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Effect of Low Adapted Temperature and Medium Composition on Growth and Erythropoietin (EPO) Production by Chinese Hamster Ovary Cells

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Temperature and medium composition were changed with the aim of increasing growth and erythropoietin (EPO) production in EPO-producing Chinese hamster ovary (CHO) cells. We used the CHO cell line, IBE, and its derivative, CO5, which over-expresses the first two enzymes of the urea cycle, carbamoyl phosphate synthetase I (CPS I) and ornithine transcarbamoylase (OTC). When supplements were added to the medium at 33 °C, the growth of IBE and CO5 cells increased by 27% and 26%, respectively and the maximum yield of EPO was increased by 40% in both cell lines. The absolute EPO concentration in the CO5 cells was always 55~60% higher than in the IBE cells. In addition, when the two cell lines were continuously cultured with supplements at 33 °C until their growth rates approached those at 37 °C, the growth rates of both IBE and CO5 cells increased by 54% and their maximum EPO levels increased by up to 73% and 56%, respectively. Therefore, the growth and EPO expression levels of CO5 cells increased 2.2-fold and 2.6-fold, respectively, compared to those of the IBE cells. These results indicate that adaptation to lower temperature as well as medium supplementation could be important for improving cell growth and EPO production.

Key words: CHO cells, erythropoietin (EPO), Temperature, Serum-free medium, Supplement

INTRODUCTION

The effects of various environmental parameters such as temperature (Yoon *et al.*, 2003; Fox *et al.*, 2004), pH (Borys *et al.*, 1993; Zanghi *et al.*, 1999), pO_2 (Lin *et al.*, 1993), pCO_2 (Kimura and Miller, 1996; Zanghi *et al.*, 1999), and osmolality (Ryu *et al.*, 2000; Kim *et al.*, 2000) on cell growth and recombinant protein production have been investigated extensively with the aim of increasing recombinant protein production in Chinese hamster ovary (CHO) cell lines. Temperature is an important parameter affecting growth and recombinant protein production. Mammalian cells, including CHO cells, are generally cultured at 37 °C and their growth is reduced at lower temperatures, although the cells remain viable for long periods of time (Yoon *et al.*, 2003).

The use of various supplements to increase growth has been also investigated. For mass production in commercial quantities, serum has shortcomings such as high cost and interference with product purification (Glassy *et al.*, 1988). Various supplements have been tried as alternatives: SITE (selenium, insulin, transferrin, ethanolamine), yeast extract, and linoleic acid-BSA (Liu *et al.*, 2001). The addition of a mixture of SITE (the most effective supplement currently), yeast extract, and linoleic acid-BSA can increase growth by up to 80% (Liu *et al.*, 2001; Raghu *et al.*, 2002).

The accumulation of ammonium ion in mammalian cell cultures is toxic and inhibits growth and the production of recombinant proteins (Butler and Spier 1984; Cruz *et al.*, 2000; Yang and Butler, 2002). It also alters glycosylation in the Golgi apparatus (Andersen and Goochee, 1995; Schneider *et al.*, 1996;Yang and Butler, 2000; Yang and Butler, 2002). Both cell growth and viability have been increased by reducing the ammonia concentration in the culture medium (Butler and Spier, 1984; Cruz *et al.*, 2000).

Previously, we reported the development of a CHO cell line (OTC) that over-expresses the first two enzymes of

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the urea cycle, carbamoyl phosphate synthetase I (CPS I) and ornithine transcarbamoylase (OTC) (Park *et al.*, 2000). The OTC cell line both reduces the ammonia concentration in the culture medium and increases growth (Chung *et al.*, 2003; Park *et al.*, 2000). The cell line CO5 was developed in our laboratory by transfection of the CPS I and OTC genes into the EPO-producing CHO cell line, IBE (Kim *et al.*, 2004). Growth and EPO production of CO5 cells were ~15-~20% and 2.5-fold higher, respectively, than those of IBE cells, and the ammonia concentration per cell in the culture medium of CO5 cells was ~15-~30% lower than that of IBE cells (Kim *et al.*, 2004).

CHO cells are popular mammalian hosts for the commercial production of therapeutic proteins, and EPO is typical of the recombinant proteins produced by cell culture. EPO, a glycoprotein produced by CHO cells, is a physiologically active protein with a substantial worldwide market. It is used in the clinical treatment of anemia associated with chronic renal failure, cancer chemotherapy, and bone marrow transplantation. A more efficient cell culture system is required to support the massive demand for EPO.

Various culture conditions have been investigated to increase EPO expression in CHO cells. When EPO-producing CHO cells were cultured at 33 °C, EPO levels increased, but growth was reduced (Yoon *et al.*, 2003). The present experiments were aimed at maximizing recombinant EPO expression in CO5 cells by increasing both growth and viability. Supplements were added to the culture medium and the cells were continuously cultured at 33 °C until their growth rate was similar to that of cells at 37 °C. The effect of temperature and supplementation on EPO expression were then determined.

MATERIALS AND METHODS

Cell lines

The IBE cell line was developed by co-transfecting the dihydrofolate reductase (DHFR) gene into DHFR-deficient CHO cells together with the human EPO gene (Kim *et al.*, 2004). OTC cells, *dhfr-* CHO cells expressing CPSI and OTC were also developed in our lab (Park *et al.*, 2000) and the CO5 cell line expressing CPSI and OTC was generated by transfecting plasmids pCIneo-CPS45 and pREP-OTC1 into IBE cells (Kim *et al.*, 2004).

Cell culture and cell growth determination

All cells were cultured in a humidified incubator at 33 and 5% CO_2. IBE and CO5 cells were maintained in Minimum Essential Medium Alpha (MEM-α) (Gibco BRL, U.S.A.) supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin (Gibco BRL; 10,000 U/mL penicillin G sodium and 100 μg/mL streptomycin sulfate in 0.85% saline), 1 mM *N*-carbamoyl-L-glutamate and 5 mM L-ornithine (Sigma, U.S.A.) as activators for CPSI and OTC, respectively. OTC cells were maintained in the same medium, except for the addition of 0.1 mM sodium hypoxanthine, and 0.016 mM thymidine (Gibco BRL). The cells were inoculated at a cell density of 1×10^5 cells/mL into six-well plates containing 2.5 mL of MEM-α medium. When the viable cell titer reached approximately 3×10^5 cells/mL, the medium was removed and the cells were incubated in serum-free medium supplemented with 1% SITE (selenium, insulin, transferrin, and ethanolamine) (Sigma), 0.3 g/L yeast extract (Duchefa, Netherlands), and 0.09% linoleic acid-BSA (Sigma). The cells could be grown at 33 °C after two successive passages at 35 °C and five successive passages at 33 °C. After the cells had been maintained at 33 °C until their growth rate approached that at 37 °C, the medium was changed to Ex-cell 301 serum-free medium (JHR, U.S.A.) with the same supplements. Culture supernatants were collected daily for 11 days and viable cells were counted with a hemocytometer in a suspension of trypan blue following trypsinization.

Ammonia assay

The supernatants were aliquoted and standard solutions containing 0, 1, 3 and 5 mM NH_4Cl were prepared in the medium and stored at -20 °C. Ammonia concentrations were determined by a modification of the previously described indophenol method (Sikdar and Sawant, 1994). To remove inhibitors of the colorimetric reaction, protein-removing reagents (1% sodium tungstate, 0.1N sulfuric acid) were mixed with each sample, and coloring reagents (reagent I: 5 g phenol and 25 mg sodium nitroprusside/500 mL, reagent I : 5 g NaOH, 53.6 g $Na_2HPO_4 \cdot 12H_2O$ and 10 mL chlorine 10%/1 L) were added. The mixtures were kept at 37 °C for 20 min, and cooled in ice-cold water for 5 min to stop the reaction. The ammonia concentration was calculated from the OD at 630 nm.

EPO assay

EPO concentrations were quantified by an enzyme-linked immunosorbent assay (ELISA). In brief, 96-well plates were coated with monoclonal antihuman EPO antibody. These were then blocked with 2% bovine serum albumin (BSA) (USB, Canada) and supernatant samples loaded into the wells. The primary antibody was polyclonal rabbit EPO antibody (R&D System, U.S.A.), and the secondary antibody was horse radish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Sigma, U.S.A.). EPO concentrations were calculated from OD values at 490 nm.

Evaluation of specific EPO productivity

Specific EPO productivity (q_{EPO}) was based on the data

collected throughout the period of the culture, and was calculated as the EPO concentration against the time integral values of the growth curve (Renard *et al.*, 1988).

RESULTS

Effect on cell growth of temperature and medium composition

The effects of culture temperature and medium composition on growth of IBE, CO5, and OTC cells were determined. The cells were cultured in serum-free medium, and serum-free medium plus supplements until the cell growth rate at 33 °C was similar to that at 37 °C. Viable cell numbers in cultures at 33 °C in the presence of serum-free medium plus supplements were higher in all cases than in serum-free medium (Fig. 1). Viable cell counts cultured at 33 °C in the presence of serum-free medium plus supplements until the cell growth rate at 33 °C was similar to that at 37 °C were also higher than in serum-free medium without supplements, in all cases (Fig. 1). The numbers of viable CO5 cells at 33 °C in the presence of serum-free medium and serum-free medium plus supplements were 25% greater than those of IBE cells (Fig. 2A, B) and 24% greater than those of IBE cells at 33 °C in the presence of serum-free medium plus supplements when grown until the growth rate became similar to that of the cells at 37 °C (Fig. 2C). The viability

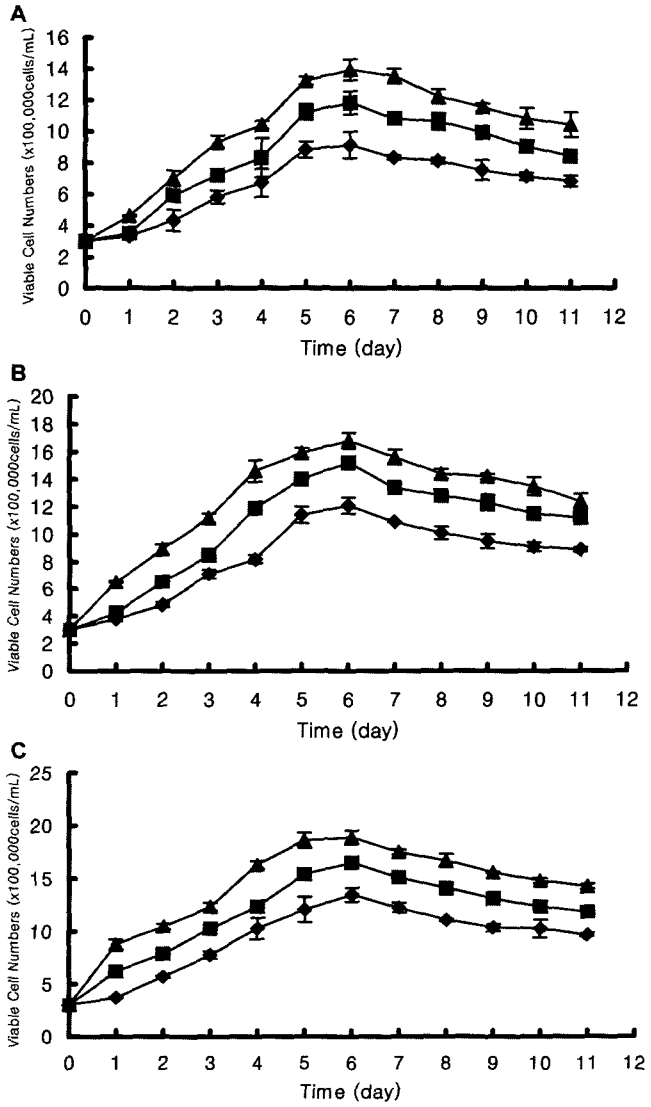

Fig. 1. Effect of temperature and medium composition on viable cell numbers. All cells were cultured at 33 °C. OTC cells served as a positive control. Values are means ± SD of duplicate cultures. The cells were cultured in the presence of serum-free medium (SFM), serum-free medium plus supplements (SFM+sup), and serum-free medium plus supplements until the cell growth rate at 33 °C was similar to that at 37 °C (A-SFM+sup). A: IBE, B: CO5, C: OTC (◆: SFM, ■: SFM+sup, ▲: A-SFM+sup).

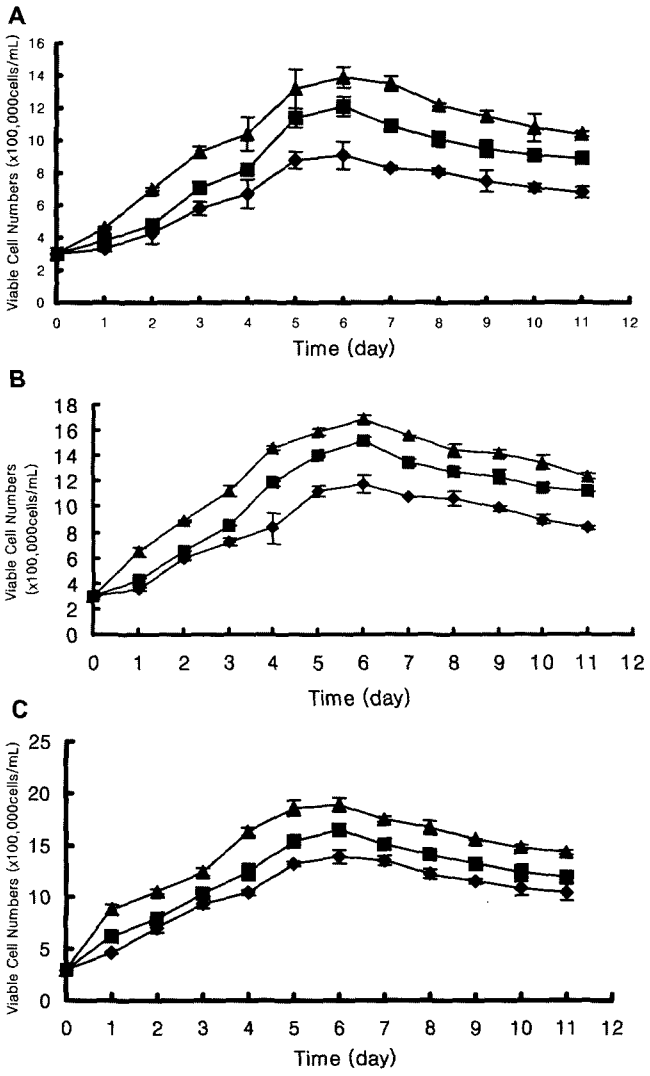

Fig. 2. Viable cell numbers in each cell line. A: SFM, B: SFM+sup, C: A-SFM+sup (◆: IBE, ■: CO5, ▲: OTC). Abbreviation are listed in the legend of Fig. 1.

of OTC cells cultured at 33 °C in the presence of serum-free medium and serum-free medium plus supplements was 39% and 42% greater than that of IBE cells, respectively (Fig. 2A, B). The viability of CO5 cells cultured at 33 °C in the presence of serum-free medium plus supplements until the cell growth rate became similar to that of the cells at 37 °C was 44% greater than that of IBE cells (Fig. 2C). These results indicate that the growth of each of the cell lines cultured in the presence of serum-free medium plus supplements, and cultured at 33 °C until their growth rates were similar to those of cells at 37 °C increased by 20-30% and 55-60% respectively, compared to those of the cells grown in serum-free medium. The viability of CO5 and OTC cells was always 20-30% and 40% higher than that of IBE cells, respectively.

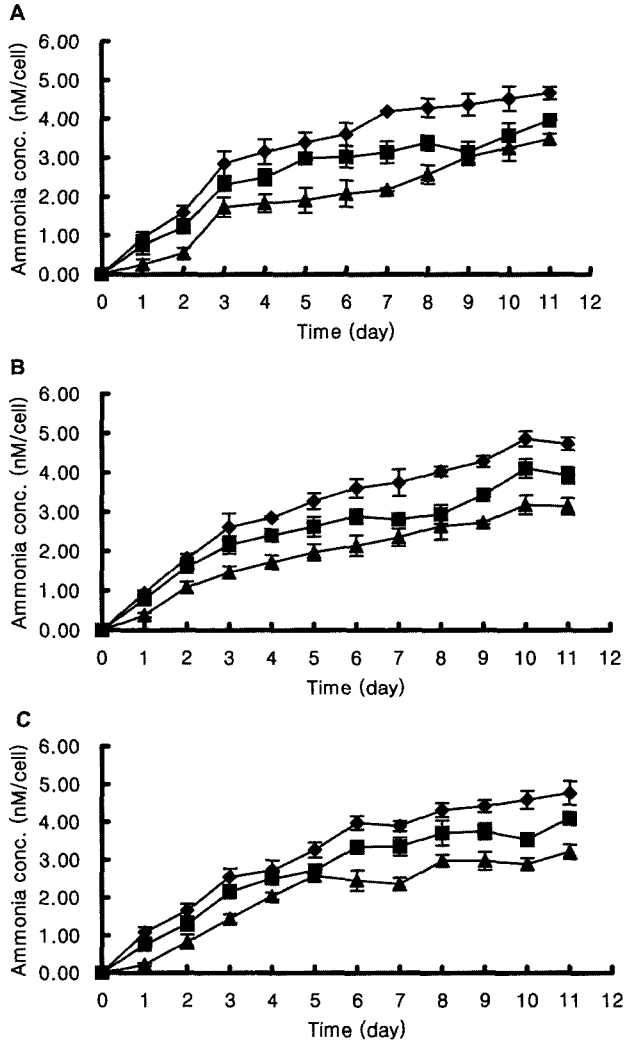

Fig. 3. Ammonia concentrations per cell in cell culture medium. Values were calculated by dividing the mean value of the ammonia concentration by the mean number of viable cells per 2.5 mL. A. Culture in the presence of serum-free medium. B. Culture in the presence of serum-free medium plus supplements. C. Culture in the presence of serum-free medium plus supplements until the growth rate at 33 °C was similar to that at 37 °C (◆ : IBE, ■ : CO5, ▲ : OTC).

Comparison of ammonia concentrations per cell

We measured ammonia concentrations in the culture media of IBE, CO5, and OTC cells. The results showed that the ammonia concentrations in the culture media (Fig. 3A, B, and C), as well as the concentrations per cell (data not shown), were not affected by temperature and medium composition and that those in CO5 and OTC cells, respectively, were 15-20% and 40% lower than in IBE cells. This suggests that ammonia concentrations are essentially unaffected by temperature and medium composition.

Comparison of EPO expression between IBE and CO5 cells

We measured the EPO levels in the supernatants of IBE and CO5 cells by EPO ELISA as described in Materials and Methods. The EPO concentrations in CO5

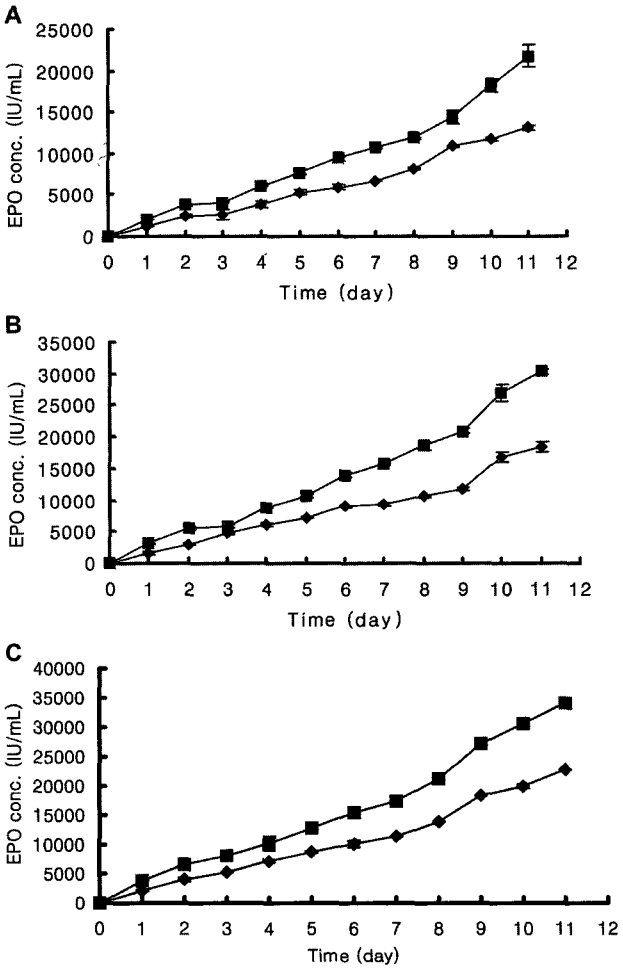

Fig. 4. EPO expression in IBE and CO5 cells. All cells were cultured at 33 °C and values are means ± SD of duplicate cultures. Other details are as listed in the Fig. 3 legend.

supernatants were in each case 55-60% higher than in those of IBE cells (Fig. 4), with no effect of temperature or medium composition.

Effect of temperature and medium composition on maximum EPO production

We compared the effect of temperature and medium composition on the levels of EPO in IBE and CO5 cells cultured at 33 °C in the presence of serum-free medium, serum-free medium plus supplements, and in serum-free medium plus supplements when grown until the growth rate was similar to that at of the cells at 37 °C. The results shown in Fig. 5 and Table I demonstrate that the maximum EPO concentrations of IBE and CO5 cells cultured at 33 °C in the presence of serum-free medium plus supplements, and serum-free medium plus supplements until the cell growth rate became similar to that of the cells at 37 °C, were up to 40-46% and 70% greater, respectively, than those in the presence of serum-free medium.

Effect of temperature and medium composition on specific EPO productivity

To compare EPO productivity, EPO was assayed by EPO ELISA in the culture supernatants as described in Fig. 5. Fig. 6 displays the plots of time integrals of number of viable cells vs. EPO concentration used to determine specific EPO productivity (q_{EPO}) for the cultures shown in Fig. 5. Values of q_{EPO} did not change significantly when temperature and medium composition were altered. The maximum q_{EPO} of IBE and CO5 cells cultured at 33 °C in serum-free medium plus supplements until the growth rate approached that of cells at 37 °C were 226.728 IU/10^5 cells/day and 501.029 IU/10^5 cells/day, respectively (Fig. 6). These values are 36% and 30% higher, respec-

Table I. Effect of temperature and medium composition on maximum EPO production

Cell line	Supplements[a]	Adapted to 33 °C[b]	Maximum EPO conc. (IU/mL)
IBE	-	-	13123 ± 329
	+	-	18333 ± 812
	+	+	22809 ± 60
CO5	-	-	21839 ± 1287
	+	-	30425 ± 403
	+	+	34120 ± 856

[a] 1% SITE (selenium, insulin, transferrin, ethanolamine), 0.3 g/L yeast extract, 0.09% linoleic acid-BSA.
[b] The cells were maintained for five successive passages at 33 °C after two passages at 35 °C in MEM-a medium, and cultured at 33 °C until their growth rate was similar to that at 37 °C.

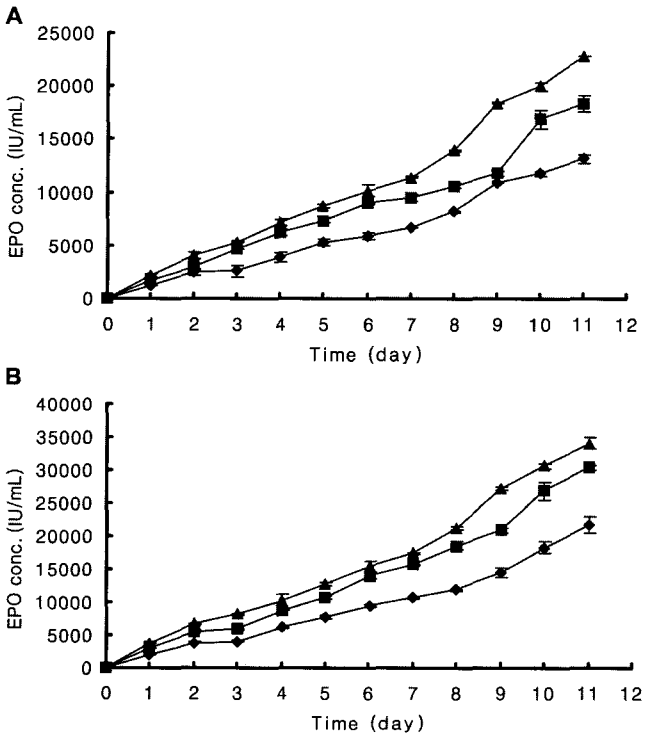

Fig. 5. Effect of temperature and medium composition on EPO production. Details are as listed in the Fig. 1 legend. A: IBE, B: CO5 (◆ : SFM, ■ : SFM+sup, ▲ : A-SFM+sup).

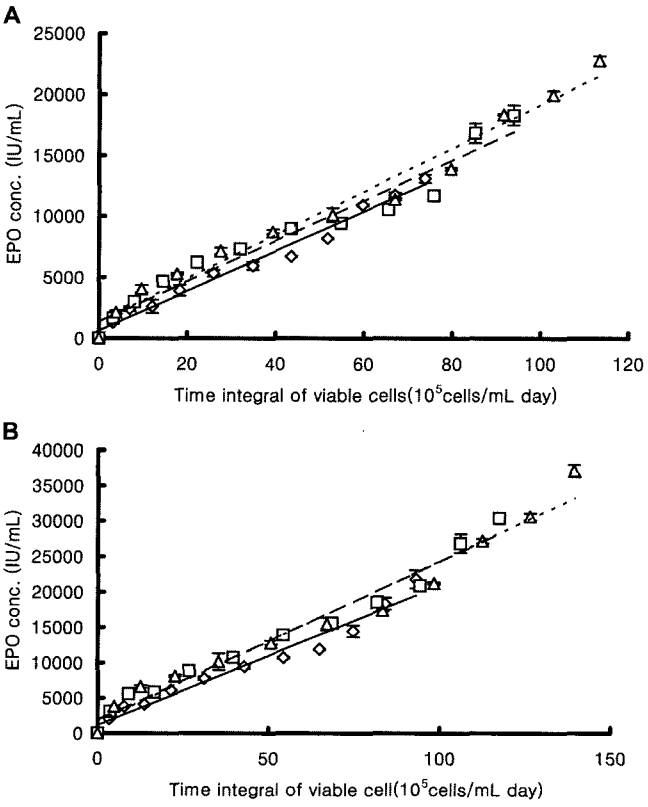

Fig. 6. The effect of temperature and medium composition on q_{EPO}. The q_{EPO} value was calculated from the slope of each plot. Details are as listed in the Fig. 1 legend. ◇: SFM, □ : SFM+sup, △ : A-SFM+sup.

tively, than those in the presence of serum-free medium; the minimum q_{EPO} of IBE and CO5 cells, respectively, was 196.717 IU/10^5 cells/day and 387.451 IU/10^5 cells/day in the presence of serum-free medium.

DISCUSSION

We have investigated whether EPO expression levels and cell numbers could be increased in EPO-producing CHO cells by lowering the growth temperature and supplementing the medium. We have shown that EPO expression levels in CO5 were 55-60% greater than in IBE (Fig. 4) and CO5 also grew better (Fig. 1), and accumulated less ammonia per cell than IBE (Fig. 3). Our findings imply that the higher cell growth and EPO production in CO5 cells are related to the reduced accumulation of ammonium ion by these cells.

The ammonia concentration in the culture medium of CO5 cells was 10-15% lower than in that of IBE cells (data not shown). The ammonia concentration in the culture medium per cell was also 15-20% lower than in that of IBE cells (Fig. 3). On the other hand, Chung *et al.* (2003) reported that the ammonia concentration in the culture medium, and in the culture medium per cell, were 15-30% and 40-60% lower, respectively, in OTC cells than in CHO cells (Chung *et al.*, 2003). Differences in the rate of removal of ammonia from the culture medium could result from use of different cell lines, culture conditions, or inocula, since the CHO and OTC cells were cultured in MEM-α with 10% FBS at 37 °C and inoculated at 1×10^5 cells/mL (Chung *et al.*, 2003). In our study, cells were cultured in serum-free medium at 33 °C and inoculated at 3×10^5 cells/mL.

As mentioned above, the numbers of viable CO5 cells were always 20-30% higher than those of IBE cells. In addition, numbers of viable cells of each of the cell lines when cultured in the presence of serum-free medium plus supplements, or in serum-free medium plus supplements until their growth rate at 33 °C was similar to that at 37 °C, were 25-30% and 55-60% greater, respectively, than those cultured in serum-free medium (Fig. 1). However, Liu *et al.* (2001), reported that viable cells in CHO cultures in the presence of serum-free medium plus supplements were 80% higher than those in serum-free medium on its own (Liu *et al.*, 2001). One possible explanation of the difference in the increase in those results and in ours is that in the study of Liu *et al.* (2001), the cells were cultured at 37 °C and inoculated at 8×10^4 cells/mL compared to 33 °C and inocula of 3×10^5 cells/mL in our case.

In the present experiments, EPO expression was 40-70% higher secondary to an increase in cell numbers (Fig. 5). Regardless of the culture temperature and medium composition, CO5 cells had 55-60% higher EPO expression levels than IBE cells (Fig. 4). The extent of this increase differed from that observed in our previous study in which EPO expression in CO5 cells was 2-2.5 fold greater than in IBE cells, perhaps because in our previous study the cells were cultured in MEM-a with 10% FBS and fresh medium was supplied at 1-, 2-, and 3-day intervals whereas in the current study, the cells were maintained in batch culture for 11 days in the presence of serum-free medium. Cell viability was lower in the current experiments because of the extended culture period and possibly because of an increased release of proteolytic enzymes from the dead cells.

We previously reported that CO5 produced 2 times more EPO than IBE. CO5 had a higher growth rate and accumulated less ammonia per than IBE, resulting in the enhancement of EPO production in CO5. Therefore, the temperature and medium composition as well as ammonia concentration and cell viability could be important factors for the enhancement of EPO production.

In conclusion, cell viability and EPO expression levels increased in both CO5 and IBE cells cultured in the presence of serum-free medium plus supplements until their growth rate at 33 °C approached that at 37 °C, suggesting that adaptation to the lower temperature as well as medium composition are important for improving cell growth and recombinant protein production. These results may be of wide applicable for increasing the production of other physiologically active proteins in mammalian cell cultures.

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