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Reduction of Ammonia Accumulation and Improvement of Cell Viability by Expression of Urea Cycle Enzymes in Chinese Hamster Ovary Cells

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Abstract Previously, we developed a CHO cell line (CHO-OTC1-A19) that expresses the first two enzymes in the urea cycle and exhibits a higher ammonia-removing ability and faster growth rate than a vector-controlled CHO cell line (CHO-neo-5). The current study was undertaken to develop a cell line with an ammonia-removing ability higher than the cell line developed previously. To accomplish this, CHO cell lines expressing the first three, first four, or all five enzymes of the urea cycle were constructed using a stable transfection method. Finally, the CHO-AS-16, CHO-AL-19, and CHO-Arg-11 cell lines expressing the first three, first four, and all five enzymes of the urea cycle, respectively, were selected and found to exhibit higher ammonia-removing ability than the CHO-OTC1-A19 cell line. Among the three selected cell lines, CHO-AL-19 showed the highest ammonia-removing ability and highest cell viability at a higher cell density, with 40% and 15% lower ammonia concentration in the culture media than that of CHO-neo-5 and CHO-OTC1-A19 cell lines, respectively. CHO-AL-19 also showed 44% and 10% higher cell viability than the CHO-neo-5 and CHO-OTC1-A19 cell lines, at a higher cell density, respectively. The ammonia concentrations in the culture media were expressed as the ammonia concentration/cell, and the CHO-AL-19 cells revealed 45–60% and 20% lower ammonia concentration/cell than the CHO-neo-5 and CHO-OTC1-A19 cells, respectively.

Key words: Ammonia concentration, transfection, cell viability

Mammalian cell cultures are extensively used in the production of therapeutic proteins, because they are more capable of producing correct post-translational modifications, including glycosylations, which are essential for many therapeutic protein activities. However, mammalian cell lines generally require high levels of glutamine (2–5 mM) as the major energy and nitrogen source. Moreover, both cellular degradation and chemical decomposition of glutamine result in the accumulation of toxic waste ammonia in culture media.

The accumulation of ammonia in mammalian cell cultures has been shown to result in a reduction of the specific growth rate and final cell density [4, 5, 9, 38]. Ammonia also induces apoptosis in cultured cells that can be detected by DNA fragmentation and a decrease in the cellular volume [37]. Apoptosis has a critical role in determining both cell growth and survival [32]. In addition, the ammonia accumulated in cell cultures has also been shown to cause specific alterations in protein glycosylation [1, 3, 34, 38], including a reduction in the content of *O*-linked glycan and decrease in the terminal sialylation of the *N*-linked glycans of erythropoietin produced in CHO cells [8, 39]. These alterations in glycosylation patterns are also evident in the IgM expressed by hybridoma [34] and G-CSF expressed by CHO cells [1].

Various techniques to prevent ammonia formation or selectively remove accumulated ammonia from culture media have already been reported. Nutrient control, such as the substitution of glutamine by glutamate or other amino acids and the controlled addition of glutamine at low concentrations, has been suggested to reduce ammonia formation [6, 13, 15, 16, 21, 23]. However, although the partial replacement of glutamine by glutamate decreases ammonia production, it also decreases cell growth [6]. In addition, engineering methods, such as natural cation exchangers [6, 7, 17, 18], hydrophobic microporous hollow fiber modules [6], and nonporous ion-exchange membranes

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[26, 33], have also been applied to eliminate the ammonia accumulated in culture media. However, none of these techniques has been successfully applied to recombinant protein production.

Previously, we reported on the development of a CHO cell line that can express the first two enzymes of the urea cycle Carbamoyl Phosphate Synthetase I (CPSI) and Ornithine Transcarbamoylase (OTC), as a means of reducing ammonia accumulation in culture media [31]. This cell line showed a reduced ammonia concentration in culture media and higher cell growth rate than vector-controlled CHO cells. In extention of the earlier study, the current study describes the establishment of CHO cells expressing the first three, first four, or all five enzymes of the urea cycle by introducing Argininosuccinate Synthetase (AS), Argininosuccinate Lyase (AL), or AS, AL, and Arginase (Arg) genes into CPSI and OTC-expressing CHO cells. The underlying hypothesis for the present study is that the ammonia-removing ability of CHO cells expressing CPS1 and OTC can be improved by the addition of the third, fourth, and fifth steps of the urea cycle, leading to the conversion of ammonia into urea. The resulting urea can then be excreted into the culture media, thereby lowering the ammonia concentration and toxic effects of ammonia. The cell lines established in the current study were characterized in terms of their ammonia-removing ability and cellular growth rates.

MATERIALS AND METHODS

DNA Manipulations

All recombinant plasmids used in the current study were prepared by following the standard protocols of Sambrooks [30]. The plasmids were isolated using a DNA purification kit (Boehringer Mannheim, Germany) and used in the construction of recombinant plasmids and transfections.

RNA Extraction and RT-PCR

Total RNA was isolated from rat liver using a High pure RNA isolation kit (Boehringer Mannheim Co., U.S.A.) and used in an RT-PCR to clone the full coding regions of the AS (Genbank accession number: X12459) and Arg cDNA (Genbank accession number: NM_017134). The cytoplasmic RNA from cultivated CHO cells was extracted after lysis with a Nonidet P40-lysis buffer [30] and used in an RT-PCR with an RNA PCR kit (catalog No.; R019A, Takara, Japan) to confirm the mRNA expressions of the urea cycle enzymes introduced to the CHO cell lines. The RT-PCR was performed with 100 ng of total RNA using a One-Step RT PCR kit-AMV (Takara Shuzo Co., Japan). The primers used in the RT-PCR are listed in Table 1. The RT-PCR products were fractionated by 0.9% agarose gel electrophoresis and then visualized by ethidium bromide staining.

Table 1. Primers used in current study.

Primer	Sequence and location	Use
CPS-u	5'-ATG CTT TCT GTG AAG GCA CAG-3' Located at 245-271of rat CPS cDNA	Expression checks
CPS-d	5'-GGG CTG ACC TCT GGG TGG AA-3' Located at 1,267-1,284 of CPS cDNA	Expression checks
OTC-u	5'-ATG CTG TCT AAT TTG AGG-3' Located at 87-104 of OTC cDNA	Expression checks
OTC-d	5'-TCA GAA CTT TGG CTT CTG-3' Located at 1,134-1,151 of OTC cDNA	Expression checks
AS-u	5'-CAA GAT GTC CAG CAA GGG-5' Located at 1-18 of AS cDNA	Cloning of a full coding AS cDNA
AS-d	5'-CTA TTT GGC GGT GAC CTT-3' Located at 1,253-1,236 of AS cDNA	Cloning of a full coding AS cDNA
AS(246)-u	5'-CAG TCC AGT GCA CTC TAT-3' Located at 246-263 of AS cDNA	Expression checks
AS(992)-d	5'-CCA GAA ACC GGT GTA TAA-3' Located at 975-992 of AS cDNA	Expression checks
AL(564)-u	5'-ACG TGA TGT TCT CTT CCC-3' Located at 565-582 of AL cDNA	Expression checks
AL(1306)-d	5'-GGC CAT GAA CAC AGC TTT-3' Located at 1,289-1,306 of AL cDNA	Expression checks
Arg-u	5'-GAG CAT GAG CTC CAA GCC-3' Located at 23-40 of Arg cDNA	Cloning of full coding Arg cDNA and expression checks
Arg-d	5'-TTA TTT CGG TGG TTT AAG-3' Located at 981-998 of Arg cDNA	Cloning of full coding Arg cDNA and expression checks

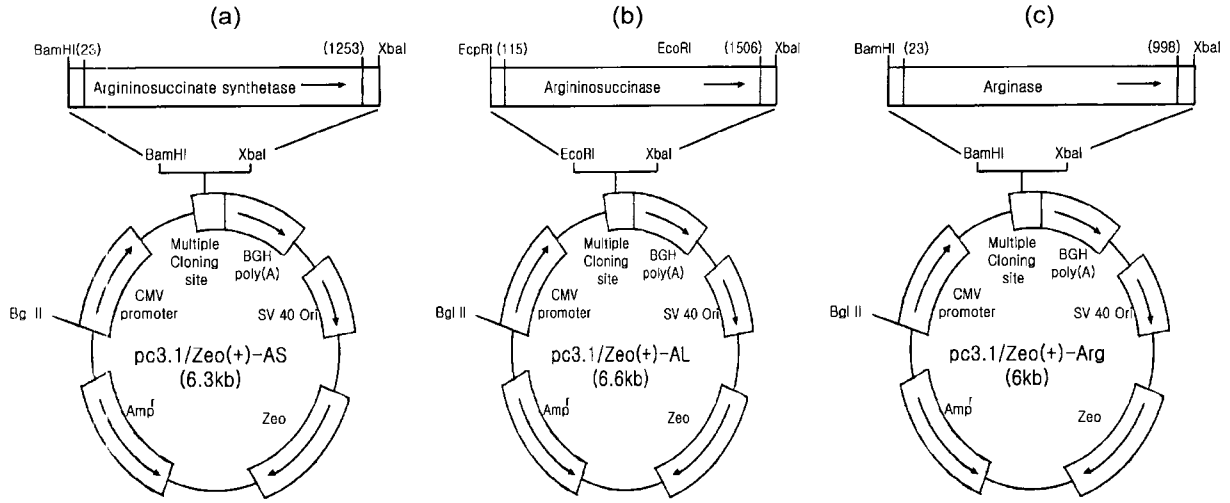

Fig. 1. Diagrams of expression plasmids used.
(A) Map of pc3.1/Zeo(+)-AS plasmid. A 1.2 kb fragment containing the full coding cDNA region of rat liver AS was inserted into the *Bam*HI and *Xba*I sites of pcDNA3.1/Zeo(+). (B) Map of pc3.1/Zeo(+)-AL plasmid. The pc3.1/Zeo(+)-AL plasmid contained a 1.6 kb full coding cDNA fragment of human liver AL in the *Eco*RI site. (C) Map of pc3.1/Zeo(+)-Arg plasmid. A 1.0 kb cDNA fragment of rat liver Arg was cloned into the *Bam*HI and *Xba*I sites of pcDNA3.1/Zeo(+).

Plasmid Preparation

To construct pc3.1/Zeo(+)-AS containing a neomycin-resistant gene, the full coding region of AS cDNA (about 1.2 kb) prepared by RT-PCR was inserted into a pcDNA3.1/V5/His-TOPO vector (catalog No.: K4800-01, Invitrogen Co., U.S.A.) using the TA cloning method and designated as pcDNA3.1(+)-AS. To subclone the AS cDNA from pcDNA3.1(+)-AS into pcDNA3.1/Zeo(+) (catalog No.: V860-20, Invitrogen Co.) containing a Zeocine-resistant gene, the pcDNA3.1(+)-AS and pcDNA3.1/Zeo(+) plasmids were both doubly-digested with *Bam*HI and *Xba*I. The 1.2 kb-*Bam*HI and *Xba*I fragment released from the digestion of pcDNA3.1(+)-AS was then ligated to the *Bam*HI and *Xba*I digested pcDNA3.1/Zeo(+), thereby completing the preparation of pc3.1/Zeo(+)-AS (Fig. 1A). The nucleotide sequence of the AS cDNA in the pc3.1/Zeo(+)-AS plasmid was determined using the Sanger dideoxy method using a 377 DNA sequencer (ABI PRISM, Perkin Elmer, U.S.A.) and confirmed by comparison with the rat AS cDNA sequence.

To prepare pc3.1/Zeo(+)-AL, the pUC19-AL plasmid (kindly provided by Dr. Roderick R. McInnes, The Hospital for Sick Children Research Institute, Toronto, Canada) [11] was digested with *Xba*I, then partially digested with *Eco*RI to isolate the human AL full coding cDNA. *Eco*RI-*Xba*I fragments (1.6 kb) of pUC19-AL were then ligated to the *Eco*RI-*Xba*I digested pcDNA3.1/Zeo(+) (Invitrogen Co.). A map of the resultant plasmid pc3.1/Zeo(+)-AL is shown in Fig. 1B. The pc3.1/Zeo(+)-Arg plasmid was prepared by a protocol similar to that used in the preparation of pc3.1/Zeo(+)-AS. About 1.0 kb of an RT-PCR product containing the full coding region of rat Arg cDNA was inserted into a pcDNA3.1/V5/His-TOPO vector (Invitrogen Co.) by the TA cloning method, resulting in the pcDNA3.1(+)-Arg plasmid. To subclone the Arg cDNA from pcDNA3.1(+)-Arg into pcDNA3.1/Zeo(+) (Invitrogen Co.), the pcDNA3.1(+)-Arg and pcDNA3.1/Zeo(+) plasmids were both doubly digested with *Bam*HI and *Xba*I. The 1.0 kb *Bam*HI-*Xba*I fragment released from the digestion of pcDNA3.1(+)-Arg was then ligated to the *Bam*HI-*Xba*I digested pcDNA3.1/Zeo(+) fragments. The resulting plasmid was named pc3.1/Zeo(+)-Arg and its map is shown in Fig. 1C. The nucleotide sequence of the Arg cDNA in pc3.1/Zeo(+)-Arg was determined using the Sanger dideoxy method using an automatic DNA sequencer and confirmed by comparison with the rat Arg cDNA sequence previously published by Kawamoto *et al.*, 1987 [19].

Cell Culture

The CHO-*dhfr*(-) was obtained from the American Type Culture Collection (ATCC) and maintained in an MEM-α medium containing 10% fetal bovine serum (catalog No.: 16000-044, Gibco BRL, U.S.A.), penicillin (100 units/ml)/streptomycin (100 ug/ml) (catalog No.: 15140-122, Gibco BRL), 0.1 mM sodium hypoxanthine, 0.016 mM thymidine (HT supplement: catalog No.: 11067-030, Gibco BRL Products Co.), and 25 mM HEPES (N-2-hydroxyethyl-piperizine-N-2-ethane sulfonic acid).

Transfection and Transfectant Selection

CHO-OTC1-A19 cells at a 50% confluency on a 60 mm plate were used for the transfections based on the $CaCl_2$ co-precipitation method. To improve the stable transfection

efficiency, the plasmids were linearized by *Bgl*II digestion. Cell lines expressing the first three enzymes of the urea cycle were developed using 10 μg of pc3.1/Zeo(+)-AS. Cell lines expressing the first four enzymes of the urea cycle were developed using 5.0 μg of pc3.1/Zeo(+)-AS and 5.0 μg of pc3.1/Zeo(+)-AL. And cells lines expressing all five enzymes of the urea cycle were developed using 3.3 μg of pc3.1/Zeo(+)-AS, pc3.1/Zeo(+)-AL, and pc3.1/Zeo(+)-Arg, respectively. Ten micrograms of linearized DNA were added to the logarithmically growing CHO-OTC1-A19 cells. After 7 h of incubation at 37°C, the cells were fed growth media, and the cells were subcultured two days later in media containing Zeocine (600 μg/ml) (Invitrogen Co.). The cells were fed again with the Zeocin-containing media every 4 days, and colonies appeared after 10- 12 days of subculture.

Sample Preparation for Ammonia Assay and Cell Growth Determination

The cells were seeded into six-well plates at a density of 1×10^5 cell/ml in a volume of 2.5-ml of medium. *N*-carbamoyl-L-glutamate (1 mM) and L-ornithine (5 mM) were added to the media as activators for CPSI and OTC, respectively. Every 24 h after seeding, the media were removed, and centrifuged for 1 min at 6,000 rpm to remove any dead cells, and the supernatants were stored at -20°C until use. After removing the culture media for an ammonia assay, the cells remaining on the plate were trypsinized and mixed with 0.4% trypan blue (W/V) at a ratio of 1:1, and the viable cells were counted on a hemacytometer.

Ammonia Assay

The ammonia concentrations were determined using a modification of the indophenol method, as described previously [31]. To remove the inhibitor for colorimetric reactions, protein-removing reagents (1% sodium tungstate and 0.1N sulfuric acid) were mixed with the sample. Then, coloring reagents (reagent I: 5 g of phenol and 25 mg sodium nitroprusside ($Na_2[Fe(CN)_5NO]_2\cdot H_2O$)/500 ml, and reagent II: 5 g of NaOH and 53.6 g of $Na_2HPO_4\cdot12H_2O$ and 10 ml chlorine 10% per liter) were added to the mixture. The reaction mixture was kept at 37°C for 20 min, then cooled by incubating in ice-cold water for 5 min to stop the reaction. The ammonia concentration was calculated based on the OD value at 630 nm.

RESULTS

Construction of Cells Expressing Enzymes for the First Three, First Four, or All Five Steps of the Urea Cycle

To develop cells expressing the enzyme genes for the first three, first four, or all five stages of the urea cycle, expression plasmids for rat AS, human AL, and rat Arg were prepared. All vectors included a cytomegalovirus promoter to direct the expression of the inserted genes and bovine growth hormone poly A signal on the 3' side of the inserted genes. AS, AS, and AL, or AS, AL, and Arg genes were introduced into CHO-OTC1-A19 cells expressing CPSI and OTC. Transfectants were selected in the selection media and propagated into cell lines. Twenty-four cell lines were then chosen from each transfection and screened for exogenous gene expressions by an RT-PCR. Thirteen of the 24 cell lines selected from the transfection with the AS expression plasmids were found to coexpress CPSI, OTC, and AS mRNA, while seventeen of the 24 cell lines coexpressed the mRNA of CPSI, OTC, AS, and AL. Only seven of the selected 24 cell lines were found to express all 5 mRNAs of the enzymes in the urea cycle.

Screening Cell Lines with Reduced Ammonia Accumulation and Increased Growth Rate

The ammonia-removing ability was examined in the 15 cell lines expressing all the introduced exogenous genes (5 cell lines from each transfection). In the current study, cell lines were selected that exhibited similar expression levels of CPSI and OTC mRNA as the parental cell line and higher expression levels of the newly introduced exogenous genes, AS, AL, and Arg. Their ammonia-removing abilities were determined by measuring the ammonia concentration in the culture media. Finally, CHO-AS-16, CHO-AL-19, and CHO-Arg-11 were chosen as the cell lines with the highest ammonia-removing abilities and highest cell viabilities from each transfection. The expressions of the introduced exogenous genes in CHO-AS-16, CHO-AL-19, and CHO-Arg-11 are shown in Fig. 2. Figure 2 also shows

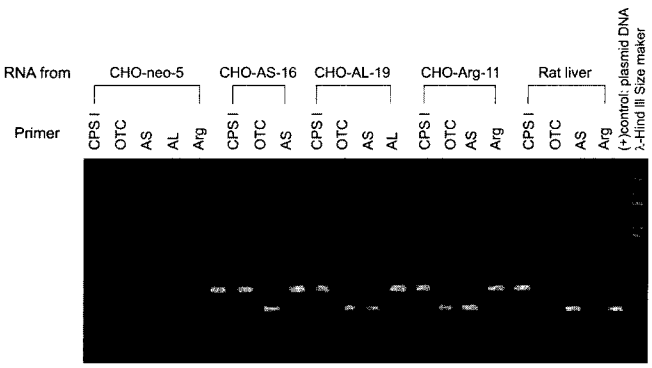

Fig. 2. Expression of introduced genes in CHO-AS-16, CHO-AL-19, and CHO-Arg-11 cell lines.

Total RNA was isolated from the cell lines of rat liver as indicated and used in the RT-PCR. The primers used for the RT-PCR are presented in Table 1. The RT-PCR products were analyzed by 0.9% agarose gel electrophoresis, and ethidium bromide stained. The total RNA from the CHO-neo-5 cells served as a negative control, while the total RNA from the rat liver was included as a positive control for examining the CPSI, OTC, AS, and Arg expressions. The pc3.1/Zeo(+)-AL plasmid was used as a positive control for examining the AL expression.

that RT-PCR products of the expected size were not detected in the total RNA isolated from CHO-neo-5, indicating that no urea cycle enzymes were expressed in the CHO-neo-5 cells. The total RNA extracted from the CHC-AS-16 cells did contain the expected RT-PCR products for the CPSI (about 1 kb), OTC (about 1 kb), and AS (about 0.7 kb) genes. Similarly, the total RNA extracted from the CHO-AL-19 and CHO-Arg-11 cells also contained RT-PCR products of the expected sizes. Accordingly, these results showed that CPSI, OTC, and AS were expressed by the CHO-AS-16 cell line, CPSI, OTC, AS, and AL by the CHO-AL-19 cell line, and CPSI, OTC, AS, AL, and Arg by the CHO-Arg-11 cell line.

Reduced Ammonia Accumulation of CHO-AS-16, CHO-AL-19, and CHO-Arg-11 Cell Lines

To examine the ammonia-removing ability of the CHO-AS-16, CHO-AL-19, and CHO-Arg-11 cell lines, the ammonia concentrations in the culture media were monitored every 24 h for 6 days. As shown in Fig. 3, all three cell lines showed 30–50% lower ammonia accumulations than the CHO-neo-5 cells, while the CHO-AS-16 cell line had a similar ammonia concentration as that of the parental cell line, CHO-OTC1-A19. However, the CHO-AL-19 and CHO-Arg-11 cell lines showed lower ammonia concentrations than the CHO-OTC1-A19 cell line by about 15%.

Growth of CHO-AS-16, CHO-AL-19, and CHO-Arg-11 Cell Lines

To examine the cell growth of the CHO-AS-16, CHO-AL-19, and the CHO-Arg-11 cell lines, the number of viable cells was counted every 24 h after seeding. As shown in Fig. 4, the number of viable CHO-AS-16 cells was higher than the number of CHO-neo-5 cells after 96 h of culture,

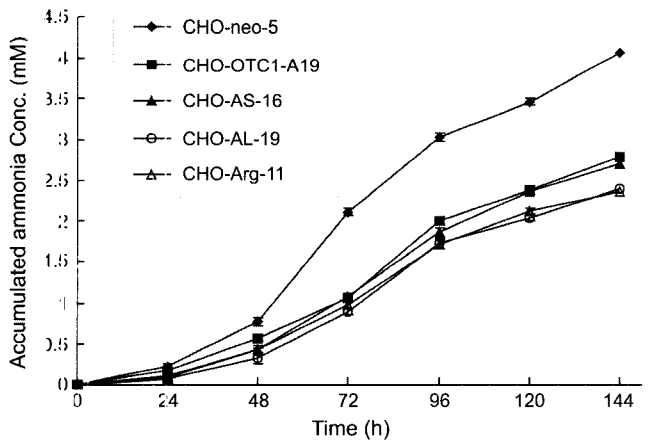

Fig. 3. Ammonia concentration in 2.5 ml culture media of CHO-AS-16, CHO-AL-19, and CHO Arg-11 cell lines.
The CHO-neo-5 and CHO-OTC1-A19 cell lines were included as negative and positive controls, respectively. Each time shown represents the mean± SD of duplicate cultures.

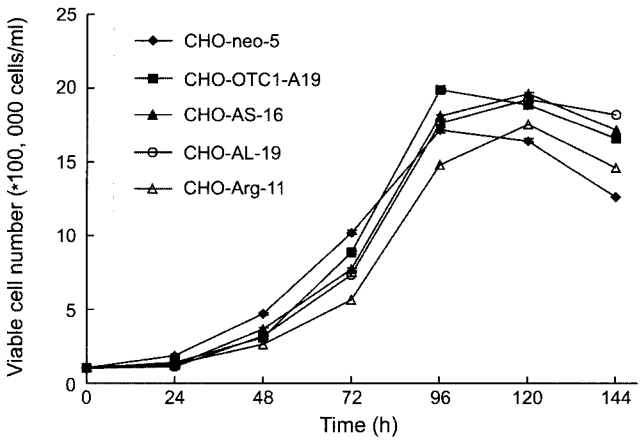

Fig. 4. Kinetics of viable cell densities of CHO-AS-16, CHO-AL-19, and CHO-Arg-11 cell lines.
The cells were inoculated into six-well plates at a density of 1×10^5 cell/ml in a medium volume of 2.5 ml. The viable cells were counted using a hematocytometer in a suspension of trypan blue following trypsinization. The values are the mean±SD of duplicate cultures.

at which time the cells had reached 100% confluency. CHO-AS-16 had a higher number of viable cells than CHO-neo-5 by 36% and a similar number of viable cells to CHO-OTC1-A19 at the end of the culture period (Fig. 4). The number of viable CHO-AL-19 cells was higher than that of the CHO-neo-5 cell line after 96 h of culture, while after 144 h of culture, the CHO-AL-19 cells showed a 44% higher viable cell number than CHO-neo-5 and 10% higher number of viable cells than CHO-OTC1-A19. CHO-Arg-11 showed higher numbers than CHO-neo-5 after 120 h of culture, while CHO-Arg-11 had more growth than CHO-neo-5 (by 16%) and less than CHO-OTC1-A19 (by 7 to 10%) after 144 h.

Accordingly, these results indicate that CHO-AS-16, CHO-AL-19, and CHO-Arg-11 had higher growth rates than the CHO-neo-5 cell line at higher cell densities. However, only the CHO-AL-19 cell line had a higher growth rate than the CHO-OTC1-A19 cell line.

Reduction of Ammonia Accumulation Per Cell

As described in the previous section, all three cell lines established in the current study exhibited lower ammonia concentrations in the culture media than the CHO-neo-5 and CHO-OTC1-A19 cell lines, and the CHO-AL-19 cell line showed higher viable cell numbers than the control cell lines. Since a cell population with a higher number of cells during culture can lead to an increase in the ammonia accumulation in the media due to a higher degree of metabolism than a cell population with a smaller number of cells, each cell's ammonia-removing ability may have been underestimated based on the ammonia concentration in the culture media. Therefore, the ammonia concentration in the media was calculated per cell, thereby

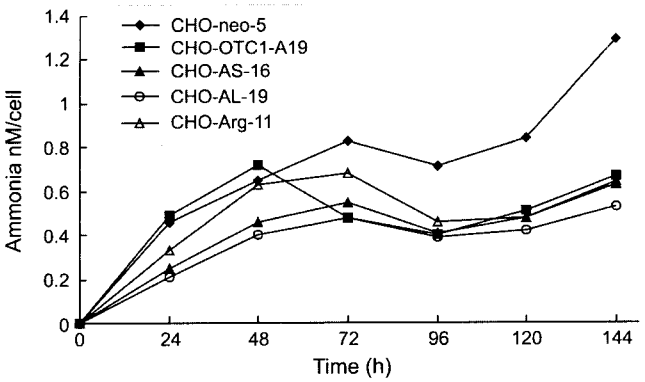

Fig. 5. Ammonia concentrations in culture media with the CHO-AS-16, CHO-AL-19, and CHO-Arg-11 expressed on per cell basis.

CHO-neo-5 and CHO-OTC1-A19 cells were included as negative and positive controls, respectively. The values were calculated by dividing the mean value of the ammonia concentration by the mean number of total viable cells in a volume of 2.5 ml, as shown in Figs. 3 and 4, respectively.

providing a more accurate measurement for monitoring the actual ammonia-removing ability of a cell line. Based on the results shown in Figs. 3 and 4, the ammonia concentrations in the culture media per cell were calculated and plotted in the graph shown in Fig. 5. After 96 h of culture, at which time the cell population reached 100% confluency, the CHO-AS-16, CHO-AL-19, and CHO-Arg-11 cells showed a 40–60% lower ammonia concentration in the media per cell than the CHO-neo-5 cells: 40–50% lower in CHO-AS-16, 45–60% lower in CHO-AL-19, and 35–50% lower in CHO-Arg-11. The CHO-AS-16 and CHO-Arg-11 cell lines both exhibited similar or slightly lower ammonia concentrations per cell than the CHO-OTC1-A19 cell line at the end of the culture period. However, the CHO-AL-19 cell line had a lower ammonia concentration per cell than the CHO-OTC1-A19 cell line, by about 20%.

Discussion

It is widely recognized that ammonia (NH_3 or NH_4^+) is one of the most important inhibitory substances that accumulates in cell cultures. Several studies have shown the adverse effects of ammonia on protein production in animal cell cultures [1, 2, 14, 19, 27].

Previously, we developed a CHO cell line expressing the first two enzymes of the urea cycle in an attempt to decrease the accumulation of ammonia. Although this cell line exhibited a reduced ammonia accumulation and higher cell growth rate than vector-controlled CHO cells, about 2.5–3 mM ammonia were present at the end of a 6-day culture period. Accordingly, the present study established CHO cells expressing the first three, first four, or all five enzymes of the urea cycle to further reduce the ammonia accumulation.

The established CHO cells expressing the first three enzymes of the urea cycle exhibited ammonia-removing ability and cellular growth rate similar to cells expressing the first two enzymes of the cycle, whereas the cells expressing the first four enzymes showed improved ammonia-removing abilities and a higher viable cell concentration. The cells expressing the enzymes from all five steps showed a lower ammonia concentration and, contrary to expectation, a lower cell viability. Therefore, the additional AS expression in cells expressing CPSI and OTC did not appear to redirect the ammonia flux to the next steps of the urea cycle. The argininosuccinate formed in the cells expressing the first three enzymes might have been further metabolized at a similar rate to the citrulline formed in the cells expressing CPSI and OTC, thereby resulting in a similar ammonia disappearance rate and similar growth rate. The arginine formed by the additional expression of AS and AL in the CPSI and OTC-expressing cells might have been actively utilized for the synthesis of proteins and compounds, such as polyamines, required to maintain cellular physiological functions, which in turn facilitated a flux from ammonia to arginine synthesis and improved the cellular growth rate. However, the presently developed CHO cell line expressing all the five urea cycle enzymes exhibited the lowest ammonia concentration among all the cell lines developed. Nevertheless, this lower ammonia accumulation did not result in a higher cellular growth rate. One possible explanation for this is that the forced overexpression of arginase induced by the use of a strong promoter degraded both the arginine formed by the introduced urea cycle enzymes as well as the arginine added to the media as an essential amino acid to support normal cellular growth, thereby causing an arginine deficiency and slow growth rate. The three cell lines established in the present study contained about 2 mM ammonia in the culture media after 6 days of culture. Although the effect of ammonia on cell growth depends on the cell line used and the culture conditions, this residual level of ammonia might have been too high. An ammonia concentration of 2–8 mM has been found to inhibit cell growth in various other cell lines, such as BHK [9], hybridoma [14], and CHO cells [21, 38]. In addition, 0.6–10 mM ammonia concentrations prevent the growth of fibroblasts [39] and kidney cells [35], or shorten the life of lymphocytes [24] and hybridoma cells [20] in a culture. Moreover, the physiological concentration is about 0–0.5 mM [6]. Thus, a further reduction in the ammonia concentration is still needed to facilitate cell respiration and perhaps increase the productivity of physiologically active proteins. The combined expression of CPSI and another enzyme, such as glutamine synthetase, could be used as an approach to further reduce ammonia accumulation in a mammalian

liver, in which CPSI removes ammonia from the periportal region first, followed by the removal of any residual ammonia by glutamine synthase in the pericentral region [9, 25, 35], leading to near-zero ammonia levels in the blood flowing from the liver.

In conclusion, CHO cells were established with introduced genes for the first three, first four, or all five stages of the urea cycle, and their ammonia-removing abilities and growth rates were characterized. Among the cell lines established, cells with introduced genes for the first four stages of the urea cycle were found to be the most effective in reducing the ammonia accumulation and improving the cellular growth rate. Accordingly, this cell line could be used to improve the productivity of many therapeutic glycoproteins.

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