte differentiation. With three N-linked and one O-linked glycosylation sites, human EPO is a highly glycosylated protein, with the carbohydrate portion accounting for more than 40% of its molecular mass [16]. Sialylated EPO has a longer serum half-life and greater in vivo potency than the non-sialylated form [17]. We co-expressed human α2,3-ST (α2,3-sialyltransferase) and CMP-SAS and also overexpressed Chinese-hamster CMP-SAT in CHO EC2-1H9 cells that produced recombinant human EPO. We investigated the effect of CMP-SAS expression on the intracellular pool of CMP-sialic acid. Furthermore, recombinant human EPO was purified from the culture supernatant of CHO cells and sialylation of the purified EPO was evaluated.

Materials and methods

Construction of the expression vectors
The genes encoding human α2,3-ST and Chinese-hamster CMP-SAT were cloned by RT-PCR (reverse transcription-PCR) (AccuPower RT/PCR PreMix kit, BioNeer, Daejeon, Korea) from human fibroblast cells and CHO EC2-1H9 cells, respectively based on previously reported sequences (GenBank® accession numbers L23768 and Y12074). For the purposes of cloning α2,3-ST, the forward (5′-ATGGG-ACCTTTGATATTTTG-3′) and reverse (5′-TCAGATGC-CACCTGATGATAGAT-3′) primers were used. In the case of CMP-SAT, the forward primer was 5′-CAGCTAGCCACCATGGCTAGGCAG-3′ and the reverse primer was 5′-TCCGAATTCCTCACACACCAATGACTCTTTGC-3′, where the introduced restriction sites are underlined, and the coding regions of the CMP-SAT are shown in bold. NheI and EcoRI restriction sites were introduced up- and downstream of the coding region for subsequent subcloning. The amplified products were sequenced by dideoxy sequencing. Human CMP-SAS gene was provided by Professor Michael Betenbaugh, Department of Chemical and Biomolecular Engineering, The Johns Hopkins University, Baltimore, MD, U.S.A. (GenBank® accession number AF397212). The genes encoding human α2,3-ST and CMP-SAS and Chinese-hamster CMP-SAT were then inserted into the mammalian expression vector pcDNA3.1/Zeo(+) (Invitrogen, Carlsbad, CA, U.S.A.) to generate pcSTz, pcCSz and pcCSATz, which expressed α2,3-ST, CMP-SAS and CMP-SAT respectively.

Cell lines and culture maintenance
The recombinant human EPO-producing CHO cell line (EC2-1H9; provided by Dr Hyo Jeong Hong, Antibody Engineering Research Unit, Korea Research Institute of Bioscience and Biotechnology, Yuseong-gu, Taejon, Korea) was constructed by introducing the cDNA encoding human EPO under the control of the cytomegalovirus promoter.

EC2-1H9 cells were transfected with pcSTz, pcCSz, and pcCSATz using Lipofectamine™ (Gibco, Grand Island, NY, U.S.A.). Drug selection was performed for 2 weeks by seeding 10⁴ cells/well in 96-well tissue-culture plates (Nunc, Roskilde, Denmark) containing MEMα (Eagle’s minimum essential medium, α modification; Gibco) supplemented with 10% (v/v) dFBS (dialysed fetal bovine serum; JRH Biosciences, Lenexa, KS, U.S.A.), 3.5 g/l glucose, 500 μg/ml zeocin (Invitrogen), 20 nM MTX (methotrexate; Sigma, St Louis, MO, U.S.A.) and 1% (v/v) antibiotic/antimycotic solution (Gibco) in a humidified atmosphere containing 5% CO₂ at 37°C. EC2-1H9 cells were also cultured in the same medium without zeocin. All cells were maintained as monolayer cultures in 75 cm² T-flasks (Nunc). Four different cell lines were produced: those that expressed α2,3-ST only (EC2-1H9-ST), those that expressed both α2,3-ST and CMP-SAS (EC2-1H9-CST), those that expressed α2,3-ST, CMP-SAS and CMP-SAT (EC2-1H9-SCST) and those transfected with the null pcDNA3.1/Zeo(+) plasmid as a negative control (EC2-1H9-Δ2-3).

Detection of α2,3-ST and CMP-SAS by RT-PCR
Total RNA was extracted from the recombinant CHO cells with TRIZol® reagent (Invitrogen) as described by the manufacturer, and 1 μg from each sample was used as the template for RT-PCR to confirm the mRNA expression of α2,3-ST and CMP-SAS. To amplify α2,3-ST, the forward primer (ST-f, 106–130 bp) 5′-GAGGAGGACTCTCCTCATGATTATGATAGAT-3′ and reverse primer (ST-r, 973–949 bp) 5′-CATAGCCTGTTCTTAAAATATGTTCTGTTTATTCGC-3′ were used. In the case of CMP-SAS, the forward primer (CSAS-f, 1–25 bp) 5′-ATGGG-ACCTTTGATATTTTG-3′ and reverse primer (CSAS-r, 1305–1281 bp) 5′-TCCGAATTCCTCACACACCAATGACTCTTTGC-3′ were used. The amplified products were electrophoretically resolved on a 1% agarose gel and visualized by ethidium bromide staining.

Western-blot assay of α2,3-ST and CMP-SAS expression
Total cell lysates were prepared and subjected to SDS/10%-(w/v)-PAGE (20 μg of total protein in each lane). The
samples were then transferred on to a nitrocellulose membrane (Amersham Biosciences, Uppsala, Sweden) overnight at 40 °C. The blots were soaked in 3 % (w/v) fat-free dried skimmed milk for 2 h at room temperature (25 °C) before probing with rabbit anti-(human α2,3-ST) polyclonal antibodies and rabbit anti-(human α2,6-ST) polyclonal antibodies. The blots were then treated with donkey anti-(rabbit IgG) polyclonal antibody–horseradish peroxidase conjugate (Amersham Biosciences). After washing the blots with TBS-T buffer [Tris-buffered saline (10 mM Tris/HCl and 150 mM NaCl, pH 7.4) containing 0.1 % Tween-20], the α2,3-ST- and CMP-SAS-specific bands were visualized using the ECL (enhanced chemiluminescence) Western-blotting system (Amersham Biosciences).

Detection of CMP-SAT transcript level by real-time PCR
Total RNA was extracted from CHO cells, and reverse transcription of 1 μg of RNA to cDNA was performed using AccuPower RT PreMix. The cDNA was used as the template in the real-time PCR. Real-time PCR was carried out using a MiniOpticon Mj Mini instrument (Bio-Rad, Hercules, CA, U.S.A.). The PCR conditions used were an initial denaturation of 95 °C for 5 min, followed by 40 cycles of 95 °C for 20 s, 58 °C for 30 s, and 72 °C for 30 s. The amplification reaction was performed in a final volume of 20 μl. Each reaction mixture contained 2 × iQTM SYBR® Green Supermix (Bio-Rad), 10 pmol of primers and 5 μl of the cDNA template. To detect CMP-SAT expression, 5′-TGATTAA GTGTTGGACTTTTAGC-3′ as the forward primer and 5′-CTTCAGTGTAGGTAACCTGG-3′ as the reverse primer. Primers for detection of CHO β-actin were 5′-AGCTGAGAGGGAAATTGTGCG-3′ as the forward primer and 5′-GCAACGGAACCGCTCATT -3′ as the reverse primer. After PCR amplification, data analysis was performed using Mj Opticon Monitor version 3.1 software (Bio-Rad).

Cell counting and EPO assay
The cells were counted using a haemocytometer after trypsinization. The viable cells were distinguished from the dead cells using the Trypan Blue dye-exclusion method. The concentration of secreted EPO was measured using a sandwich ELISA method. Nunc 96-well plates were coated with anti-(human EPO) monoclonal antibodies (R&D Systems, Minneapolis, MN, U.S.A.) and blocked with BSA. EPO standard and culture supernatant samples were loaded into the wells and treated with rabbit anti-(human EPO) polyclonal antibody (Sigma). Goat anti-(rabbit IgG) polyclonal antibody–horseradish peroxidase conjugate was used as the secondary antibody. The Amax of the coloured substrate OPD (o-phenylenediamine; Sigma) was measured using an ELISA plate reader (Bio-Rad).

Measurement of intracellular CMP-sialic acid in CHO cells
Nucleotide sugars were extracted from CHO cells, and intracellular CMP-sialic acids were quantified as described by Tomiya et al. [18]. Cells were lysed in ice-cold 75 % (v/v) ethanol using a sonicator (Sonifier Cell Disruptor 350; Branson, Danbury, CT, U.S.A.; 50 % amplitude at 1 W; ten bursts of 20 s each, with 90 s cool-down on ice; repeated four times). Soluble fractions were collected and freeze-dried. The samples were resuspended in 40 mM phosphate buffer, pH 9.2, to stabilize CMP-sialic acids, and then centrifuged (15 000 g for 10 min at 4 °C). The supernatants obtained were filtered through a 10 000-molecular-mass-cut-off membrane (Millipore, Bedford, MA, U.S.A.). Nucleotide-sugars were separated using a CarboPac PA-1 column with a PA-1 guard column ( Dionex, Sunnyvale, CA, U.S.A.) and detected by their A260 (model 486 tunable UV-visible absorbance detector; Waters, Milford, MA, U.S.A.).

Production and purification of EPO
The cells were cultivated as monolayer cultures in 175 cm2 T-flasks (Nunc) in MEMα supplemented with 10 % (v/v) dFBS, 3.5 g/l glucose, 1 % (v/v) antibiotic/antimycotic solution and 20 mM MTX. Exponentially growing cells were seeded at a concentration of 1.2 × 10⁷ cells. After 3 days, the culture medium was replaced with a commercially available serum-free medium (CHO-S-SFMII; Gibco). After 2 days, the culture supernatant containing EPO was harvested, filtered through a 0.45-μm-pore-size membrane and stored at −70 °C.

To isolate EPO, the culture supernatant was concentrated and dialysed with PBS by ultrafiltration (Amicon Ultra; Millipore). Briefly, the supernatant was loaded on to an immunoaffinity column consisting of CNBr-activated Sepharose 4B (Amersham Biosciences) coupled with anti-(human EPO) monoclonal antibodies (R&D Systems) that had previously been equilibrated with PBS. After sample application and washing, the bound EPO was eluted with 0.1 M glycine/0.5 M NaCl (pH 2.8), and the fractions were immediately neutralized with 1.0 M Tris/HCl (pH 9.0). The purified EPO was further evaluated by SDS/PAGE. It was then dialysed and freeze-dried in order to evaluate the sialylation profile. The purity of the preparation was analysed using SDS/12.5 %-PAGE followed by 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 [19]. Sialic acid was released from the purified EPO under mild acidic conditions and derivatized with OPD dihydrochloride (Sigma). The derivatized sialic acid was then analysed on a C18 reversed-phase column (Shim-pack CLC-ODS; Shimadzu, Kyoto,
Japan). The monosaccharide derivatives were detected with a model 474 fluorescence detector (Waters) 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 [20,21]. Briefly, a trypsin (trypsin/substrate protein, 1:100, w/w) digest of EPO (≈250 μg) was treated with glycoamidase F (3 units; Roche, Mannheim, Germany) in 25 mM sodium phosphate, pH 6.5, at 37 °C overnight, and the mixture was passed through a Dowex 50-X2 (H⁺) column (Dow Chemical, Midland, MI, U.S.A.). The purified glycans were subsequently lyophilized and derivatized by reductive amination with 2-AP (2-aminopyridine) and sodium cyanoborohydride [22,23] and the PA (pyridylamino)-derivatized glycans were purified by gel filtration on a Sephadex G-15 (Amersham Biosciences) column (1.0 cm × 40 cm) using 10 mM NH₄HCO₃. The PA-labelled glycans were then separated using an anion-exchange column (TSKgel DEAE-5PW; 7.5 mm × 75 mm; Tosoh, Tokyo, Japan), and the degree of sialylation was determined from the elution position [23]. Elution was achieved using 10 % acetonitrile, pH 9.5 (E1) and 3 % (v/v) (acetic acid/triethylamine)/acetonitrile (90:10, v/v), 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 using the model 474 fluorescence detector set at the following wavelengths: excitation, 310 nm; emission, 370 nm.

Results
Expression of α2,3-ST, CMP-SAS and CMP-SAT in CHO EC2-1H9 cells
The three expression vectors used in the present study were pcSTz, pcCSz and pcCSATz, which expressed α2,3-ST, CMP-SAS, and CMP-SAT respectively. pcSTz was transfected into CHO EC2-1H9 cells that produced recombinant human EPO, and eight candidate transfectants (EC2-1H9-ST) were selected with zeocin. pcSTz and pcCSz were co-transfected into the same host, and nine clones (EC2-1H9-CST) were selected with zeocin. As mentioned above, RT–PCR was carried out to detect the transcription of α2,3-ST and CMP-SAS. Among the EC2-1H9-CST clones, cells expressing both α2,3-ST and CMP-SAS mRNA (EC2-1H9-SCST1 and EC2-1H9-SCST3) were selected (Figure 2). To screen the subclones for CMP-SAT overexpression, it was necessary to detect the transcript level of CMP-SAT more precisely, because the CMP-SAT gene is expressed endogenously in CHO cells. Real-time PCR was chosen to detect the overexpression of CMP-SAT, since this method has been shown to provide distinct advantages over traditional PCR detection. Real-time PCR analysis has a wide dynamic range of quantification of 7–8 logarithmic decades, a high technical sensitivity and high precision [24]. cDNAs were extracted from the clones expressing both α2,3-ST and CMP-SAS mRNA and used as the template in the real-time amplification of a 0.9 kb band (Figure 1). For the EC2-1H9-CST clones, those cells that expressed both α2,3-ST and CMP-SAS (EC2-1H9-CST6) were selected on the basis of the amplification of 0.9 kb and 1.3 kb bands (Figure 1).

pcSTz, pcCSz and pcCSATz were co-transfected into EC2-1H9 cells, and 12 candidate transfectants (EC2-1H9-SCST) were selected with zeocin. As mentioned above, RT–PCR was carried out to detect the transcription of α2,3-ST and CMP-SAS. Among the EC2-1H9-SCST clones, cells expressing both α2,3-ST and CMP-SAS mRNA (EC2-1H9-SCST1 and EC2-1H9-SCST3) were selected (Figure 2). To screen the subclones for CMP-SAT overexpression, it was necessary to detect the transcript level of CMP-SAT more precisely, because the CMP-SAT gene is expressed endogenously in CHO cells. Real-time PCR was chosen to detect the overexpression of CMP-SAT, since this method has been shown to provide distinct advantages over traditional PCR detection. Real-time PCR analysis has a wide dynamic range of quantification of 7–8 logarithmic decades, a high technical sensitivity and high precision [24]. cDNAs were extracted from the clones expressing both α2,3-ST and CMP-SAS mRNA and used as the template in the real-time amplification of a 0.9 kb band (Figure 1). For the EC2-1H9-CST clones, those cells that expressed both α2,3-ST and CMP-SAS (EC2-1H9-CST6) were selected on the basis of the amplification of 0.9 kb and 1.3 kb bands (Figure 1).
PCR reaction. The threshold cycle value \((C_t)\) was defined by the amplification plot of real-time PCR. The amount of CMP-SAT transcript was calculated based on \(C_t\) and normalized with the transcript level of the housekeeping gene coding for \(\beta\)-actin. Figure 3 shows the fold increase in CMP-SAT transcript with respect to the control cells (EC2-1H9). The CMP-SAT expression level of EC2-1H9-SCST1 and EC2-1H9-SCST3 increased about 5- and 8-fold respectively compared with that of the control cells. In the case of null vector cells (EC2-1H9-\(\Delta 2\)-3), the transcript level was similar to that of the control cells. To analyse the sialylation of EPO, EC2-1H9-SCST1 and EC1-1H9-SCST3 cells were selected and cultured to produce recombinant EPO.

**Cell growth and EPO production**

The cells were cultivated as monolayer cultures in 6 ml of MEM supplemented with 10\% (v/v) dFBS, 3.5 g/l glucose, 1\% (v/v) 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). Figure 4 shows cell growth and EPO production in the EC2-1H9-ST9, EC2-1H9-CST6 and EC2-1H9-SCST3 cells. The transfected cell lines showed similar growth rates compared with the control (EC2-1H9). The cumulative EPO concentration in the EC2-1H9-ST9, EC2-1H9-CST6 and EC2-1H9-SCST3 cells was similar to that of the EC2-1H9 cells in serum-free medium. The null vector cells (EC2-1H9-\(\Delta 2\)-3) showed similar profiles compared with the control cells. These results indicated that the expression of \(\alpha 2,3\)-ST, CMP-SAS, and CMP-SAT in CHO cells under serum-free conditions did not significantly affect the cell growth and EPO production.

**Quantification of intracellular CMP-sialic acid content**

To investigate the effect of the introduced-gene expression on the intracellular pool of CMP-sialic acid, exponentially growing CHO cells were harvested and nucleotide-sugars were extracted from the CHO cells. The nucleotide-sugars were separated by HPLC with a UV detector measuring \(A_{260}\). The peak of CMP-sialic acid was identified by comparing with a standard. The concentration of CMP-sialic acid was calculated against a standard of known concentration and normalized with respect to the cell number (Figure 5).

As Figure 5 shows, the intracellular CMP-sialic acid level was increased 1.5-fold in the CHO cells that expressed both \(\alpha 2,3\)-ST and CMP-SAS (EC2-1H9-CST6) compared with the control cells (EC2-1H9). Also, similar levels were shown in both EC2-1H9-SCST1 and EC2-1H9-SCST3 cells that expressed \(\alpha 2,3\)-ST, CMP-SAS and CMP-SAT simultaneously. Although there was a slight increase in the intracellular CMP-sialic acid level when \(\alpha 2,3\)-ST was expressed alone in EC2-1H9-ST9 cells, it was not significant.
Figure 5  Intracellular CMP-sialic acid contents in EC2-1H9 (EC2), EC2-1H9-Δ2-3 (Null vector), EC2-1H9-ST9 (ST9), EC2-1H9-CST6 (CST6), EC2-1H9-SCST1 (SCST1) and EC2-1H9-SCST3 (SCST3) cells

The CMP-sialic acid contents were normalized with respect to cell number. Results are means ± S.D. for duplicate experiments.

compared with that in EC2-1H9-CST6, EC2-1H9-SCST1 and EC2-1H9-SCST3 cells. These results indicate that the synthesis of CMP-sialic acid was enhanced by CMP-SAS expression, thereby elevating somewhat the intracellular pool of CMP-sialic acid in CHO cells. However, the proportion of the increase in CMP-sialic acid content was limited probably due to the feedback inhibition of the single bifunctional enzyme EK (UDP-N-acetylglucosamine 2-epimerase/N-acetylmannosamine kinase) by CMP-sialic acid [25]. The null vector cells (EC2-1H9-Δ2-3) showed similar CMP-sialic levels compared with the control cells.

Sialylation analysis of EPO in genetically engineered CHO cells

For EPO production, cells were cultivated in serum-free medium for 2 days and the supernatant was harvested from each culture. To analyse the sialylation of EPO, EPO was purified by immunoaffinity chromatography. The purified EPO was then subjected to SDS/PAGE with Coomassie Blue staining under reducing conditions. No difference in the molecular size was observed for EPO among the samples (results not shown).

The sialic acid content of the purified EPO was measured as mol of sialic acid/mol 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 mol of EPO [26]. Figure 6 shows the sialic acid content of each CHO cell type. The sialic acid content was higher in EC2-1H9-ST9 than in EC2-1H9 (increased from 6.1 to 6.9). However, there was a marginal increase in EC2-1H9-CST6 compared with EC2-1H9-ST9 (from 6.9 to 7.1), although the intracellular CMP-sialic acid level was increased 1.5-fold. When CMP-SAT was overexpressed, sialylation further increased. Sialic acid content was higher in material from EC2-1H9-SCST1 and EC2-1H9-SCST3 cells than from EC2-1H9-CST6 cells (increased from 7.1 to 7.6 and 8.0 respectively). The sialic acid content of EC2-1H9-Δ2-3 cells was similar to that of EC2-1H9 cells.

To evaluate the sialylation pattern of EPO, the N-glycans from each purified EPO sample were isolated and labelled with 2-AP as described above. The PA-glycans were then applied to an anion-exchange column (DEAE-SPW) and separated, using HPLC, on the basis of the number of attached sialic acid residues that carried a negative charge. The PA-glycans were identified as neutral- (asialo-), mono-, di-, tri-, and tetra-sialylated glycans. Figure 7 shows the elution profile of the PA-glycans from each sample. The relative amounts of sialylated glycans were quantified on the basis of peak area (Table 1). As expected, relative sialylation increased in EC2-1H9-ST9 and EC2-1H9-CST6 compared with the control (EC2-1H9). The amount of trisialylated glycans increased in both EC2-1H9-ST9 (from 27.0 to 32.1%) and EC2-1H9-CST6 cells (from 27.0 to 33.0%). The proportion of monosialylated glycans in EC2-1H9-ST9 decreased from 27.2 to 23.5 and 23.0% respectively. It is noteworthy that the expression of both α2,3-ST and CMP-SAS did not increase the relative sialylation significantly compared with the expression of α2,3-ST alone. However, when CMP-SAT was co-expressed with both α2,3-ST and CMP-SAS, the sialylation substantially increased in both EC2-1H9-SCST1 and EC2-1H9-SCST3 compared with EC2-1H9-CST6 cells. Instead, the relative amount of monosialylated glycans in both EC2-1H9-SCST1...
Figure 7  Sialylation profile (DEAE-SPW column) of the PA-glycans from the EPO produced by EC2-1H9 (EC2), EC2-1H9-Δ2-3 (Δ2-3), EC2-1H9-ST9 (ST9), EC2-1H9-CST6 (CST6), EC2-1H9-SCST1 (SCST1)) and EC2-1H9-SCST3 (SCST3) cells cultured in serum-free medium

The PA-glycans were separated on the basis of the number of attached sialic acid residues. Asialoglycans (neutral) to tetrasialylated glycans were detected among the EPO glycans from the CHO cells.

Table 1  Relative amount of sialylated glycan (SG) in the recombinant EPO produced by EC2-1H9 (EC2), EC2-1H9-Δ2-3 (Δ2-3), EC2-1H9-ST9 (ST9), EC2-1H9-CST6 (CST6), EC2-1H9-SCST1 (SCST1) and EC2-1H9-SCST3 (SCST3) cells

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<th>CST6</th>
<th>SCST1</th>
<th>SCST3</th>
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<td>9.8</td>
<td>9.1</td>
<td>7.7</td>
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<td>Tetra-</td>
<td>6.8</td>
<td>6.6</td>
<td>7.5</td>
<td>8.7</td>
<td>14.4</td>
<td>15.5</td>
</tr>
</tbody>
</table>

Discussion

In the present study we found that the co-expression of both α2,3-ST and CMP-SAS did not lead to an increased sialylation of recombinant EPO compared with the expression of α2,3-ST alone, although there was an increase in the intracellular CMP-sialic acid level. However, increased sialylation was achieved by the overexpression of CMP-SAT coupled with the co-expression of α2,3-ST and CMP-SAS. This implies that the translocation of CMP-sialic acid into the Golgi was enhanced by the overexpression of CMP-SAT, which in turn resulted in the increased sialylation of EPO produced in CHO cells.

One of us [27] has previously shown that EPO sialylation was improved by the expression of α2,3-ST in a different CHO cell line. Although the extent of increase in the present study was comparatively lower than that found in the previous one [27], it is suggested that the expression of α2,3-ST is effective for the enhancement of EPO sialylation. If cell lines showing higher sialyltransferase activity were selected by measuring the activity, the sialylation may have been further enhanced. CHO-derived glycoproteins differ from human-derived glycoproteins with regard to the type of sialic acid linkage. Natural human glycoproteins usually contain sialic acids in both α2,3 and α2,6 linkage [26], whereas CHO-derived glycoproteins have only α2,3-linked sialic acid [28,29] owing to the lack of a functional copy of the gene for α2,6-ST (α2,6-sialyltransferase) in CHO cells. Many research groups have attempted to overexpress α2,3- and/or α2,6-ST, resulting in the increased sialylation of glycoproteins, although the extent of increase varied [10,30–32]. On the basis of these results, the extent of sialylation improved by the overexpression of sialyltransferase seems to be protein-dependent and cell-line-specific.

Despite an elevation in the intracellular level of CMP-sialic acid, there was no increase in sialylated glycans in EPO when both α2,3-ST and CMP-SAS were co-expressed compared with the expression of α2,3-ST alone. In the process of sialic acid biosynthesis, UDP-GlcNAc (UDP-N-acetylglucosamine) is epimerized to N-acetylmannosamine.
(ManNAc) by UDP-GlcNAc-2 epimerase. ManNAc is subsequently phosphorylated by ManNAc kinase to yield ManNAc 6-phosphate. In mammalian cells, these two activities are present in EK, in which the N-terminal domain contains the 2-epimerase activity and the C-terminal domain includes the kinase activity [33,34]. The ManNAc 6-phosphate is then converted into sialic acid through several steps, eventually to CMP-sialic acid. Baker et al. [6] reported that ManNAc supplementation increased dramatically the CMP-sialic acid content of CHO cells (over 12-fold), with no apparent increase in recombinant glycoprotein sialylation. Our results concur with those obtained in this previous study, even though the extent of CMP-sialic acid increase was relatively low in the present case (1.5-fold) compared with ManNAc-supplementation-induced increases reported previously [5,6]. The results from both studies imply that the availability of CMP-sialic acid substrate within the Golgi lumen may be a limiting factor. EK is inhibited by CMP-sialic acid in a feedback-dependent manner [25]. The feedback mechanism can be by-passed in the case of ManNAc supplementation, because ManNAc is incorporated into the sialylation pathway beyond EK [35]. This suggests that ManNAc supplementation could be a more effective method of increasing the intracellular CMP-sialic acid than enhancing the CMP-SAS activity. However, considering the high cost of ManNAc, the overexpression of CMP-SAS in CHO cells may still be a more economically viable strategy than ManNAc supplementation. Thus CMP-SAS over-expression may be beneficial if the feedback inhibition is circumvented.

When α2,3-ST and/or CMP-SAS were expressed, the proportion of trisialylated glycans of recombinant EPO showed a significant increase, whereas that of tetrasialylated glycans remained relatively unchanged. These results are in accordance with our previous results [27]. Previously it was also reported that the sialylation of human interferon-γ in CHO cells was improved by the transfection of sialyltransferases [31]. However, the amount of tetrasialylated glycans was slightly increased. Thus, it seems that there may exist a certain steric hindrance when human α2,3-ST transfers CMP-sialic acid to trisialylated glycans, which inhibits the production of fully sialylated glycans in CHO cells [10]. Previous studies revealing that several sialyltransferases have different branch specificities supports this hypothesis [36,37]. Interestingly, our studies showed that tetrasialylated glycans increased markedly upon the overexpression of CMP-SAT with α2,3-ST and CMP-SAS. CMP-sialic acid substrate availability, elevated in the Golgi by the over-expression of CMP-SAT, might reduce the steric hindrance by the concentration effect. However, clarification of the mechanism involved awaits further study.

A dramatic increase in sialylation was not achieved in the present study or in previous ones [5–7,10,15,27,30–32]. This may be for the following reasons. First, under-
References


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