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Effect of Mild-Thiol Reducing Agents and α2,3-Sialyltransferase Expression on Secretion and Sialylation of Recombinant EPO in CHO Cells

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We have previously reported that *N*-acetylcysteine (NAC) not only delayed apoptosis but also enhanced the production of recombinant erythropoietin (EPO) in Chinese hamster ovary (CHO) cell culture. To investigate the production enhancement mechanism, the effects of similar thiol-reducing agents were studied. Intriguingly, all mild reducing agents examined including mercaptoethanesulfonic acid (MESNA), thiolactic acid (TLA), and thioglycolate (TG) were shown to block apoptosis and increase EPO production. A pulse-chase study of EPO secretion revealed that all four thiol-reducing agents increased the EPO secretion rate; among them TLA showed the highest rate. In terms of product quality, the sialic acid content of the glycoprotein is one of the most important factors. It was reported that a number of glycoproteins produced by CHO cells often have incomplete sialylation, particularly under high-producing conditions. Human α2,3-sialyltransferase (α2,3-ST) was introduced into EPO-producing CHO cells in order to compensate for the reduced sialylation during supplementation with NAC. When α2,3-ST was expressed in the presence of NAC, reduced sialylation was restored and an even more sialylated EPO was produced. Thus, our study is significant in that it offers increased EPO production while still allowing the prevention of decreased sialylation of EPO.

Key words: CHO cells, erythropoietin, sialylation, sialyltransferase, thiol-reducing agent

Numerous strategies have been proposed to enhance the productivity of cell culture processes, in addition to the conventional methods such as cell line selection and process optimization. Controlling the cell cycle with relevant genes or growth factor genes has been reported [15]. Controlling apoptosis in cell culture by employing apoptotic genes or compounds has also been examined [3]. The idea of delaying apoptosis in cell culture has lately attracted the attention of many process development scientists. Supplementation with compounds that alter the physiology of the cell has also been considered as an attractive strategy to increase productivity. The best example perhaps would be sodium butyrate (NaBu). However, certain concentrations of NaBu induce apoptosis, even though it enhances the production levels of various recombinant proteins [6].

We have previously reported that the progression of apoptosis induced by NaBu in cell culture was partially inhibited by *N*-acetylcysteine (NAC), and at the same time the production of recombinant erythropoietin (EPO) was significantly enhanced 2-fold compared with the control [6]. This effect has been recently confirmed by a study on the production of recombinant interferon-β in Chinese hamster ovary (CHO) cells [19]. However, the functional mechanism of NAC is still unclear.

For therapeutic glycoproteins, an important quality criterion is sialylation, since sialic acid residues play a major role in determining the circulatory lifespan of glycoproteins. Sialic acids occupy the terminal position on oligosaccharide chains, thereby masking the penultimate sugar, galactose, from recognition and uptake by the hepatocyte asialoglycoprotein receptor [27]. Therefore, it is desirable to maximize the sialic acid content of glycoproteins used as therapeutic agents to ensure their quality and consistency. For example, sialylated EPO has a longer serum half-life and greater *in vivo* potency than the non-sialylated form [9]. However, it was reported that a number of glycoproteins produced by CHO cells often have incomplete sialylation, particularly under high-productivity conditions [21].

In this study, we investigated whether mild thiol reducing agents share the characteristics of NAC and how they influence the secretion of recombinant EPO. Furthermore,

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we analyzed the sialylation of EPO produced in NAC-treated cultures and introduced human α2,3-sialyltransferase (α2,3-ST) into EPO-producing CHO cells to improve the sialylation.

Materials and Methods

Cell Lines and Culture Conditions

Recombinant EPO-producing CHO cell lines (EC1, EC2-2C5, and EC2-1H9) were kindly provided by Dr. H. J. Hong of the Korea Research Institute of Bioscience and Biotechnology. The cell lines were constructed by introducing cDNA-encoding human EPO under the control of a cytomegalovirus (CMV) promoter. Cells were cultured in 6-well plates (Nunc, Roskilde, Denmark) in MEMα (Gibco, Grand Island, NY, USA), supplemented with 10% dialyzed fetal bovine serum (dFBS, Gibco) and 3.5 g/l glucose containing antibiotic-antimycotic solution (Gibco), in a humidified atmosphere of 5% CO_2 at 37°C.

Quantitative EPO Assay

EPO concentrations of the supernatants were measured using an indirect ELISA (enzyme-linked immunosorbant assay). Anti-EPO monoclonal antibody was purified from the supernatant of a hybridoma (ATCC HB 82C9 5F3AD) culture using a Protein G column (GE Biosciences, Uppsala, Sweden). Recombinant EPO was purchased from Roche and used as a standard. Anti-mouse monoclonal antibody conjugated to horseradish peroxidase (Promega, Madison, WI, USA) was used as a secondary antibody. The absorbance of the colored substrate (*o*-phenylenediamine; Sigma, St. Louis, MO, USA) was measured at 490 nm using an ELISA plate reader (Bio-Rad, Hercules, CA, USA).

Metabolic Labeling and Chase Conditions

Thiol-reducing agents, *N*-L-acetylcysteine (NAC), mercaptoethanesulfonic acid (MENSA), thiolactic acid (3-mercaptopropionic acid, TLA), and thioglycolic acid (TG), were purchased from Sigma. EC2-2C5 cells were grown on 40 mm plates in MEMα supplemented with 10% dFBS, 3.5 g/l glucose, and antibiotic-antimycotic solution, for 32 h. The cell monolayer was washed twice with methionine- and cysteine-free Dulbecco's modified Eagle's medium (Gibco) and was then preincubated in the same medium for 30 min at 37°C. Each monolayer was pulse-labeled for 15 min at 37°C with a 70 μCi Promix [^{35}S] methionine and [^{35}S] cysteine protein labeling mixture (GE Biosciences) in 250 μl of pre-warmed medium. The pulse was stopped by adding 1 ml of pre-warmed normal MEMα with various reducing agents (NAC, MESNA, TLA, TG) and chased for 30 min and 1 h. Culture supernatants were removed and cells were transferred to ice and washed twice with ice-cold phosphate-buffered saline (PBS) to prevent formation or rearrangement of protein disulfide bonds. Each dish of cells was lysed with 1 ml of ice-cold lysis buffer (25 mM Tris-HCl, pH 7.4, containing 0.5% Triton X-100, 50 mM NaCl, 2 mM EDTA, 20 mM NEM, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml each antipain, chymostatin, leupeptin, and pepstatin, and 10 μg/ml soybean trypsin inhibitor). Lysates were adjusted to 1.5 ml with lysis buffer, and then spun for 15 min at 12,000 ×*g* to pellet nuclei and cell debris. The supernatants were removed, mixed with lysis buffer, and frozen at −70°C.

Immunoprecipitation

Each 1 ml solution containing 10 ml of sample and 10% SDS was preincubated with 5 μl of protein G-Sepharose 4B (Sigma) for 1 h at 4°C to preclear the samples of protein G-binding components. Precleared samples were each incubated with 10 μl of protein G-Sepharose 4B and 10 μl of rabbit anti-EPO antibody (Sigma) for 15 h at 4°C. The complexes were washed three times with fresh immunoprecipitation buffer [50 mM Tris-HCl, pH 7.4, containing 1% Triton X-100, 150 mM NaCl, 2 mM EDTA, and 0.02% (w/v) sodium azide], and prepared for SDS-PAGE analysis.

SDS-Polyacrylamide Gel Electrophoresis

Immunoprecipitation pellets were resuspended in 30 μl of SDS-PAGE sample buffer [0.25 M Tris-HCl, pH 6.8, containing 2% (w/v) SDS, 20% (v/v) glycerol, and 0.004% (w/v) bromophenol blue]. All the samples were boiled for 5 min prior to electrophoresis. Cooled samples were loaded into the wells of a 12.5% SDS-polyacrylamide gel for electrophoresis. Gels were stained, fixed, dried under vacuum, and imaged using a Fujix Bas2000 phosphoimager. The labeled protein was quantitatively analyzed with a Tina20 image analyzer.

Construction of α2,3-ST Expression Vector

The gene encoding human α2,3-ST was cloned by reverse transcription-polymerase chain reaction (RT-PCR; AccuPower RT/PCR PreMix kit; Bioneer, Daejeon, Korea) from human fibroblast cells, based on the previously reported sequence (GenBank Accession No. L23768). The forward primer was 5'-ATGGGACTCTTGGTA TTTGT-3' and the reverse primer was 5'-TCAGATGCCACTGCT TAGAT-3'. The amplified products were sequenced by dideoxy sequencing. The gene encoding human α2,3-ST was then inserted into the mammalian expression vector pcDNA3.1/Zeo(+) (Invitrogen, Carlsbad, CA, USA) to generate pcSTz.

Screening of α2,3-ST Expressing Clones

EC1 cells were transfected with pcSTz using Lipofectamine (Gibco). Drug selection was performed for 2 weeks by seeding 10^4 cells/well in 96-well tissue culture plates (Nunc) containing MEMα supplemented with 10% dFBS, 3.5 g/l glucose, 500 μg/ml zeocin (Invitrogen), and 1% antibiotic-antimycotic solution, in a humidified atmosphere containing 5% CO_2 at 37°C. Stable transfectants were selected by zeocin treatment (EC1-ST).

Detection of α2,3-ST Expression by RT-PCR

Total RNA was extracted from EC1 and EC1-ST 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. To amplify α2,3-ST, a forward primer (ST-f, 106–130 bp) 5'-GAGGAG GACTCCAATTCAGTGGTTC-3' and a reverse primer (ST-r, 973–949 bp) 5'-CATAGCCAAATCCTGCGACTGCCAC-3' were used. The amplified products were electrophoresed on a 1% agarose gel and visualized by ethidium bromide staining.

Western Blot Assay of α2,3-ST Expression

Total cell lysates were prepared and subjected to 10% SDS-PAGE (20 μg of total protein in each lane). The samples were then transferred to a nitrocellulose membrane (GE Biosciences) 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. The blots were then treated with anti-rabbit IgG donkey polyclonal antibody-HRP conjugate (GE Biosciences). After washing the blots with TBS-T buffer (Tris-buffered saline containing 0.1% Tween-20), the α2,3-ST-specific bands were visualized using the ECL Western blotting system (GE Biosciences).

Purification of EPO

The culture supernatant was concentrated and dialyzed with PBS by ultrafiltration (Amicon Ultra, Millipore, Bedford, MA, USA). Briefly, the supernatant was loaded onto an immunoaffinity column consisting of CNBr-activated Sepharose 4B (GE Biosciences) coupled with monoclonal anti-human EPO (R&D Systems, Minneapolis, MN, USA) 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). 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.

Isolation and Analysis of *N*-Glycans from Recombinant EPO

N-Glycans of the recombinant EPO were prepared as described previously [1, 7]. Briefly, a trypsin [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, USA). The purified glycans were subsequently lyophilized and derivatized by reductive amination with 2-aminopyridine (2-AP, pyridylamino-, PA-) and sodium cyanoborohydride [18, 29], and the PA-derivatized glycans were purified by gel filtration on a Sephadex G-15 (GE 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 a gradient of 0–20% E2 for 40 min. The PA-derivatized glycans were monitored by a fluorescence detector (474, Waters, Milford, MA, USA) set at the following wavelengths: excitation, 310 nm; emission, 370 nm.

RESULTS

Cell Growth and EPO Production in Media Supplemented with NAC

We have previously shown that NAC with NaBu not only delayed the apoptosis induced by NaBu but also exerted a synergistic effect on the production of EPO [6]. When NAC alone was added to the media, cell growth was retarded, but nevertheless, the viability of the cells was similar to untreated cells (Fig. 1). Strikingly, cells treated with NAC showed up to a 2.5-fold higher EPO titer on day 6 compared with the untreated batch, even though the cells were much lower.

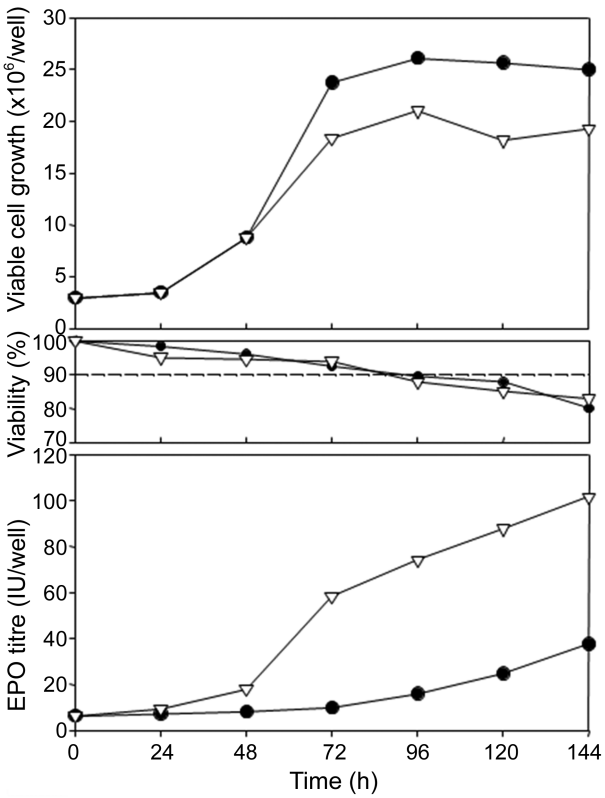

Fig. 1. Cell growth, viability, and production of EPO in the presence or absence of NAC.
The closed circles (●) indicate the culture supplemented with 20 mM NAC, and the open triangles (▽) indicate the control.

Increased EPO Production in Media Supplemented with Thiol-Reducing Agents

Three additional thiol-reducing agents that share the properties of NAC were found through a literature search [10, 25, 30]. The three candidates, mercaptoethanesulfonic acid (MESNA), thiolactic acid (TLA), and thioglycolate (TG) were shown to have anti-apoptotic and cell protective properties. EPO production was measured after addition of the thiol-reducing agents to the culture medium. EC1 cells (0.3×10^6) were inoculated in a 6-well plate containing 5 ml of MEMα and 10% dFBS supplemented with various reducing agents. After 24 h (day 1) and 72 h (day 3), culture media were withdrawn and EPO titers were measured by ELISA (Fig. 2). At day 1, EPO titers from all samples were similar. At day 3, however, samples from cultures that were incubated with reducing agents showed higher EPO production than cultures without reducing agents. The highest EPO titer was observed in the cultures supplemented with TLA, whereas the cultures supplemented with MESNA had the lowest titer. (The concentration of each reducing agent was determined according to an optimization process.)

To establish whether the effect could be generalized to all CHO cells, two CHO cell transformants (EC2-2C5 and

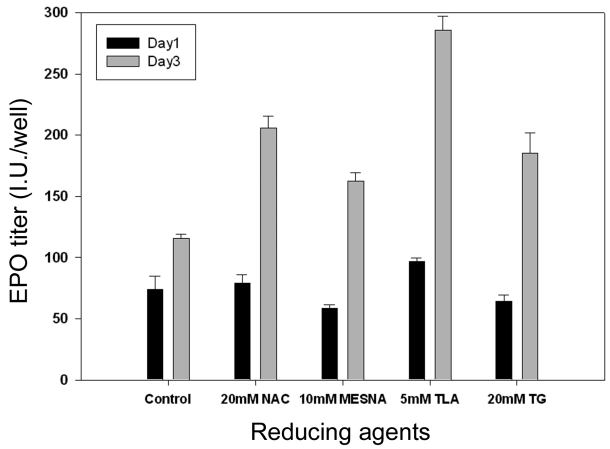

Fig. 2. Thiol-reducing agents increase production of EPO in CHO cells.
Cells were grown in 5 ml of medium in 6-well plates. EPO titers were measured after 24 h (day1) and 72 h (day3) of cell culture. The titers are means of triplicate assays, and the error bars represent the standard deviation.

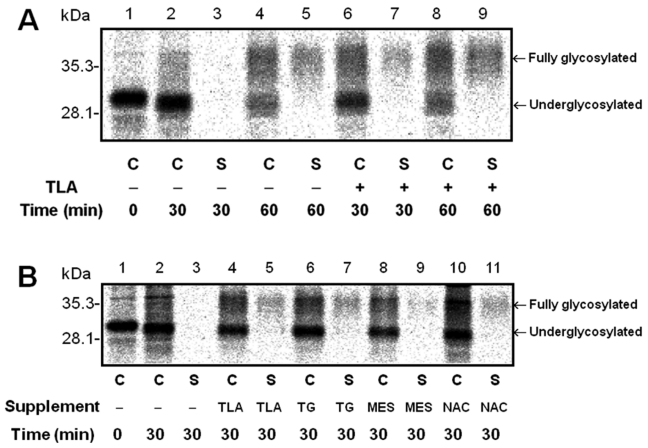

Fig. 3. Thiol-reducing agents induce EPO secretion.
(**A**) TLA-induced EPO secretion at different time points (30 and 60 min). (**B**) Secretion of EPO in the presence of various reducing agents at 30 min. The gels were stained, fixed, dried under vacuum, and imaged using a Fujix Bas2000 phosphoimager. C: cell lysate; S: supernatant; + and -: culture with or without thiol-reducing agents. The arrows indicate EPO bands with different molecular masses due to glycosylation.

EC2-1H9) were used for comparative studies. Although EPO titers were lower than in EC1 cells owing to clonal variation, the effect of enhancing productivity with reducing agents was similar (data not shown).

Thiol-Reducing Agents Increase EPO Secretion

Protein secretion studies were greatly facilitated by pulse-chase experimentation. We employed pulse-chase to verify the differences in secretion rates between untreated cells and those treated with thiol-reducing agents. Cells were pulsed for 15 min with [^{35}S] methionine and [^{35}S] cysteine, and then chased for 30 min and 60 min in the presence of TLA. Cell lysates and supernatants were then precipitated with rabbit anti-EPO antibody and protein G-Sepharose 4B, and run on SDS-PAGE gels. In Fig. 3, the labeled EPO was present in two major forms. A considerable amount of EPO detected in the cell lysates had a low molecular mass due to incomplete post-translational modification (lane 1). EPO has carbohydrate moieties that constitute 40% of the molecular mass. A fully glycosylated EPO band with a high molecular mass was observed in the cells after 30 min of chase but was not detected in the supernatant (lanes 2, 3). After 60 min, fully glycosylated EPO was observed in the supernatant. However, when chased in the presence of TLA, fully glycosylated EPO was readily observed in the supernatant after 30 min and yielded a more concentrated band at 60 min. When other mild reducing agents were included in the chase, they all produced bands that indicated the secretion of EPO after 30 min of chase (Fig. 3B). NAC, TLA, and TG were strong inducers of secretion, whereas MESNA was a relatively weak inducer. We do not think that TLA and the other reducing agents enhanced the synthesis of EPO during the 30 min chase. Instead, it is more probable that they facilitated the secretion of protein that had been retained in the cell. These results show that reducing agents increase EPO production by increasing the secretion rate of proteins.

Although it remains unclear exactly how this facilitation of secretion is accomplished, several previous reports may provide a hint. Alberini *et al*. [2] showed that B lymphocytes synthesize, but do not secrete, IgM subunits, and that only the polymeric form of IgM is secreted by plasma cells. They also found that the μ heavy chain C-terminal cysteine of secreted IgM is involved in the intracellular retention of unpolymerized IgM subunits, and that free thiol groups prevent the unhindered transport of proteins through the secretory pathway. They suggested that use of a reducing agent would change the intracellular redox potential and prevent the interaction of thiol groups with themselves and/or with any unknown system in the ER to eventually allow IgM subunits to be secreted. On the other hand, Lodish and Kong [17] suggested the involvement of protein folding. They reported that treatment of cells with a strong reducing agent like DTT leads to a strong reducing environment in the ER, resulting in protein retention in the ER. In this case, proteins are not assembled owing to reduction of their thiol groups and secretion cannot proceed.

Other factors besides facilitated secretion are involved in the increase in protein production. One of our cell lines, EC1, produces enormous amounts of recombinant EPO. It is quite possible that a portion of this non-native protein accumulates in the ER because of overexpression. As mentioned previously, protein retained in the cell is

immediately cleared by degradation, and we believe that this process makes a difference in the final EPO titers.

Thiol-reducing agents may alter the redox state of the ER from an oxidative state to a slightly reduced state, thereby facilitating protein folding and enhancing the secretion rate of proteins that would otherwise be retained in the cell. In other words, the shortened retention time prevents protein degradation. The mechanism has yet to be clarified, but the fact that an increased EPO secretion rate enhances EPO production is highly persuasive. The mechanism behind protein retention in the ER under normal conditions remains to be studied.

Expression of α2,3-ST in CHO EC1 Cells

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 [23]. Many groups have attempted to overexpress α2,3- and/or α2,6-sialyltransferase, in order to increase the sialylation of glycoproteins [5, 11, 13, 26].

The vector pcSTz was constructed to express α2,3-ST. pcSTz was introduced into CHO EC1 cells by transfection. Nine transfectant candidates (EC1-ST) were selected with zeocin. To detect α2,3-ST expression in the clones, RNA was isolated from each transfectant to perform RT-PCR. No α2,3-ST transcript was detected in the control cells (EC1). The expression of α2,3-ST was verified by western blotting. Among the candidates clones, EC1-ST2 cells showed a higher level of α2,3-ST mRNA expression than EC1-ST9 cells. Similarly, western blot analysis showed that EC1-ST2 expressed a higher level of protein (Fig. 4). Other candidates showed lower or negligible expression levels compared with EC1-ST2 (data not shown). To analyze the sialylation of EPO, EC1-ST2 cells were selected and cultured to produce recombinant EPO.

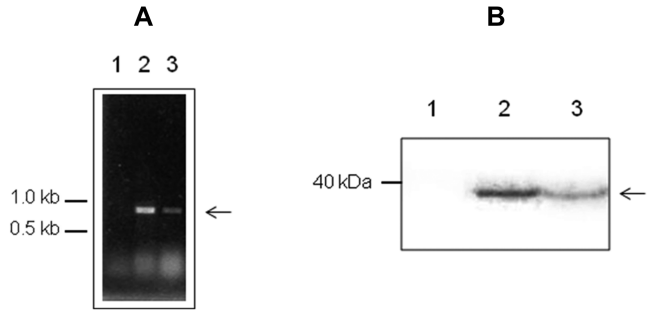

Fig. 4. RT-PCR (**A**) and western blot analysis (**B**) of a2,3-ST.
Total RNA isolated from transfected cells was amplified by RT-PCR. The cell lysates were subjected to 10% SDS-PAGE and western blot analysis. EC1 cells were used as a negative control. Lane 1, EC1; Lane 2, EC1-ST2; Lane 3, EC1-ST9.

Sialylation of Recombinant EPO in the CHO Cells Overexpressing α2,3-ST in the Presence of NAC

For EPO production, EC1 cells were cultivated in serum-free media with (EC1-NAC) or without (EC1) NAC for 12 h under a humidified atmosphere of 5% CO_2 at 37°C, and the supernatant was harvested from each culture. EC1-ST2 cells were cultivated with NAC under the same media and conditions as EC1 cells (EC1-ST2-NAC). To analyze the difference in sialylation of EPO, samples of EPO produced by each culture were purified by immunoaffinity chromatography. The purified EPO samples were then subjected to SDS-PAGE with Coomassie staining under reduced conditions. However, the gel staining did not show differences in the sizes of the EPO samples (data not shown).

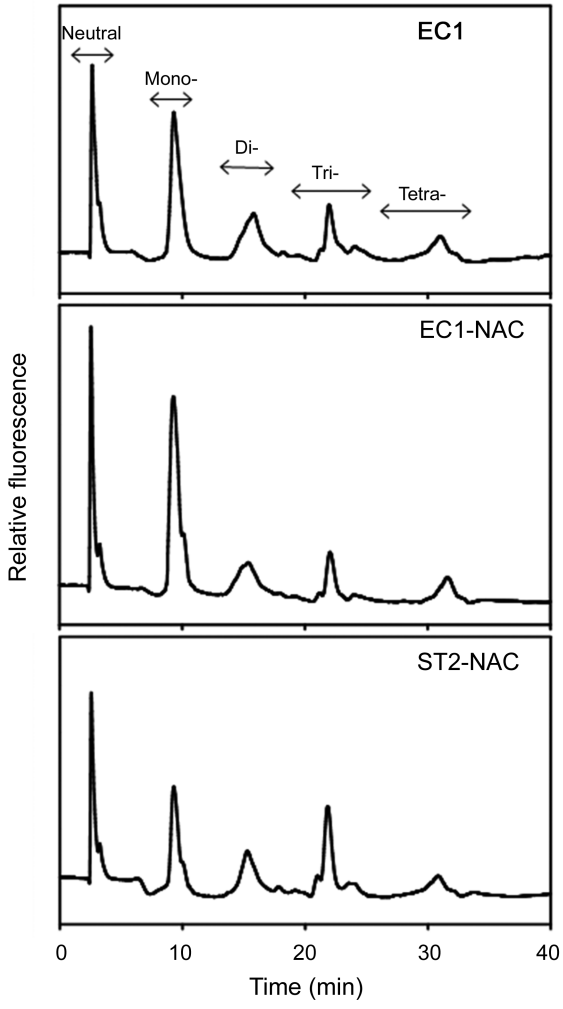

Fig. 5. Sialylation profiles (DEAE-5PW column) of the PA-glycans from EPO produced by EC1, EC1-NAC, and EC1-ST2-NAC cells.
The PA-glycans were separated based on the number of attached sialic acid residues. Asialo- (neutral) to tetra-sialylated glycans were detected among the EPO glycans from the CHO cells.

Table 1. Relative levels of sialylated glycans in recombinant human EPO produced by EC1, EC1-NAC, and EC1-ST2-NAC cells[a].

Sialylated glycans (%)	EC-1 (control)	EC1-NAC (NAC)	EC1-ST2-NAC (NAC/α2,3-ST)
Neutral (asialo)	22.3 ± 2.4	28.8 ± 3.0	21.4 ± 5.4
Mono-	29.5 ± 5.8	35.0 ± 3.3	21.6 ± 1.1
Di-	21.1 ± 1.2	14.9 ± 1.1	19.1 ± 3.2
Tri-	16.5 ± 2.2	13.2 ± 4.2	26.8 ± 1.4
Tetra-	10.6 ± 0.8	8.1 ± 1.0	11.1 ± 1.8

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

To evaluate the sialylation pattern of EPO, the *N*-glycans from each purified EPO sample were isolated and labeled with 2-AP, as described above. The fluorescently labeled PA-glycans were then applied to an anion-exchange column (DEAE-5PW) and separated based on the number of sialic acid residues, which are negatively charged. The PA-glycans were separated into neutral- (asialo-), mono-, di-, tri-, and tetra-sialylated glycan groups (Fig. 5). Although the quantity of the glycans varied according to the recombinant strains, the recombinant EPO produced by the CHO cells in our study exhibited *N*-glycans ranging from asialo- to tetra-sialylated, similar to those found in human EPO; thus the glycosylation machinery of the CHO cells is similar to that of human cells.

The relative amounts of sialylated glycans were quantified based on the area of the peaks (Table 1). An increase in the proportion of more highly sialylated to less highly sialylated glycans indicates an increase in sialylation levels. When NAC was added to the culture media (EC1-NAC), EPO sialylation was reduced. Compared with the control sample (EC1), the proportion of neutral (22.3% to 28.8%) and mono-sialylated (29.5% to 35.0%) glycans increased, whereas that of di- (21.1% to 14.9%), tri- (16.5% to 13.2%), and tetra-sialylated (10.6% to 8.1%) glycans decreased. The extent of sialylation can alternatively be interpreted as site sialylation that indicates the number of molecules of sialic acid per *N*-glycan. This was computed from the relative amounts of sialylated glycans [28].

Site sialylation (molecules of sialic acid per *N*-glycan) = 0.01[0(%0S) + 1(%1S) + 2(%2S) + 3(%3S) + 4(%4S)]
where %0S, %1S, %2S, %3S, and %4S are the percentages of neutral, mono-, di-, tri-, and tetra-sialylated glycans, respectively. The site sialylation of EPO was reduced from 1.64 to 1.37 (16.5%) by the treatment with NAC. These data imply that the rate of sialylation metabolism in the cell may not be capable of supporting the increased secretion rate of EPO in the presence of NAC.

The terminal sialylation step mediated by sialyltransferase is known to occur in the distal Golgi shortly before secretion [24]. In this study, we assumed that the endogenous sialyltransferase activity in our CHO cells may not be sufficient to support the increased secretion rate of the EPO, which eventually leads to release of undersialylated EPO into the media. In order to overcome this problem, overexpression of the sialyltransferase was performed by introducing the α2,3-ST gene into our CHO cells. When α2,3-ST was expressed in the presence of NAC (EC1-ST2-NAC), glycan sialylation was restored to levels even higher then those of untreated EC1 cells (Table 1).

The relative amounts of neutral (28.8% to 21.4%) and mono-sialylated (35.0% to 21.6%) glycans decreased, whereas those of di- (14.9% to 19.1%) and tetra-sialylated (8.1% to 11.1%) glycans increased, compared with the EPO samples produced in EC1 cells supplemented with NAC (EC1-NAC). In particular, the pool of tri-sialylated glycans was augmented significantly from 13.2% to 26.8%. In the case of site sialylation, there was a 35% increase (1.37 to 1.85).

Discussion

Many attempts have been made to increase recombinant protein production levels in mammalian cells. A number of these attempts have involved altering the culture conditions or supplementing the culture medium with chemicals [6, 20]. However, these efforts have often resulted in reduced or incomplete sialylation [8, 21]. We have previously found that the addition of butyrate to CHO cells led to decreased sialylation [8, 16], and a similar case has been reported by another group [21]. Treatment with NAC for the purpose of improving productivity also resulted in reduced sialylation of EPO.

The presence of terminal sialic acid increases the *in vivo* circulatory half-life of glycoproteins, as sialic acid terminated glycans are not recognized by asialoglycoprotein receptors [27] that otherwise target glycoproteins for degradation. Hence, achieving maximum and consistent sialylation on these recombinant glycoproteins is another important goal of recombinant glycoprotein production. Sialylation levels of recombinant proteins in CHO cells have been successfully increased by feeding a direct intracellular precursor for sialic acid synthesis [12], transfection of glycosyltransferase [14], overexpression of CMP-sialic acid transporter [28], and overexpression of sialuria-mutated rat GNE/MNK [4, 22]. In this study, we employed the strategy of α2,3-ST

overexpression. We have demonstrated that the overexpression of α2,3-ST not only compensated for the reduced sialylation levels caused by NAC treatment but also significantly increased sialylation levels relative to untreated CHO cells. This report is the first to show repletion of the sialylation of EPO under NAC-mediated production enhancement. Thus, this study is significant in that it offers the possibility of preventing decreased sialylation of glycoproteins while still allowing increased glycoprotein production.

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REFERENCES

1. Ailor, E., N. Takahashi, Y. Tsukamoto, K. Masuda, B. A. Rahman, D. L. Jarvis, *et al*. 2000. *N*-Glycan patterns of human transferrin produced in *Trichoplusia ni* insect cells: Effects of mammalian galactosyltransferase. *Glycobiology* **10:** 837–847.
2. Alberini, C. M., P. Bet, C. Milstein, and R. Sitia. 1990. Secretion of immunoglobulin M assembly intermediates in the presence of reducing agents. *Nature* **347:** 485–487.
3. Arden, N. and M. J. Betenbaugh. 2004. Life and death in mammalian cell culture: Strategies for apoptosis inhibition. *Trends Biotechnol*. **22:** 174–180.
4. Bork, D., W. Reutter, W. Weidemann, and R. Horstkorte. 2007. Enhanced sialylation of EPO by overexpression of UDP-GlcNAc 2-epimerase/ManAc kinase containing a sialuria mutation in CHO cells. *FEBS Lett*. **581:** 4195–4198.
5. Bragonzi, A., G. Distefano, L. D. Buckberry, G. Acerbis, C. Foglieni, D. Lamotte, *et al*. 2000. A new Chinese hamster ovary cell line expressing alpha2,6-sialyltransferase used as a universal host for the production of human-like sialylated recombinant glycoproteins. *Biochim. Biophys. Acta* **1474:** 273–282.
6. Chang, K. H., K. S. Kim, and J. H. Kim. 1999. *N*-Acetylcysteine increases the biosynthesis of recombinant EPO in apoptotic Chinese hamster ovary cells. *Free Radic. Res*. **30:** 85–91.
7. Choi, O., N. Tomiya, J. H. Kim, J. M. Slavicek, M. J. Betenbaugh, and Y. C. Lee. 2003. *N*-Glycan structures of human transferrin produced by *Lymantria dispar* (gypsy moth) cells using the LdMNPV expression system. *Glycobiology* **13:** 539–548.
8. Chung, B. S., Y. T. Jeong, K. H. Chang, J. Kim, and J. H. Kim. 2001. Effect of sodium butyrate on glycosylation of recombinant erythropoietin. *J. Microbiol. Biotechnol*. **11:** 1087–1092.
9. Dordal, M. S., F. F. Wang, and E. Goldwasser. 1985. The role of carbohydrate in erythropoietin action. *Endocrinology* **116:** 2293–2299.
10. Ferrari, G., C. Y. Yan, and L. A. Greene. 1995. *N*-Acetylcysteine (D- and L-steroisomers) prevents apoptotic death of neuronal cells. *J. Neurosci*. **15:** 2857–2866.
11. Fukuta, K., T. Yokomatsu, R. Abe, M. Asanagi, and T. Makino. 2000. Genetic engineering of CHO cells producing human interferon-γ by transfection of sialyltransferases. *Glycoconj. J*. **17:** 895–904.
12. Gu, X. and D. I. C. Wang. 1998. Improvement of interferon-γ sialylation in Chinese hamster ovary cell culture by feeding of *N*-acetylmannosamine. *Biotechnol. Bioeng*. **58:** 642–647.
13. Jassal, R., N. Jenkins, J. Charlwood, P. Camilleri, R. Jefferis, and J. Lund. 2001. Sialylation of human IgG-Fc carbohydrate by transfected rat α2,6-sialyltransferase. *Biochem. Biophys. Res. Commun*. **286:** 243–249.
14. Jeong, Y. T., O. Choi, H. R. Lim, Y. D. Son, H. J. Kim, and J. H. Kim. 2008. Enhanced sialylation of recombinant erythropoietin in CHO cells by human glycosyltransferase expression. *J. Microbiol. Biotechnol*. **18:** 1945–1952.
15. Kaufmann, H., X. Mazur, R. Marone, J. E. Bailey, and M. Fussenegger. 2001. Comparative analysis of two controlled proliferation strategies regarding product quality, influence on tetracycline-regulated gene expression, and productivity. *Biotechnol. Bioeng*. **72:** 592–602.
16. Kim, N. S., K. H. Chang, B. S. Chung, S. H. Kim, J. H. Kim, and G. M. Lee. 2003. Characterization of humanized antibody produced by apoptosis-resistant CHO cells under sodium butyrate-induced condition. *J. Microbiol. Biotechnol*. **13:** 926–936.
17. Lodish, H. F. and N. Kong. 1993. The secretory pathway is normal in dithiothreitol-treated cells, but disulfide-bonded proteins are reduced and reversibly retained in the endoplasmic reticulum. *J. Biol. Chem*. **268:** 20598–20605.
18. Nakagawa, H., Y. Kawamura, K. Kato, I. Shimada, Y. Arata, and N. Takahashi. 1995. Identification of neutral and sialyl *N*-linked oligosaccharide structures from human serum glycoproteins using three kinds of high-performance liquid chromatography. *Anal. Biochem*. **226:** 130–138.
19. Oh, H. K., M. K. So, J. Yang, H. C. Yoon, J. S. Ahn, J. M. Lee, *et al*. 2005. Effect of *N*-acetylcystein on butyrate-treated Chinese hamster ovary cells to improve the production of recombinant human interferon-β-1a. *Biotechnol. Prog*. **21:** 1154–1164.
20. Oster, T., C. Thioudellet, I. Chevalot, C. Masson, M. Wellman, A. Marc, *et al*. 1993. Induction of recombinant human gamma-glutamyl transferase by sodium butyrate in transfected V79 and CHO Chinese hamster cells. *Biochem. Biophys. Res. Commun*. **193:** 406–412.
21. Santell, L., T. Ryll, T. Etcheverry, M. Santoris, G. Dutina, A. Wang, *et al*. 1999. Aberrant metabolic sialylation of recombinant proteins expressed in Chinese hamster ovary cells in high productivity cultures. *Biochem. Biophys. Res. Commun*. **258:** 132–137.
22. Son, Y. D., Y. T. Jeong, S. Y. Park, and J. H. Kim. 2011. Enhanced sialylation of recombinant human erythropoietin in Chinese hamster ovary cells by combinatorial engineering of selected genes. *Glycobiology*. **21:** 1019–1028.
23. Takeuchi, M., S. Takasaki, H. Miyazaki, T. Kato, S. Hoshi, N. Kochibe, *et al*. 1988. Comparative study of the asparagine-linked sugar chains of human erythropoietins purified from urine and the culture medium of recombinant Chinese hamster ovary cells. *J. Biol. Chem*. **263:** 3657–3663.
24. Thorens, B. and P. Vassalli. 1986. Chloroquine and ammonium chloride prevent terminal glycosylation of immunoglobulins in plasma cells without affecting secretion. *Nature* **321:** 618–620.

25. Troy, C. M., D. Derossi, A. Prochiantz, L. A. Greene, and M. L. Shelanski. 1996. Downregulation of Cu/Zn superoxide dismutase leads to cell death *via* the nitric oxide-peroxynitrite pathway. *J. Neurosci*. **16:** 253–261.
26. Weikert, S., D. Papac, J. Briggs, D. Cowfer, S. Tom, M. Gawlitzek, *et al*. 1999. Engineering Chinese hamster ovary cells to maximize sialic acid content of recombinant glycoproteins. *Nat. Biotechnol*. **17:** 1116–1121.
27. Weiss, P. and G. Ashwell. 1989. The asialoglycoprotein receptor: Properties and modulation by ligand. *Prog. Clin. Biol. Res*. **300:** 169–184.
28. Wong, N. S. C., M. G. S. Yap, and D. I. C. Wang. 2006. Enhancing recombinant glycoprotein sialylation through CMP-sialic acid transporter over expression in Chinese hamster ovary cells. *Biotechnol. Bioeng*. **93:** 1005–1016.
29. Yamamoto, S., S. Hase, S. Fukuda, O. Sano, and T. Ikenaka. 1989. Structures of the sugar chains of interferon-gamma produced by human myelomonocyte cell line HBL-38. *J. Biochem*. **105:** 547–555.
30. Yan, C. Y., G. Ferrari, and L. A. Greene. 1995. *N*-Acetylcysteine-promoted survival of PC12 cells is glutathione-independent but transcription-dependent. *J. Biol. Chem*. **270:** 26827–26832.