Enhancement of recombinant erythropoietin production in CHO cells in an incubator without CO₂ addition

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Abstract

The effect of low levels of carbon dioxide (CO₂) in the gas phase on the production of recombinant human erythropoietin (EPO) in CHO cells was explored. A T-flask culture in an incubator without CO₂ addition showed a slow cell growth initially followed by the cessation of growth, while other cultures incubated under 0.5–5% CO₂ concentrations grew normally at the same rate during the entire period of cultivation. Interestingly, the production of EPO in the culture incubated under no CO₂ supply was highest among the tested cultures. The cell specific secretion rate of EPO (q_{EPO}) of the culture under no CO₂ supply was about 3 times higher than that of the culture under 5% CO₂ supply. Western blot analysis and *in vivo* bioassay of EPO showed no apparent changes in EPO quality between the two cases of different CO₂ environments (air vs. 5% CO₂), suggesting robust glycosylation of EPO by CHO cells even under very reduced CO₂ environment. Various combinations of the two extreme cases, with 5% CO₂ supply (suitable for cell growth) and no CO₂ addition (better for EPO production), were made in order to maximize the volumetric productivity of EPO secretion (P_V) in CHO cells. The P_V of the cultures programmed with initial incubation under 5% CO₂ followed by no CO₂ supply was about 2 times superior to that of the culture incubated only under no CO₂ supply. The P_V of the culture under no CO₂ supply was slightly lower than that of culture grown under 5% CO₂. However, the q_{EPO} of the no CO₂ supply case was more than 5 times higher than that of the culture under 5% CO₂ supply. In conclusion, we have demonstrated that a simple programming of CO₂ supply to an incubator can enhance the production of EPO in CHO cells remarkably, without any apparent change of the EPO quality.

Introduction

Most medium used in mammalian cell culture is buffered with bicarbonate in equilibrium with carbon dioxide (CO₂) in the gas phase. CO₂ is known to play an essential role in mammalian cell culture *in vitro* not only to maintain the culture pH at normally optimum range of 6.9–7.4 but also to regulate many cellular activities (McLimans, 1972). Typically mammalian cell cultures are incubated under about 5% CO₂ to achieve the desired pH (Parker, 1950; Kieler and Gromek, 1967). However, 5% CO₂ may not be an optimal level in many cases of mammalian cell cultivation since the percentage has been selected on the

basis of its being the usual concentration found in the alveolar spaces of the lung.

During scale-up in large scale bio-reactors, the CO₂ partial pressure (pCO₂) is easily built up to levels higher than the physiological range (31–54 mmHg), which causes significant problems in mammalian cell culture. Reduced cell growth and/or productivity were observed at 120 mmHg pCO₂ for the cultivation of CHO cells (Drapeau et al., 1990) and NS/O myeloma cells (Aunins et al., 1991). pCO₂ levels of more than 105 mmHg resulted in CHO cell growth inhibition and dramatic reduction in productivity (Gray et al., 1996). Growth inhibition of CHO cells by CO₂ in a dose-dependent manner was reported (Kimura and Miller,

Figure 1. Effect of different CO₂ gas phase concentrations on CHO cell growth, culture pH, and EPO production (○: 5% CO₂, □: 3% CO₂, △: 1% CO₂, ▽: 0.5% CO₂, ●: 0.03% CO₂). (a) Viable cell number in T-flask, (b) pH, (c) EPO concentration in the medium, (d) intracellular EPO level per 10⁶ cells. EPO analysis was repeated three times for each sample to ensure a reproducibility of assay results. Values are means $\pm$ S.D. of duplicate cultures.

1996). Reduction of specific productivity in BHK-21 cells was observed at a pCO₂ of 50 mmHg or higher concentration (Taticek et al., 1998). The growth rate of Hybridoma AB2-143.2 cells was decreased with increasing pCO₂ (deZengotita et al., 1998).

Enhanced levels of pCO₂ in mammalian cell cultures also affect the quality of recombinant glycoproteins. Grampp et al. (1994) reported that the percentage of N-glycolylneuraminic acid present in recombinant proteins expressed by CHO cells decreased with increasing pCO₂. The decrease of CHO cell polysialylation was also observed at elevated pCO₂

(Zanghi et al., 1999), implying high levels of dissolved CO₂ or [HCO₃⁻] have negative impact on protein glycosylation in mammalian cells. These detrimental effects of elevated pCO₂ should be overcome if one would like to obtain higher productivity and better quality of the product from the mammalian cell culture. Especially, in the production of recombinant glycoprotein biopharmaceuticals, it is essential to obtain well and consistently glycosylated protein products to ensure their biological activity and stability.

The negative effects of high pCO₂ levels on mammalian cell growth, productivity, and product quality

Figure 2. Western blot analysis of two EPOs produced under air and 5% CO₂ in the gas phase. Supernatants were taken from T-flasks, 48 h after cultivation under 5% CO₂ (lane 1) and air environment (lane 2). The Bio-RAD pre-stained molecular weight standard was used (lane 3).

may be overcome by reducing the CO₂ concentration in the gas supplied to the cell culture. Reducing the CO₂ concentration of the gas down to 2% appears to have no obvious effect on cell growth, if appropriate buffers like Hepes are added (Butler, 1991). Garnier et al. (1996) observed a significant improvement of TGF β receptor yield when baculovirus infected *sf-9* cells were incubated under 0.03% CO₂ environment. Except this report, however, very little work has been done on the influence of extremely reduced CO₂ environment on the production of recombinant glycoproteins in mammalian cells.

In this study, we examined the effects of reduced CO₂ levels in the gas phase on the production of recombinant human erythropoietin (EPO) in CHO cells. Once obtaining two individual optimum concentrations of CO₂ in the gas phase, one of which ensures the highest cell growth rate and the other one gives the highest EPO productivity, various combinations of the two different CO₂ levels were investigated to further increase the volumetric productivity of EPO secretion in CHO cells.

Materials and methods

Cell line and media

A Chinese Hamster Ovary (CHO) cell line manipulated to secrete recombinant human erythropoietin

(EPO) was used in this study. The EPO secreting CHO cells, which were coamplified with dihydrofolate reductase (DHFR), had been described previously (Yoon et al., 1998). Iscove's Modified Dulbecco's medium (IMDM, Cat. No. 12200, GIBCO, U.S.A.) supplemented with 10% (v/v) fetal bovine serum (FBS, GIBCO, U.S.A.) was used as the CHO cell growth medium. The serum free medium, SF2 medium (Yoon et al., 1998), was used for EPO production. 36 mM sodium bicarbonate was used in both cell growth and EPO production media.

Cell cultivation: Comparison experiments under reduced CO₂ levels in the gas phase

Cells were inoculated in T-flasks (25 cm², NUNC, DENMARK) at a density of 5×10^5 cells per T-flask and incubated at 37 °C under 5% CO₂ and 95% relative humidity conditions. The CHO cell growth medium was used to increase cell density. After the cell density reached approximately 1×10^6 cells per T-flask, the growth medium was removed and cells were washed once with Dulbecco's phosphate buffered saline (D-PBS, GIBCO, U.S.A.). The T-flasks were then filled with the SF2 medium for EPO production and incubated under different concentrations of CO₂ in the gas phase (0.03–5%).

Cell cultivation: Combination of 5% CO₂ and no CO₂ supply

Cells were inoculated in T-flasks (25 cm², NUNC, DENMARK) at a density of 2.5×10^5 cells per T-flask and incubated in an incubator at 37 °C under 5% CO₂ and 95% relative humidity conditions for two days. At this stage the CHO cell growth medium was used to obtain a sufficient cell density. Cells were then further incubated for three days either under air (pCO₂-0.03%) or 5% CO₂. Once the CHO cell growth medium was removed and cells were washed with D-PBS, the SF2 medium for EPO production was added to the T-flasks. Cells were then incubated for three days either under air or 5% CO₂. The six combinations of incubation under two different CO₂ concentrations are described schematically in Figure 3.

Cell cultivation: Influence of different levels of sodium bicarbonate in the medium

Cells were inoculated in T-flasks (25 cm², NUNC, DENMARK) containing the CHO cell growth medium at a density of 1×10^6 cells per T-flask and

Figure 3. Schematic diagram of six different cases of gas combinations. T-flasks were incubated under various combinations of air and 5% CO₂ environments.

incubated in an incubator at 37 °C under 5% CO₂ and 95% relative humidity conditions. After the cell density reached approximately 2.5×10^6 cells per T-flask, the CHO cell growth medium was removed and cells were washed once with D-PBS. The T-flasks were then filled with three different SF2 media containing 15, 36, and 55 mM of sodium bicarbonate and kept in an incubator at 37 °C for 78 h either under air or 5% CO₂. To exclude the osmolality effect of sodium bicarbonate on the cell growth and EPO production, the media were initially adjusted around 290–293 mOsm kg⁻¹ with 4 M NaCl.

Assays

Medium samples were taken at 12–24 h intervals and the culture pH was measured with pH meter (Cole-

Parmer, U.S.A.). After centrifugation the supernatant was kept at –75 °C for later analysis. Glucose and lactate concentrations were determined using YSI 2700 biochemistry analyzer (Yellow Spring Instruments, U.S.A.).

The viable cell number was measured by trypan blue dye exclusion. In each cell sample, the viable cell number was counted three times and the mean value and the standard deviation were calculated from duplicate cultures. Cells in each T-flask were washed once with D-PBS, trypsinized, and centrifuged to obtain the cell pellet sample. To obtain the cell lysate, 100 µl of cell lysis buffer (Schroder and Friedl, 1997) was added to a total of $0.5\text{--}4 \times 10^6$ cells. After incubation at 4 °C for 2 h the cell lysate was centrifuged. The supernatant and cell lysate were kept at –75 °C for the later analysis of intracellular EPO content.

Figure 4. Cell growth and EPO production under various combinations of air and 5% CO₂. After two days of cultivation under 5% CO₂ (Phase I), CHO cells were further cultivated for three days under either air or 5% CO₂ (Phase II). The CHO cell growth medium was then replaced with the SF2 medium for EPO production, and cells were incubated for three days more under either air or 5% CO₂ (Phase III). The viable cell number (a) and EPO concentration in the medium (b) were monitored (○: 1, ●: 2, ■: 3, ▲: 4, ▼: 5, □: 6, same combination numbers as used in Figure 3). EPO analysis was repeated three times for each sample to ensure a reproducibility of assay results. Values are means $\pm$ S.D. of duplicate cultures.

Quantitative analysis of EPO

The intra- and extracellular recombinant human EPO concentrations were quantified by using an enzyme-linked immunosorbent assay (ELISA). The 96 well plates (NUNC, DENMARK) were coated with a monoclonal mouse anti-human EPO antibody (R&D Systems Inc., U.S.A.) in D-PBS, for 2 h. The plates were blocked with blocking buffer (1% (w/v) bovine serum albumin in D-PBS) for 3 h and incubated for 2 h after adding the internal EPO standard, supernatant samples, and/or cell lysates. The plates were then treated for 1 h with a polyclonal rabbit anti-human EPO antibody (R&D Systems Inc., U.S.A.) in a diluent solution (1% (w/v) bovine serum albumin in D-PBS plus 0.03% (v/v) Tween 20). They were further treated with a horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG (ICN, U.S.A.) for 1 h. In every treatment, plates were washed 4 times with a washing buffer (D-PBS plus 0.03% (v/v) Tween 20). The detection of bound HRP was performed using TMB peroxidase substrate kit (BIO-RAD, U.S.A.). Absorbance was measured at 450 nm using an ELISA plate reader (Molecular Devices, U.S.A.). All incubations were done at 37 °C. In each assay, samples were pre-diluted at two different dilutions and analyzed. EPO analysis was repeated three times for each sample to ensure a reproducibility of assay results.

Cell cycle analysis

Cells were taken from T-flasks, centrifuged, and washed twice with 1 ml of D-PBS. The cell pellet sample, typically 2×10^6 cells, was resuspended in 2 ml of 70% ice-cold ethanol and kept at -20 °C for later analysis. Prior to analysis, the cells were stained with 50 μ l of 1 mg ml⁻¹ propidium iodide (SIGMA, U.S.A.) and incubated at least for 30 min in the dark. Flow cytometric analysis was carried out using a Becton-Dickinson FACScan fitted with an argon laser operating at 488 nm for determination of cellular DNA content. The software program MOD-FIT LT (Verity Software House Inc., U.S.A.) was used to quantify the cell cycle distribution of each sample.

Determination of in vivo bioactivity of EPO

The *in vivo* bioactivity of secreted EPO was evaluated by the reticulocyte measurement assay. The culture supernatant was dialyzed overnight against D-PBS buffer at 4 °C and diluted 10, 40, and 160 times with diluent. The diluent that was used for the dilution of

EPO samples as well as the EPO international standard (European Pharmacopoeia Biological Reference Preparations, Batch No. 1, Order code E1515000) consisted of D-PBS with 0.25% (w/v) human serum albumin (SIGMA, U.S.A.). The 12.5, 50, and 200 U ml⁻¹ of EPO standards were prepared and used for the EPO bioassay calibration. Three groups of six female B6D2F1 hybrid mice were dosed subcutaneously with diluted EPO samples or standards (six mice per each diluted sample). On day 5, blood was taken from mice by cardiac puncture and the reticulocyte count was done as described by Barbone et al. (1994) using flow cytometry analysis.

Western blot analysis

Prior to Western blot analysis, 20 μ l of culture supernatant sample was diluted 2 times with the sample buffer (0.125 M Tris, 30% glycerol, 3% sodium dodecylsulfate (SDS), 0.0025% (w/v) bromophenol blue, 15% mercaptoethanol, pH 6.8). Half of diluted sample mixture (20 μ l) was applied to 12% polyacrylamide gel. The separation conditions were adapted from Laemmli (1970). The protein bands in the gel were transferred to a 0.45 μ m nitrocellulose membrane (NOVEX, U.S.A.) soaked with transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3) for 90 min at 40 V. After blocking the unoccupied protein binding sites on the nitrocellulose membrane with blocking buffer (1% (w/v) bovine serum albumin/D-PBS), the blotted membrane was then incubated with a polyclonal rabbit anti-human EPO antibody (R&D Systems Inc., U.S.A.) and subsequently treated with a HRP conjugated goat anti-rabbit IgG (ICN, U.S.A.). The EPO bands were finally visualized by using the 4-chloro-naphthol substrate (BIO-RAD, U.S.A.).

Calculation of the specific glucose consumption rate, the specific lactate production rate, the specific secretion rate of EPO, and the volumetric productivity of EPO secretion

The cell specific EPO secretion rate (q_{EPO}) was determined by plotting the EPO concentration at each sampling point versus the integral over the time of viable cell number curve (Renard et al., 1988; Bibila et al., 1994). The specific glucose consumption rate (q_{glc}) and the specific lactate production rate (q_{lac}) were calculated by a method described elsewhere (Lee et al., 1991). The volumetric productivity of EPO secretion (P_V) was calculated by the following formula: $P_V = P_{EPO}/T_F$, where P_{EPO} and T_F are concentration

of EPO in the medium ($\mu\text{g ml}^{-1}$) and cultivation time (h), respectively.

Results

Comparison experiments under reduced CO₂ levels in the gas phase

Viable cell number, culture pH, intra- and extracellular levels of recombinant human EPO produced in CHO cells under 0.03–5% CO₂ concentrations in the gas phase were monitored after the T-flask culture medium was switched from the CHO cell growth medium to the SF2 medium for EPO production. A T-flask culture in an incubator without CO₂ supply showed a slow cell growth initially followed by the cessation of growth, while the other cultures incubated under 0.5–5% CO₂ grew normally at the same rates for the entire period of cultivation (Figure 1a). The cell densities reached maximum levels of 1.53×10^7 cells per T-flask after 72 h of incubation under 0.5–5% CO₂.

When cells were grown in an incubator under 5% CO₂ in the gas phase, the culture pH was stable initially and started to decrease gradually due to lactate produced during cell growth. Under reduced CO₂ levels, rather significant pH fluctuation occurred initially followed by a gradual decrease of culture pH to the levels below the initial value (Figure 1b). Interestingly, the extracellular levels of EPO varied with the CO₂ levels in an incubator (Figure 1c). The EPO production in the culture incubated under no CO₂ supply was higher than those of the cultures performed under 0.5–5% CO₂ supply. The cell specific secretion rate of EPO (q_{EPO}) of the culture done without CO₂ supply was about 3 times higher than that of the culture performed under 5% CO₂ supply. The intracellular level of EPO per 10^6 viable cells without CO₂ supply was much higher than those obtained under 0.5–5% CO₂ conditions (Figure 1d). The metabolic parameters and specific EPO secretion rate of the CHO cells incubated under 0.03–5% CO₂ in the gas phase are summarized in Table I. The data indicate that the reduction of CO₂ concentration in the gas phase down to 1% CO₂ level did not apparently influence the key metabolic parameters of CHO cell cultivation as well as the EPO production level. However, it is clearly shown that under an extremely reduced CO₂ environment (i.e. no CO₂ supply) the cell specific EPO secretion rate was increased tremendously although the yield of glucose to viable cell number in this case was lower than those in the other cases (0.5–5% CO₂).

To investigate the cause of enhanced EPO production under extremely reduced CO₂ level in the gas phase, the cell cycle distribution of the cells incubated under no CO₂ addition was compared with that of the cells grown under 5% CO₂ in the gas phase (Table II). The percentage of cells in the G₀/G₁ phase under no CO₂ supply increased from 36 to 75%, while the percentage of cells in the G₀/G₁ phase remained relatively unchanged when cells were grown under 5% CO₂ level in the gas phase. The percentage of cells in S phase decreased gradually from 52 to 25% when cells were incubated under no CO₂ supply.

Quality of EPO produced under extremely reduced CO₂ level in the gas phase

To check the quality of the EPO produced by CHO cells grown under extremely reduced CO₂ level in the gas phase, *in vivo* bioactivity of EPO obtained after 48 h of cell cultivation was determined by using the reticulocyte measurement assay (Barbone et al., 1994). The level of secreted EPO in the medium under no CO₂ supply was about 2 times higher than that of secreted EPO under 5% CO₂. However, the specific bioactivity of the EPO secreted under no CO₂ supply was not apparently different from that of the EPO secreted under 5% CO₂ (Table III). Western blot analysis of the EPOs produced under these two different CO₂ conditions also showed no apparent difference in their molecular weight distribution patterns (Figure 2).

Combination of 5% CO₂ and no CO₂ supply conditions

In many preliminary experiments in our laboratory, the CHO cells incubated under no CO₂ supply (i.e. air environment) grew slower but produced higher amounts of EPO in comparison to the CHO cells grown under 5% CO₂. Thus, we hypothesized that if cells were sufficiently grown under the normal 5% CO₂ condition and then switched to the air environment, the EPO productivity from these CHO cells would be much higher than the productivity obtained from the cells incubated only under air environment during the entire period of cultivation.

For preliminary comparison prior to the combination experiment, the CO₂ environment for the cells grown in the SF2 medium for EPO production was switched to a different gas condition in the middle of the cultivation. The EPO level in the culture medium under the forward combination of gas environment (5% CO₂ → air) was significantly higher than

Table 1. The specific EPO secretion rates and metabolic parameters of CHO cells incubated under various CO₂ concentrations in gas phase

CO ₂	Specific EPO secretion rate	Specific glucose consumption rate	Specific lactate production rate	Y ^a _{lac/glc}	Y ^b _{cell/glc}
(%)	q _{EPO} (μg 10 ⁶ cells ⁻¹ h ⁻¹)	q _{glc} (μmol 10 ⁶ cells ⁻¹ h ⁻¹)	q _{lac} (μmol 10 ⁶ cells ⁻¹ h ⁻¹)	(mol mol ⁻¹)	(10 ⁶ cells μmol ⁻¹)
0.03 (air)	0.20	0.25	0.47	1.93	0.12
0.5	0.08	0.23	0.35	1.55	0.17
1	0.06	0.20	0.31	1.55	0.18
3	0.07	0.19	0.30	1.64	0.17
5	0.06	0.18	0.29	1.58	0.18

^a Yield coefficient of glucose to lactate.

^b Yield coefficient of glucose to viable cells.

The values (viable cell number, EPO concentration in the medium, glucose in the medium, and lactate in the medium) used for the calculation of q_{EPO}, q_{glc}, and q_{lac} were averages.

that under the backward combination of gas environment (air → 5% CO₂). This preliminary result (data not shown) suggested that the cell status at the gas switching point was important to enhance the EPO productivity. Thus, a more sophisticated gas supply program from the starting point of the cell cultivation was designed to maximize the EPO volumetric productivity (Figure 3). All cultures in six combination cases were initially grown in the CHO cell growth medium for two days under 5% CO₂ until the viable cells reached to the target cell density of 10⁶ cells T-flask⁻¹ (Phase I). Cells were then incubated in the CHO cell growth medium for three days under various combinations of headspace gas (Phase II) (Figure 3). Finally, after switching the CHO cell growth medium to the SF2 medium for EPO production, cells were further incubated under air or 5% CO₂ for three days (Phase III). Results of the six different cases of gas combination are summarized in Table IV. The volumetric productivity of EPO secretion (P_V) in three cultures programmed to grow at least for one day under 5% CO₂ at Phase II and further incubated at least for three days under air environment at Phase III (combination 2–4) was about 2 times higher than that of the culture manipulated to grow only under air (combination 5). The P_V of the culture incubated for two days under 5% CO₂ at Phase II followed by further incubation for four days under air environment at Phase III (combination 3) appears to be superior to all other cases. The P_V of the culture incubated only under air during the Phases II and III (combination 5) was slightly lower than that of the culture grown under 5% CO₂. However, the cell specific secretion rate of EPO

Table 2. Percentage of cells in different phases of the cell cycle, incubated under air or 5% CO₂

Time (h)	Percentage of cells in different phases of the cell cycle (%)					
	Air			5% CO ₂		
	G ₀ /G ₁	S	G ₂ /M	G ₀ /G ₁	S	G ₂ /M
0	35.7	51.5	12.9	35.7	51.5	12.9
23	51.9	31.8	15.6	42.9	44.6	11.8
48	75.1	23.7	0.2	39.3	51.8	7.0
72	73.9	24.6	0.3	47.7	27.5	20.7

The cell cycle distribution was determined by using a FACS (as described in the Section Materials and Methods). The values are the averages of two measurements.

Table 3. *In vivo* bioactivity of EPO secreted from the CHO cells under air and 5% CO₂

CO ₂	EPO concentration in the medium ^a	Specific <i>in vivo</i> EPO bioactivity ^b
(%)	(μg ml ⁻¹)	(× 10 ⁵ IU mg ⁻¹)
0.03 (air)	17.3±1.2	1.27±0.1
5	7.8±0.4	1.34±0.1

^a Samples were obtained after 48 h of cultivation. Values are means±S.D. of duplicate cultures.

^b n = 6.

(q_{EPO}) of this case was over 5 times higher than that of the culture incubated under 5% CO₂.

The cell growth patterns shown in Figure 4a clearly indicate that incubation under 5% CO₂ at Phase II gave a significantly higher viable cell number. In particular, incubation under air environment at Phase II appears to be harmful to cell growth. A continuous supply of

Table 4. Maximum viable cell number, EPO concentration in the medium, specific EPO secretion rate, and volumetric productivity of EPO (P_V) obtained under different combinations of CO_2 environments as described in Figure 3

Combination number	Maximum viable cell number ($\times 10^6$ cells T-flask $^{-1}$)	EPO concentration in the medium at $t = 190$ h ($\mu\text{g ml}^{-1}$)	Specific EPO secretion rate q_{EPO} ($\mu\text{g } 10^6 \text{ cells}^{-1} \text{ h}^{-1}$)	Volumetric productivity of EPO ($\mu\text{g ml}^{-1} \text{ h}^{-1}$)
1	23.2 $\pm$ 0.9	32.9 $\pm$ 4.4	0.08	0.17
2	20.0 $\pm$ 0.4	64.4 $\pm$ 2.4	0.20	0.34
3	16.7 $\pm$ 0.8	71.1 $\pm$ 1.7	0.24	0.37
4	8.6 $\pm$ 0.8	62.4 $\pm$ 5.4	0.47	0.33
5	3.1 $\pm$ 0.1	25.5 $\pm$ 3.4	0.45	0.13
6	9.0 $\pm$ 0.7	22.6 $\pm$ 4.0	0.19	0.12

EPO analysis was repeated three times for each sample to ensure a reproducibility of assay results. Values are means $\pm$ S.D. of duplicate cultures.

5% CO_2 both at Phases II and III resulted in an average EPO production, although the viable cell number was highest among all tested cases (Figure 4b). The P_V was lowest in the case of the combination of air supply without CO_2 at Phase II followed by the incubation under 5% CO_2 at Phase III.

The increase of EPO production level by the combination of gas supply was also indirectly confirmed by Western blot analysis of the culture supernatants after the Phase III incubation (Figure 5). The intensities of EPO bands in Western blot analysis gel indicate that the EPO levels of three cultures programmed with initial incubation for two days under 5% CO_2 during Phase II followed by further incubation under air environment during Phase III were much higher than those of the three other cases of the combination experiments. In addition, similar EPO band patterns observed in the Western blot analysis suggest that the combination of gas supply does not have any impact on the macroheterogeneity of EPO.

Influence of different levels of sodium bicarbonate in the medium

To investigate whether the fluctuation of culture pH caused by the extremely low level of CO_2 in the gas phase was responsible for the increase of EPO production in CHO cells, the culture pH was intentionally fluctuated by using different concentrations of sodium bicarbonate in the SF2 medium (Figure 6a). The intra- and extracellular levels of EPO were not changed when the cells were cultivated under 5% CO_2 . However, they were significantly increased in a dose-dependent manner of sodium bicarbonate when CO_2

was not supplied (Figures 6b and c). The pH fluctuations by sodium bicarbonate under air were much higher than those under 5% CO_2 . Interestingly, the EPO level in 36 mM sodium bicarbonate under 5% CO_2 was lower than that in 15 mM sodium bicarbonate under air, although the pH fluctuations of both cases were similar to each other (Figures 6a–c). The cells with sodium bicarbonate grew normally under 5% CO_2 but their growth was strongly inhibited if CO_2 was removed from the gas phase (Figure 6d). Cells with 55 mM sodium bicarbonate without CO_2 supply produced the highest amount of intra- and extracellular EPO, although the cell growth was the lowest among the tested groups.

Discussion

As shown in Figure 1, a significant enhancement of EPO production from CHO cells in T-flask incubated under air without any CO_2 supply was observed, although there was a strong inhibition of cell growth under this extremely reduced CO_2 environment. In fact, high levels of CO_2 are well known to inhibit the cell growth as well as the production of recombinant proteins in large scale mammalian cell cultivation (Drapeau et al., 1990; Aunins et al., 1991; Gray et al., 1996; Kimura and Miller, 1996; Taticek et al., 1998; deZengotita et al., 1998) but little work has been done about the effect of very low levels of CO_2 on the mammalian cell cultivation. It is only known that a minimum amount of CO_2 is essential to most of cells (Freshney, 1986).

Figure 5. Western blot analysis of EPOs taken from the cultures incubated for 72 h under various combinations of air and 5% CO₂ (M: pre-stained molecular weight standard, lanes 1–6: lane number corresponds to the number used in Figure 3).

In laboratory incubators, a supply of 5% CO₂ in the gas phase is a generally accepted practice for mammalian cell cultivation since CO₂ plays an essential role to maintain the culture pH at the normally optimum range of 6.9–7.4. In bioreactors, the CO₂ partial pressure may sometimes inevitably build up to at least 170 mmHg (36 mmHg is equivalent to 5% CO₂ level in the gas phase) due to the mass transfer limitation, which motivates most researchers to focus their studies on the alteration of cell growth, protein production, and protein glycosylation due to high CO₂ partial pressure (Drapeau et al., 1990; Aunins et al., 1991; Aunins and Henzler, 1993; Grampp et al., 1994; Gray et al., 1996; Kimura and Miller, 1996, 1997; Taticek et al., 1998; Zupke and Green, 1998; deZengotita et al., 1998; Zanghi et al., 1999). There has been no need, however, to cultivate recombinant mammalian cells under the very reduced level of CO₂ in the gas phase. As a previous study noted (Gray et al., 1996), CHO cells normally fail to grow in an incubator without CO₂ supply.

To the best of our knowledge, no one has reported the dramatic increase of a recombinant protein production in CHO cells cultivated under a very low level of CO₂ in the gas phase (i.e. under air environment). Only Garnier et al. (1996) noted the increase of TGF β receptor production in *sf-9* cells grown under air environment. Our finding of enhanced recombinant human EPO production in CHO cells incubated under air environment was by accident. A significantly

higher EPO concentration in the culture medium was detected when CO₂ was accidentally removed from an incubator. We postulated two possible reasons for this peculiar phenomenon: firstly, cell cycle arrest by the low CO₂ level in the gas phase may be responsible for the increase of both intra- and extracellular EPO concentration; secondly, a significant pH fluctuation by the low level of CO₂ supply from 5% to almost 0% may indirectly affect the increase of EPO production.

As seen in Table II, the percentage of CHO cells in the G₀/G₁ phase significantly increased from 36 to 75%, when CO₂ was not supplied to T-flask cultures. This means that the extremely low level of CO₂ in the gas phase is able to modulate the cell cycle. Previous studies demonstrated that the synthesis and secretion of proteins by mammalian cells occurred in the late G₁ and early S phases (Ramirez and Mutharasan, 1990; Al-Rubeai and Emery, 1990; Jenkins and Hovey, 1993). Hence the productivity of recombinant proteins produced from CHO cells could be enhanced if the cell population is arrested in the G₁ phase or if the G₁ phase is elongated by any means. It was reported that mammalian cells were arrested in the G₁ phase when butyric acid was added to the culture medium (Darzynkiewicz et al., 1981; Xue and Rao, 1981), culture temperature was shifted down from 37 to 30 °C (Moore et al., 1997), high osmotic pressure was applied to the cells (Oh et al., 1994), or tumor suppressor genes were overexpressed (Fussenegger et al., 1997). We showed that our switching of the gas enviro-

Figure 6. Effect of sodium bicarbonate on culture pH (a), intra- (c) and extracellular EPO production (b), and CHO cell growth (d) when cells were incubated under air (closed symbols) or 5% CO₂ (open symbols). The sodium bicarbonate was added to the medium prior to cultivation (○: 15 mM, □: 36 mM, △: 55 mM). EPO analysis was repeated three times for each sample to ensure a reproducibility of assay results. Values are means ± S.D. of duplicate cultures.

onment from 5% CO₂ to an almost CO₂ free condition could also arrest the CHO cells in the G₁ phase, which appeared to be a more convenient way for arresting cells in the G₁ phase, than any other method suggested so far. Several previous reports demonstrated that the mammalian cells ceasing proliferation and arrested in the G₁ phase by chemical or physical means produced higher amounts of proteins than cells in normal division (Loskutoff and Paul, 1978; Jenkins and Hovey, 1993; Fussenegger et al., 1997; Kaufmann et al., 1999; Cherlet and Marc, 2000). Although additional experiments are required, our observations strongly suggest that the increased EPO production in CHO cells under

air environment is mainly due to the cell cycle arrest in the G₀/G₁ phase which is caused directly or indirectly by the extremely reduced level of CO₂ in the gas phase.

The second postulation was that CO₂ may affect the CHO cell metabolism via culture pH changes. If this was true, the EPO production had to be augmented by the initial increase of culture pH regardless of CO₂ supply. The intentional fluctuation of culture pH by sodium bicarbonate resulted in no EPO production change under 5% CO₂, but the pH change by sodium bicarbonate under air environment appeared to affect the EPO production in a dose dependent manner, as

shown in Figure 6. These observations suggest that the fluctuation of culture pH by CO₂ level changes in the gas phase was not the only reason for the changes in the EPO production but that there were additional indirect factors modifying the CHO cell metabolism. This hypothesis was also supported by the observation that the EPO level in 36 mM sodium bicarbonate under 5% CO₂ was lower than that in 15 mM sodium bicarbonate under air, although the pH fluctuations of both cases were similar. The drawback of this analysis was that the pH fluctuation in 36 mM sodium bicarbonate under the limited CO₂ supply was too high to be compared with the addition of sodium bicarbonate under 5% CO₂, which means that the effect of elevated pH on EPO production, cell growth, and cell metabolism could not be dissociated from that of low pCO₂ level.

The quality of EPO secreted from the CHO cells incubated under extremely low level of CO₂ in the gas phase was examined both by Western blot analysis and *in vivo* activity assay of reticulocyte measurement. According to the Western blot gel shown in Figure 2, the glycosylation pattern of EPO secreted under air environment appears to be similar to that of EPO secreted under 5% CO₂ although the amount of EPO secreted under air environment is more than 2 times higher than that of EPO secreted under 5% CO₂. However, this Western blot analysis does not provide any information about the terminal sialylation of EPO, which is very important for circulatory half-life and *in vivo* bioactivity of EPO (Fukuda et al., 1989). The glycosylation of human protein therapeutics is a major concern in the bio-industry since any alteration of their structure could influence the efficacy of the products (Jenkins et al., 1996). In previous reports, it was shown that elevated CO₂ partial pressure (pCO₂) has a minor effect on the glycosylation of recombinant tPA secreted by CHO cells (Kimura and Miller, 1997) but the sialylation of the neural cell adhesion molecule is very sensitive to culture conditions (Zanghi et al., 1998a,b). Because sialylation of proteins can be altered under elevated pCO₂ (Zanghi et al., 1999), there may be a possibility that extremely reduced pCO₂ can alter terminal sialylation on EPO. Instead of examining the sialic acid content of EPO, we directly measured the *in vivo* bioactivity of EPO. The data shown in Table III indicate that the alteration of sialylation on EPO may not occur or may not be significant when CHO cells are incubated under air environment. Nevertheless, more detailed investigations on the terminal sialylation of EPO produced in almost CO₂ free environment is needed in the future.

The interesting phenomenon that the CHO cells incubated under air environment grow much slower, but produce higher amounts of recombinant human EPO than the cells grown under 5% CO₂ had motivated us to design a sophisticated CO₂ gas supply program for further enhancement of the EPO production. We showed that an appropriate CO₂ gas switching for CHO cell cultivation in T-flask could increase the EPO production level at least 2 times in comparison to the normal EPO production level obtained under 5% CO₂ (Table IV). We believe that this simple technique of CO₂ gas switching is very economical to enhance the recombinant human EPO production from CHO cells. The CO₂ gas switching technique is not only useful for the EPO production in T-flask but also could be applicable to the roller bottle or bioreactor operations. Moreover, same approaches for the production of other recombinant proteins in mammalian cells could also result in the similar enhancement of their productivity. Further studies for other protein production in various mammalian cell lines are to be done in the future.

Conclusion

We found that the CHO cells in T-flask under air environment grow slower but produce higher amount of EPO than the CHO cells under 5% CO₂ in the gas phase. We also demonstrated that a simple on-off programming of CO₂ supply to an incubator can enhance the production of EPO in CHO cells remarkably, without any apparent change of the EPO quality.

Cell cycle analysis showed that most of CHO cells under air environment are arrested in the G₀/G₁ phase. The simulation study of pH elevation with sodium bicarbonate under 5% CO₂ resulted in no increase of EPO production in CHO cells, while the elevated pH by sodium bicarbonate under air environment positively affected the EPO production in a dose dependent manner. None of these observations, however, give full explanation to the increase of EPO production in CHO cells under limited CO₂ gas supply. Further studies for the understanding of this interesting phenomenon are to be done in the future.

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