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Enhancement of Erythropoietin Production from Chinese Hamster Ovary (CHO) Cells by Introduction of the Urea Cycle Enzymes, Carbamoyl Phosphate Synthetase I and Ornithine Transcarbamylase

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Abstract Efficient mammalian erythropoietin (EPO)-expression systems are required for therapeutic applications. The accumulation of ammonia is a major problem in the production of recombinant proteins in cultured animal cells. To counter this problem we introduced the first two genes of the urea cycle, carbamoyl phosphate synthetase (CPSI) and ornithine transcarbamylase (OTC), into IBE Chinese Hamster Ovary (CHO) cells by stable transfection. The resulting cell line, CO5, had a higher growth rate and accumulated less ammonia per cell than the parental cell line, IBE. In addition, it produced 2 times more EPO than the parent, and the purified EPO contained a higher proportion of acidic isoforms with approximately 15% more sialic acid.

Key words: Erythropoietin, urea cycle enzymes, ammonia concentration, sialylation

Mammalian cells are used extensively for producing therapeutic proteins. Post-translational modification of many such proteins, especially their glycosylation, is extremely important for their therapeutic efficacy. Erythropoietin (EPO) is a representative recombinant therapeutic protein produced by cell culture, and recombinant human EPO (rhEPO) is the top-selling biopharmaceutical product in the world [38]. It was developed to treat anemia associated with chronic renal failure, as well as normal anemia induced by HIV infection, cancer chemotherapy, and bone marrow transplantation [7, 12, 24]. A more efficient mammalian expression system is urgently needed to support the massive demand for EPO [17, 19, 22, 26, 34].

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The ammonia that accumulates in mammalian cell culture media tends to inhibit cell growth and affect the production of recombinant proteins [4, 11, 36]. It also affects glycosylation taking place in the Golgi apparatus [2, 30, 36, 37]. Hence, it is important to reduce ammonia accumulation, especially as the majority of recombinant human proteins are glycoproteins and their carbohydrate moieties are important for protein folding, protease resistance, and receptor binding [14, 28, 31, 32, 35].

There have been numerous approaches to eliminating ammonia accumulation [5, 6, 8, 16, 20, 23] but no one approach is applicable to all cell culture systems. We previously developed a Chinese Hamster Ovary (CHO) cell line that expresses urea cycle enzymes, as a means of reducing ammonia accumulation [10, 26]. This cell line in fact accumulated less ammonia, and had a higher growth rate than CHO cells transfected with only expression vector. The present study is based on the idea that the production of recombinant human EPO could be improved by introducing urea cycle enzymes into the producing strain and so reducing the ammonia concentration. We therefore established a cell line, CO5, expressing the first two enzymes of the urea cycle by transfection with plasmids carrying the genes for carbamoyl phosphate synthetase (CPSI) and ornithine transcarbamylase (OTC) into the EPO-producing CHO cell line, IBE. The latter expresses the *epo* gene at a high level together with the amplifiable marker gene, dihydroflate reductase (DHFR).

We report here a comparison of the cell viability, ammonia concentration per cell, and EPO production of the parental cell line, IBE, and its CO5 derivative. We also purified the EPO made by each, and determined their sialic acid contents and p*I* on the grounds that the biological activity of EPO depends on the extent of its glycosylation, particularly its sialylation.

MATERIALS AND METHODS

Cell Line

The IBE cell line was generated by cotransfecting the *DHFR* gene into *DHFR*-deficient CHO cells together with the human *epo* gene. Stable IBE cells were selected on 10^{-6} M methotrexate (MTX) to amplify *epo*. The OTC cell line, that is, CHO *DHFR*$^-$ cells carrying a 4,500-bp fragment of the CPSI gene and a 1,064-bp fragment of the OTC gene, was made previously in our laboratory [26]. The CO5 cell line expressing CPSI and OTC was generated by transfecting plasmids pCIneo-CPS45 and pREP-OTC1 into IBE cells [26] (see Results). These 3 cell lines are growing as adherent cultures.

Culture Media

IBE and CO5 cells were maintained in Minimum Essential Medium Alpha (Gibco BRL, U.S.A.) supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin (Gibco BRL, U.S.A.; 10,000 U/ml penicillin G sodium and 100 µg/ml streptomycin sulfate in 0.85% saline), 25 mM HEPES (*N*-2-hydroxy-ethylpiperazine-*N*-2-ethanesulfonic acid), 1 mM *N*-carbamoyl-L-glutamate, and 5 mM L-ornithine (Sigma, U.S.A.); the latter two compounds were added as activators for CPSI and OTC to promote the formation of carbamoyl phosphate from NH_4^+ and HCO_3^- via the urea cycle. The OTC cells were maintained in the same medium except for addition of 0.1 mM sodium hypoxanthine and 0.016 mM thymidine (Gibco BRL, U.S.A.). All cells were routinely cultured in a humidified incubator at 37°C in 5% CO_2.

RNA Extraction and RT-PCR

Total RNA was extracted with a SNAP™ total RNA isolation kit (Invitrogen, U.S.A.) and RT-PCR was performed with a Personal Cycler (Biometra, Germany) equipped with a Titan™ one-tube RT-PCR system (Boehringer Mannheim, Germany) [10, 26].

Sample Preparation for Ammonia and EPO Assays, and Measurement of Cell Growth

The cells were seeded at 1×10^5 cells/ml into six-well plates containing 2.5 ml medium. The NH_4Cl standard solutions were prepared in this medium and stored at -20°C. Ammonia was assayed by a modification of the indophenol method as described previously [26, 33]. For ammonia assay and EPO assay, three sets of culture supernatants were collected at 1-, 2-, and 3-day intervals, respectively, over a period from 3 to 9 days after seeding to see the effects of different accumulated ammonia. After collection of media, fresh media with 10^{-6} M MTX were supplied to amplify the *epo* gene from 3 to 9 days after seeding at 1-, 2-, and 3-day intervals. After removing the culture media, the cells remaining on the plate were trypsinized and cells were counted on a hemocytometer, with a standard error of ±5%.

SDS-Polyacrylamide Gel Electrophoresis

EPO samples were subjected to 12.5% PAGE in the presence of sodium dodecyl sulfate according to the method of Laemmli [21]. Separated proteins were visualized by silver staining according to the manufacturer's directions (Amersham Pharmacia Biotech., Sweden).

Protein Assay and Enzyme-Linked Immunoabsorbent Assay (ELISA) for EPO

Protein was determined with a Coomassie brilliant blue binding assay according to Bradford [3] using epoetin (Roche, Germany) as reference and EPO was measured by an ELISA using Quantikine IVD (R&D Systems, U.S.A.).

Purification of rhEPO by Heparin and DEAE Chromatography

1×10^6 IBE or CO5 cells were seeded in 20 ml of MEM-α with 10^{-7} M MTX (Sigma, U.S.A.) in 75-cm^2 T-flasks. When the cells were approximately 70% confluent they were transferred to fresh medium containing 10^{-6} M MTX to amplify *epo*. Supernatant were collected for 5 days, centrifuged for 8 min at 8,000 rpm, and filtered with a 10 kDa cutoff before being passed through a 0.22 µm membrane. The filtered samples were dialyzed against phosphate buffer (10 mM $NaH_2PO_4{\cdot}2H_2O$, 50 mM NaCl pH 6.0) at 4°C, and the dialyzates loaded onto Heparin columns at a flow rate of 1 ml/min [9] followed by elution with a linear gradient of NaCl (from 0.05 M to 1 M). The EPO-containing fraction identified by SDS-PAGE and ELISA was equilibrated with 10 mM Tris-HCl, pH 8.0, and loaded onto a diethylaminoethyl (DEAE)-Sepharose CL-6B (Pharmacia Biotech, Sweden) column [13, 18]. After washing with the same buffer until the absorbance at 280 nm reached baseline, the protein was eluted with a linear gradient of NaCl (from zero to 0.5 M) at a flow rate of 1 ml/min at room temperature. Fifty fractions of 2 ml were collected and the EPO-containing fractions were dialyzed with phosphate buffered saline (PBS) containing 50 mM NaCl and concentrated to 5 ml for immunopurification of the rhEPO.

Immunopurification of rhEPO

The immunosorbent column was prepared by coupling 1.5 mg of anti-EPO (R&D Systems, U.S.A.) with CNBr-activated Sepharose 4 fast-flow gel, and washing with equilibrating buffer (PBS, 50 mM NaCl). The 5 ml EPO fractions from the DEAE-sepharose chromatography were recirculated 20 times with the aid of a peristaltic pump. The gel was washed with 15 ml of equilibrating buffer and rhEPO was eluted with 5 ml of 0.1 M glycine at pH 2.8. The eluted material was brought to pH 7.5 rapidly by adding 1 M Tris-Cl, pH 9.0.

Isoelectric Focusing (IEF)

IEF was performed on a Bio-Rad Mini-cell using pre-cast IEF gels (p*I* range: 3- 10; Bio-rad, U.S.A.) according to the

manufacturer's instructions, and separated proteins were visualized by silver staining (Amersham Pharmacia Biotech., Sweden). The low p*I* range was used as a standard marker.

Quantitative Analysis of Sialic Acid

Sialic acid released by hydrolysis of the purified EPO glycoproteins was measured by the resorcinol method (*European Pharmacopoeia*, 3rd Edition, pp. 658–659) using *N*-acetyl neuramic acid (Sigma, U.S.A.) as the standard.

Results

Co-Expression of CPSI and OTC in IBE Cells

To generate an EPO-producing cell line expressing CPSI and OTC, IBE cells were transfected with plasmids pCIneo-CPS45 and pREP4-OTC [26], selecting with G418 and hygromycin B. Twenty-four transfectants were screened by RT-PCR, and the RNA from twelve clones that expressed CPSI and OTC strongly yielded the expected RT-PCR CPSI (1,030 bp) and OTC (1,065 bp) products. Figure 1 shows the results with five of these, including the line finally chosen, CO5. We measured the EPO produced by the 12 cell lines by EPO ELISA and five of them produced more EPO than the IBE parent (data not shown). Examination of these five isolates revealed that CO5 had the greatest ability to reduce the medium ammonia concentration and the highest growth rate.

Comparison of Cell Growth and Ammonia Concentration

Cell growth and ammonia accumulation were compared in cultures of the IBE, CO5, and OTC cell line (see

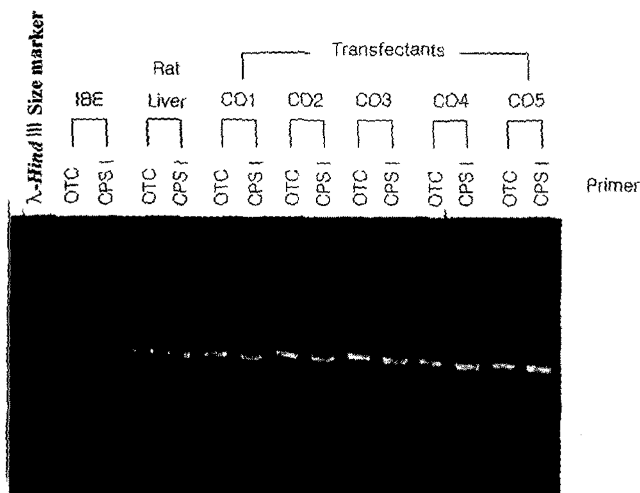

Fig. 1. Expression of CPSI and OTC genes in transfectants.
Each transfectant was given the designation CO together with an Arabic numeral. Total RNA was isolated from the parental cell line, the transfectants, and rat liver. The RNA was amplified by RT-PCR using primers for CPSI and OTC, and the products were analyzed on 0.9% agarose gels stained with ethidium bromide. RNA from IBE cells served as negative control, and the rat liver as positive control.

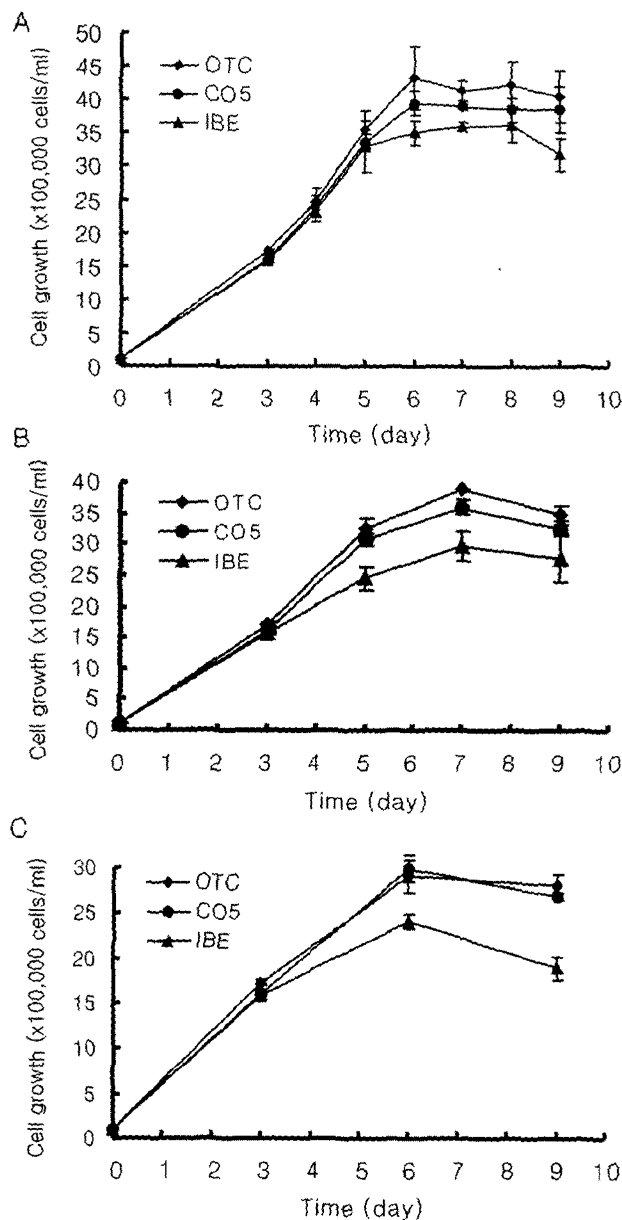

Fig. 2. Growth of IBE, CO5, and OTC cells.
Cells of each strain were inoculated in 2.5 ml medium into six-well plates at a density of 1×10^5 cells/ml and viable cells were counted with a hemocytometer following trypsinization in the presence of trypan blue. Values are means±SD of triplicate cultures. The fresh medium was supplemented with: A, 1-day interval; B, 2-day interval; C, 3-day interval.

Materials and Methods). Three sets of each cell type were seeded in six-well plates. At 1-, 2-, and 3-day intervals, respectively, over the period from 3 to 9 days after seeding, the supernatants were stored, and fresh medium containing 10^{-6} M MTX were supplied. As shown in Fig. 2, the viable cell counts of the CO5 cells were higher than those of the IBE cells but a little lower than those of the OTC cells. The viable cell number of the CO5 cells at the time of the cells reaching maximum growth

exceeded those of the IBE cells by 12.6%, 21.6%, and 24% in the cultures resuspended in fresh medium at 1-, 2-, and 3-day intervals, respectively. Also, the ammonia concentrations in the culture media were monitored under the same condition as mentioned above. As shown in Fig. 3, CO5 cells had similar or lower ammonia accumulation in the culture media than IBE cells, although cell density of CO5 cells was higher than IBE cells. Conversely, the OTC cell line showed much lower ammonia accumulation than either CO5 or IBE cells. To monitor the actual ammonia-removing ability of a cell line, the ammonia concentration in the media per cell is required for a more accurate measurement, since a cell population with a higher number of cells during culture can lead to an increase of ammonia accumulation in the media due to a higher degree of metabolism than a cell population with a smaller number of cells. Based on the results shown in Figs. 2 and 3, the ammonia concentrations in the culture media per cell were calculated and plotted in the graph shown in Fig. 4. CO5 cells had 15%, 26%, and 33% lower ammonia concentration

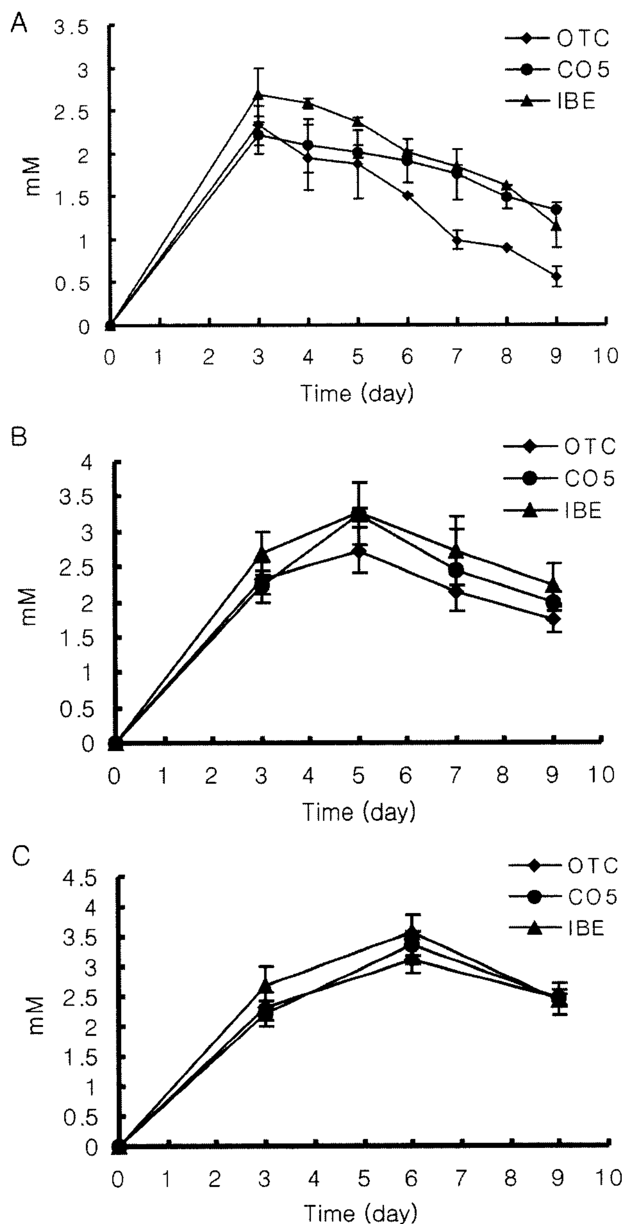

Fig. 3. Ammonia concentrations in cell culture media.
Cells were grown and treated as in the legend for Fig. 2. Values are means±SD of triplicate cultures. Fresh medium was supplemented and supernatants were collected with: A, 1-day interval; B, 2-day interval; C, 3-day interval for ammonia assay.

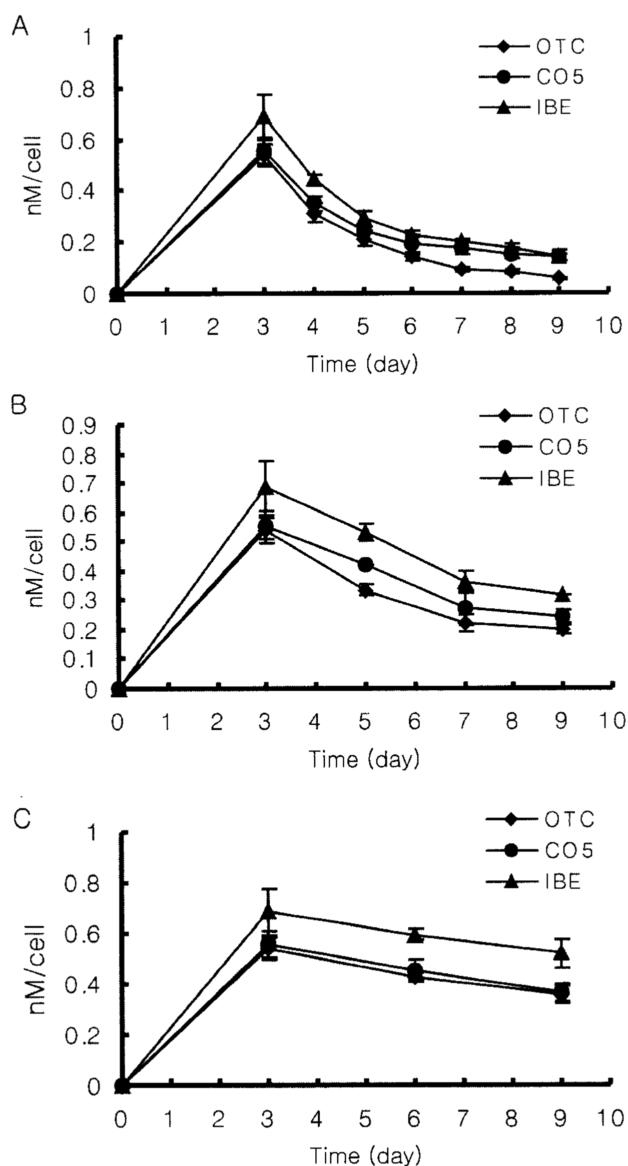

Fig. 4. Ammonia concentrations in the cell culture media per cell.
The culture conditions are the same as in Fig. 2. Values are calculated by dividing the mean ammonia concentrations by the mean number of viable cells in a volume of 2.5 ml, as shown in Figs. 2 and 3, respectively.

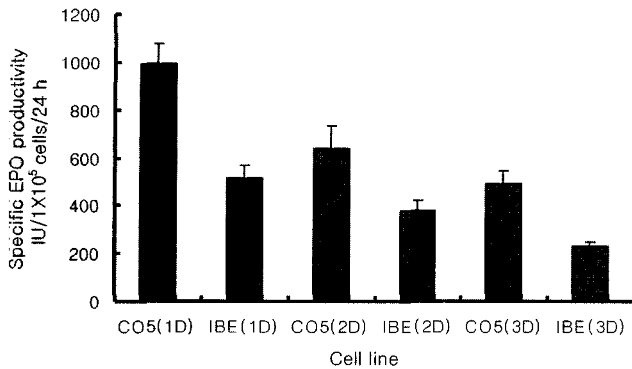

Fig. 5. The specific EPO productivity of IBE and CO5 cells.
The cells were inoculated into six-well plates at a density of 2.5×10^5 cells per well in 6-well plates and cultured in MEM supplemented with 1 mM *N*-carbamoyl-L-glutamate and 5 mM L-ornithine as activators for CPSI and OTC, the first two enzymes in the urea cycle, for 9 days. After removing the culture media for an EPO assay, fresh media were supplemented at 1-day intervals (1D), 2-day intervals (2D), and 3-day intervals (3D). EPO was assayed in the supernatants obtained at each change of medium.

in the media per cell than IBE, at the time of the cells reaching a high density when the media were changed at 1-, 2-, and 3-day intervals, respectively. CO5 cells exhibited similar or slightly higher ammonia concentration per cell than OTC cells, especially when the media were replaced at 3-day interval. These results presented that CO5 cells expressing CPSI and OTC had lower ammonia concentration than IBE cells despite of the higher cell density.

Comparison of the Specific EPO Productivity Between the IBE and CO5 Cells

To compare EPO productivity by the IBE and CO5 cells, EPO was assayed by EPO ELISA in the supernatants used

Table 1. Sialic acid contents of EPO purified from IBE and CO5 cells.

	Sialic acid content (mol sialic acid/mol EPO)	
Sample	from 3 day to 7 days	from 5 day to 9 days
IBE	12.7±1.13	10.7±1.21
CO5	14.6±1.06	12.2±1.20

Values are means±SD of triplet experiments.

in Figs. 3 and 4. Fig. 5 shows the specific EPO productivity (q_{EPO}) that was calculated from EPO production against the integral values of the growth curve [29]. The specific EPO productivity was not significantly different when media were shifted at 1-, 2-, and 3-day intervals although the ammonia concentration was lower when media were shifted at 1-day intervals than 2- and 3-day intervals. As shown in Fig. 5, the q_{EPO} of CO5 cells was 2-fold higher than IBE cells when media were shifted at 1-, 2-, and 3-day intervals. CO5 cells expressing CPSI and OTC, the first two steps of urea cycle enzymes showed 2-fold higher q_{EPO} than IBE cells with higher cell density (12.6-24%) and lower ammonia concentration (15-33%).

Purification of EPO and Comparison of Sialic Acid Content

To compare the properties of the EPO produced by the IBE and CO5 cells, we purified the EPO with Heparin DEAE and affinity chromatography as described in Materials and Methods. After further purification by affinity chromatography,

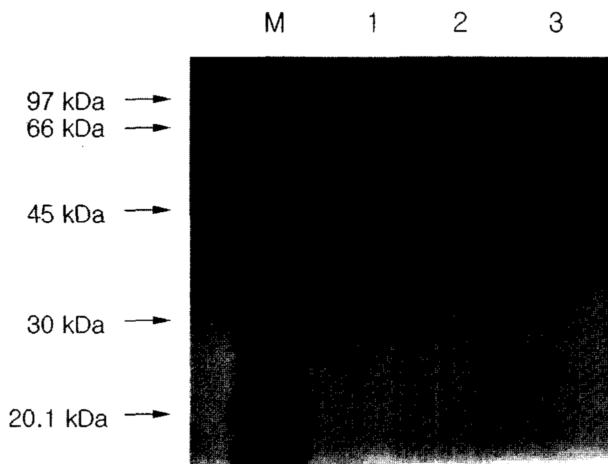

Fig. 6. SDS-polyacrylamide gel electrophoresis of EPO.
M: Molecular mass standards (Amersham Pharmacia, U.S.A.); Lane 1: sample applied to the immunoabsorbent column; Lane 2: flow-through fractions; Lane 3: eluted EPO.

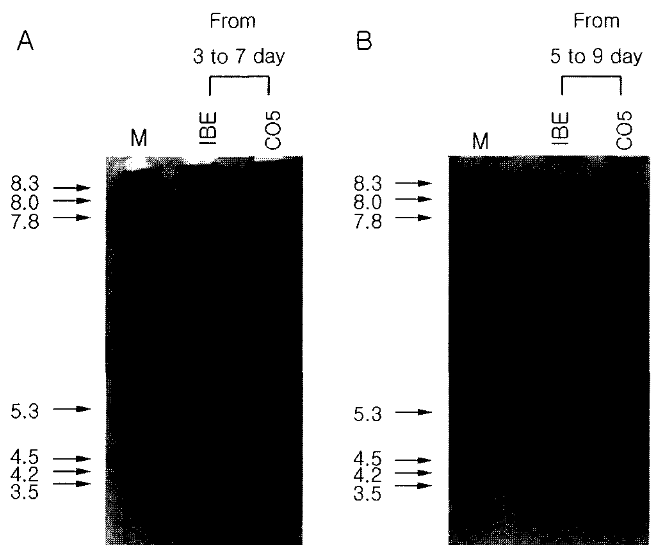

Fig. 7. IEF-gel electrophoretic patterns of EPO from IBE and CO5 cells.
Samples containing 8 μg of EPO from the immunoabsorbent columns were applied to an IEF polyacrylamide gel and analyzed as described in Materials and Methods. M: p*I* marker proteins (Serva, Germany). A, Samples collected daily from 3 to 7 days after seeding. B, Samples collected daily from 5 to 9 days after seeding.

the EPO from the IBE cells appeared as a fuzzy band of around 36 kDa on SDS-PAGE gels (Fig. 6) due to variability in the extent of sialylation. The EPO purified from the CO5 cells gave a similar result.

To compare their quality, we performed a quantitative sialic acid analysis of the EPO in the IBE and CO5 cell culture media. The medium of IBE and CO5 cells was sampled every day from 3 to 7 days and from 5 to 9 days after seeding. As shown in Table 1, the sialic acid content of the EPO (mol sialic acid/ mol EPO) from the IBE cells was 10.7- 12.7, and that from the CO5 cells was 12.2- 14.6. When analyzed by IEF to compare p*I* values, the EPO from the CO5 cells migrated formed a slightly lower band than that from the IBE cells, with an apparent p*I* between 3 and 5; it thus contained somewhat more acidic isoforms than the IBE enzyme (Fig. 7), in agreement with the quantitative sialic acid analysis. This superiority in sialic acid contents (15% higher) and p*I* range of EPO isoforms in spite of the higher EPO production in CO5 cells implies that the CO5 cell line with reduced ammonia concentration in culture media and per cell in culture is more effective on EPO glycosylation than the IBE cell line, considering that the CO5 cell line has 2-fold higher EPO productivity than IBE cells.

DISCUSSION

Mammalian cells harboring a valuable foreign gene in a stably integrated form have been widely used to produce recombinant human glycoproteins for the clinic. The accumulation of ammonia in the culture medium is a major obstacle to achieving high yields of recombinant proteins [1, 15, 25], and the aim of the current study was to reduce the accumulation of ammonia by introducing genes for the first two urea cycle enzymes, CPSI and OTC, into the producing strain. We have shown that the q_{EPO} of the CO5 cell line was 2-fold higher than the parental cell line, IBE cells (Fig. 5). It also grew better (Fig. 2) and accumulated less ammonia per cell (Figs. 3, 4). Our findings imply that the higher cell growth and EPO production of CO5 cells are related to their reduced accumulation of ammonia.

We also tried to develop an EPO-producing cell line producing an additional urea cycle enzyme, argininosuccinate synthetase (AS) or argininosuccinate lyase (AL), but it grew poorly (data not shown). We did not attempt to express all five enzymes of the urea cycle because it had been previously shown that although such cells produced little ammonia they also grew very poorly [10]. One possible explanation of this is that the forced expression of several big sized exogenous genes as well as amplified production of EPO could be a significant biological burden for the cells, causing the cells to require more additional energy and thereby resulting in the slow cell growth. Expression of only the first two enzymes of the urea cycle is a more efficient and economical strategy to reduce ammonia concentrations in the culture system.

The carbohydrate moieties of glycoproteins are important for achieving the correct protein conformation during biosynthesis, avoiding trapping and degradation, and activating target cell receptors [14, 28, 31, 32, 35]. Since the accumulation of ammonia in the culture medium tends to inhibit terminal sialylation, and this may lead to an increase in p*I* value [9, 39], it was important to measure the sialic acid content and p*I* distribution of the EPO produced. Our results showed that the sialic acid content of EPO purified from the CO5 cell line did not exhibit any reduction in spite of higher EPO production than the IBE cell line (Table 1, Fig. 7). When we consider these results, our expression system established by the introduction of CPSI and OTC is desirable to enhance EPO productivity with increased sialylation by reduction of the ammonia concentrations in culture media. Furthermore, our results showed that EPO productivity was improved by 100%, whereas cell growth was enhanced by at most 24%. Although the molecular mechanism for this increase of EPO production has not been currently elucidated, it is assumed that enhancement of EPO productivity is caused by not only higher cell density but also elevated secretion efficiency of EPO at high expression level in condition of lower ammonia concentrations. In conclusion, the findings reported here confirmed the superiority of our culture system in both quality and quantity, as EPO productivity with effective glycosylation was enhanced with a reduction in ammonia concentrations in culture media and per cell in culture and improvement of cell growth. This approach could be widely applied for the improvement and development of pharmaceuticals in mammalian cell culture.

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