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Screening of High-Productivity Cell Lines and Investigation of Their Physiology in Chinese Hamster Ovary (CHO) Cell Cultures for Transforming Growth Factor-β1 Production

CHUN, GIE-TAEK*, JOO BUOM LEE, SANG UK NAM[1], SE WON LEE, YEON HO JEONG[2], EUI YUL CHOI[3], IK HWAN KIM[4], YONG-SEOB JEONG[5], AND PYEONG-HYEUN KIM

Division of Life Sciences, Kangwon National University, Chunchon 200-701, Korea
[1]Department of Science Educations, Kangwon National University, Chunchon 200-701, Korea
[2]Division of Food and Biotechnology, Kangwon National University, Chunchon 200-701, Korea
[3]Department of Genetic Engineering, Hallym University, Chunchon 200-701, Korea
[4]Graduated School of Biotechnology, Korea University, Seoul 136-701, Korea
[5]Division of Biotechnology, Chonbuk National University, Chonju 561-756, Korea

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Abstract Using recombinant Chinese hamster ovary (CHO) cells, strategies for developing high producers for the recombinant human Transforming Growth Factor-β1 (TGF-β1) protein are proposed and their physiological characteristics in cell cultures were investigated. TGF-β1 is a pleiotrophic polypeptide involved in various biological activities, including cell growth, differentiation, and deposition of extracellular matrix proteins. The CHO cells included human TGF-β1 cDNA in conjunction with a dihydrofolate reductase (DHFR) gene, which was cotransfected into the cells to amplify the transfected TGF-β1 cDNA. As a first-round screening of the transfected cells, a relatively high TGF-β1-producing cell line was selected, and then, it acquired a resistance to increasing concentrations of methotrexate (MTX) up to 60 μM, resulting in a significant improvement in its TGF-β1 biosynthetic ability. After applying a monoclonal selection strategy to the MTX-resistant cells, more productive cells were screened, including the APP-3, App-5, and App-8 cell lines. These high producers were compared with two other cell lines (AP-1 cell line without amplification of transfected TGF-β1 cDNA and nontransfectant of TGF-β1 cDNA) in terms of cell growth, TGF-β1 productivity, sugar uptake, and byproduct formation, in the presence or absence of MTX in the culture medium. Consequently, both monoclonal selection as well as an investigation of the physiological characteristics were found to be needed for the efficient screening of higher TGF-β1 producers, even after the transfection and amplification of the transfected gene.

*Corresponding author
Phone: 82-33-250-8547; Fax: 82-33-241-4627;
E-mail: gtchun@cc.kangwon.ac.kr

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Transforming Growth Factor-βs (TGF-βs) are a family of related and generally homodimeric proteins. As a multifunctional agent, TGF-βs exhibit a broad range of biological activities, including stimulation or inhibition of cellular proliferation, matrix deposition, and metabolic effects, depending on the system [7, 11, 15, 16, 22]. Three TGF-β isoforms (TGF-β1, β2, and β3) have been purified from various sources [1, 9, 17]. TGF-β1, originally isolated from human platelets [1] and placenta [9], is considered to be the prototype for the TGF family of proteins, therefore, various structural and biological studies with TGF-β proteins have been performed using TGF-β1. TGF-β1 is synthesized in cells as a 390-amino acid molecule composed of a 29-amino acid leader peptide and 361-amino acid precursor protein [18, 22, 29]. Mature active TGF-β1 containing a C-terminal 112-amino acids is derived by the proteolysis of the precursor protein, which is a typical mammalian phosphoglycoprotein [8, 10]. The active species is a 25-kDa homodimer containing identical disulfide-linked subunits [1, 10].

Clinical interest in TGF-β1 as a potential therapeutic agent has greatly increased during the last decade. Potential applications of this protein have been suggested in the fields of wound healing, repair of bone cartilage and cardiac injury, and the suppression of the lymphocyte

function [3, 25, 26]. It has also been observed that TGF-β1 action is correlated with certain disease states, including kidney, liver, and lung fibrotic conditions. Unfortunately, TGF-β1 can only be obtained from natural sources in small quantities. The most abundant natural sources of TGF-β1 are platelets [1] and demineralized bone [24], which can also be the sources for disease-causing virus infection. Accordingly, to overcome the above-mentioned problems related to the development of TGF-β1 as a potential therapeutic protein, a recombinant method using mammalian cell culture technology needs to be explored for the mass production of this complex protein.

A mammalian cell culture has been proven to be a powerful means for the efficient production of complex human therapeutic proteins due to its various merits as explained below [4, 12, 27, 30]. In the last decade, much focus has been centered on the use of *E. coli* or other simple cell types to produce large quantities of proteins from cloned human genes [20]. However, it has been found that post-translational modifications, such as glycosylation, amidation, phosphorylation, and cleavage of protein precursors, can not be carried out correctly in bacterial cells, thereby resulting in inactive human proteins [5]. However, culture of mammalian cells for producing functional eukaryotic proteins can clearly overcome many of these problems, since post-translational processing generally occurs correctly in mammalian cells [4, 27]. As such, better mammalian cell expression systems coupled with more efficient means for mammalian cell cultures have been developed for the mass production of complex human proteins [12, 13, 30]. The current study proposes strategies for developing high producers for the recombinant human Transforming Growth Factor-β1 (TGF-β1) protein using Chinese hamster ovary (CHO) cells, and investigates the resulting physiological characteristics in the cell cultures. The CHO cells transfected with human TGF-β1 cDNA in conjunction with the dihydrofolate reductase (DHFR) gene were used [2, 19, 23]. A monoclonal selection strategy was used to test the methotrexate (MTX)-resistant cell groups to efficiently screen the high producing cell lines. In addition, the high producers selected were compared with other cell lines in terms of cell growth, TGF-β1 productivity, sugar uptake, and byproduct formation rates, in the presence or absence of MTX, a cytotoxic drug, in the cell culture medium.

MATERIALS AND METHODS

Permanent Cell Lines Expressing TGF-β1 cDNA

The TGF-β1-producing CHO cell line was originally manufactured by Dr S.U. Nam (from the Department of Science Educations, Kangwon National University, Korea) as follows: a dihydrofolate reductase deficient ($DHFR^-$) CHO cell line was purchased from ATCC (CRL-9096) (Rockville, U.S.A.), which was then transfected with human TGF-β1 cDNA in conjunction with a dihydrofolate reductase (DHFR) gene. The DHFR gene was cotransfected into the cells to amplify the transfected TGF-β1 cDNA. The DHFR gene was cut from a pSV-dhfr plasmid, which had a strong SV early promoter for the efficient expression of the DHFR gene. For the constitutive expression of the TGF-β1 protein, a cytomegalovirus promoter was located in front of the TGF-β1 cDNA. As a selectable marker, a neomycin-resistant gene was also transferred into the cell so that the transfected cells could be easily selected by treatment with G418, a neomycin analogue.

Medium for CHO Cell Cultures

The following optimized medium was utilized for the recombinant CHO cell cultures: 1:1 mixture of DMEM and Ham's Nutrient F-12 Medium (DMEM 6.7 g/l, F-12 5.35 g/l), glucose 1.349 g/l, L-glutamine 0.222 g/l, HEPES 2.383 g/l, $NaHCO_3$ 2.438 g/l, 1% (v/v) antibiotic mixture (penicillin 5,000 U/ml, streptomycin 50 mg/ml), and 10% (v/v) fetal bovine serum (FBS) (GibcoBRL, Gaithersburg, U.S.A.). The medium was sterilized using a membrane filtration method (pore size 0.22 μm). All the medium components, except for the FBS, were purchased from Sigma Chemical Co. (St. Louis, U.S.A.).

Amplification of Dihydrofolate Reductase Gene Through Treatment with a Cytotoxic Drug, Methorexate (MTX) (DHFR-MTX system)

To amplify the TGF-β1 cDNA together with the DHFR gene, a 5 mM stock solution of MTX (Sigma, St. Louis, U.S.A.) was prepared and then used as a dilute solution. The recombinant CHO cells were gradually adapted to increasing concentrations of MTX supplemented in the cell culture medium. During the initial adaptation period (final MTX concentrations ranging from 0.1 to 1.0 μM), the increase in the MTX concentration was 0.2 μM for each increment. After the cells acquired resistance to 1.0 μM of MTX, more resistant cell lines were selected through increasing the MTX concentration by 5.0 μM for each increment. It took about 2 weeks for the cells to adapt to the increased MTX concentration for each increment.

Measurement of Cell Concentration

When the cells formed a complete monolayer in the culture dish, they were suspended with 0.05% trypsin (Sigma, St. Louis, U.S.A.). Ten ml of CMF-HBSS (GibcoBRL, Gaithersburg, U.S.A.) was added to the detached cells and mixed well, then a 100 μl sample was taken and the cell concentration was measured using a hemocytometer. Before counting, the sample was mixed well with the same amount of 0.1% trypan blue (1:1 ratio), as only live cells would not be dyed.

Monoclonal Screening

Monoclonal screening was also performed to select more TGF-β1-producing cell lines. A serial dilution method was applied to obtain just one cell per well in a 96-well microculture plate. Two hundred and fifty μl of the cell culture medium was distributed into each well, which was incubated at 37°C in a humidified CO_2 incubator (Forma Scientific, Marietta, U.S.A.). Complete monolayers were formed approximately on day 5, at which point the spent medium was replaced with a new one. After 3.5 weeks of incubation, the fast growing cells were selected to be transferred to a 24-well culture plate, and then further incubated for about 2 weeks. At this stage, the more productive cell lines were screened by comparing the TGF-β1 productivity among the rapid growing cells, and then transferred to a large-sized cell culture dish (35×10 mm) for further incubation (about 1 week). After the formation of complete monolayers, the high yielding cells were again transferred to a still larger-sized cell culture dish (60×15 mm).

Enzyme-Linked Immunosorbant Assay (ELISA) to Measure TGF-β1 Concentration

The TGF-β1 was detected using a modified ELISA [6, 14]. The anti-TGF-β1 monoclonal antibody 1D11.16 was added at 1.2 μg/ml in a 0.05 M carbonate buffer, pH 9.3, to 96-well microtiter plates (Becton Dickinson, Oxnard, U.S.A.). The plates were washed three times with PBS containing 0.05% Tween 20 followed by overnight incubation at 4°C, and blocked for 1 h with a 2.7% ELISA blocking reagent (Roche, Mannheim, Germany). After being washed three times, 50 μl of standard porcine TGF-β1 and the culture supernatants were distributed to each well and incubated for 1 h at 37°C. The plates were then washed and incubated for 1 h at 37°C with 5 μg/ml of a chicken anti-TGF-β1 polyclonal antibody (R&D Systems, Minneapolis, U.S.A.). A horseradish peroxidase conjugated rabbit anti-chicken antibody (Sigma, St. Louis, U.S.A.) was also added to each well and incubated for 1 h. The plates were then washed and 0.2 mM ABTS [2-2'-azino-di(3-ethylbenzothiazoline sulfonate)] was added. After 30 min of incubation, the colorimetric reaction was measured at 405 nm using an automatic microplate reader (Model 450, Bio-Rad, Burlington, U.S.A.). To prepare a standard curve for determining the concentration of TGF-β1, porcine-TGF-β1 purchased from R&D Systems (Minneapolis, U.S.A.) was utilized.

Determination of Glucose, Lactate, and Ammonium Concentrations in Cell Culture Medium

The concentrations of residual glucose and lactate in the medium were determined using an HPLC system (Waters, Milford, U.S.A.). An Aminex HPX-87H ion exclusion column (Bio-Rad, Burlington, U.S.A.) and Refractive Index (RI) detector were utilized at room temperature to measure the glucose. The mobile phase was 0.01 N H_2SO_4 and the flow rate was 0.6 ml/min. The same column was also used for the lactate measurement. In this case, the column temperature was 65°C, with a UV detector at 210 nm and 0.6 ml/min flow rate in the mobile phase (0.01 N H_2SO_4 solution) were used. The ammonium concentration was determined using an ammonium electrode (Model 95–12) supplied from Orion Research Inc. (Boston, U.S.A.).

RESULTS AND DISCUSSIONS

Initial Selection of High-Producing Cell Lines of Recombinant TGF-β1 Protein

For the mass production of the TGF-β1 protein, both the development and screening of high-producing cell lines are required along with the optimal operation of a bioreactor system. The current study used recombinant CHO cells with the following characteristics: The cells were transfected with TGF-β1 cDNA in conjunction with a dihydrofolate reductase (DHFR) gene. The DHFR gene was cotransfected into the cells to amplify the transfected TGF-β1 cDNA. For the constitutive expression of the TGF-β1 protein, a cytomegalovirus promoter was located in front of the TGF-β1 cDNA. As a selectable marker, a neomycin-resistant gene was also transferred into the cells so that transfected cells could be easily selected by treatment with G418, a neomycin analogue.

On the basis of the characteristics of the producer cell lines, as stated above, the primary recombinant CHO cells were initially treated with 1 mg/ml of G418 and their TGF-β1 productivities were compared for the first level screening (Fig. 1). Clone A4 producing 1.5 ng/ml of TGF-β1

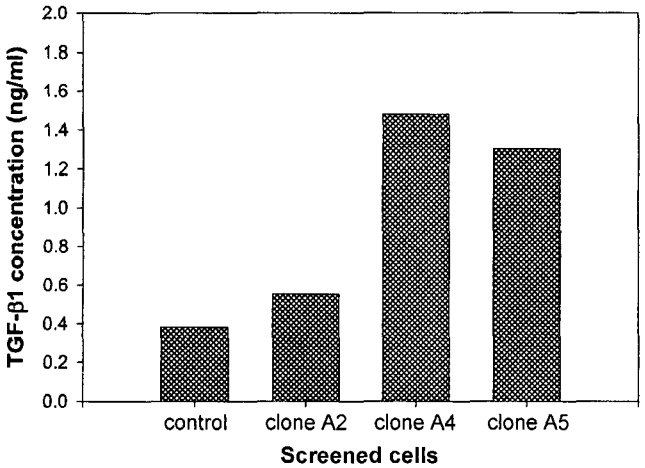

Fig. 1. Secretion of TGF-β1 by CHO cells transfected with human TGF-β1 cDNA. The cloned transfectant cells were plated on 35-mm petri plates (6×10^5 cells/ml and 2 ml/plate) and incubated for 2 days in a DMEM/F12 medium supplemented with 10% dialyzed FBS.

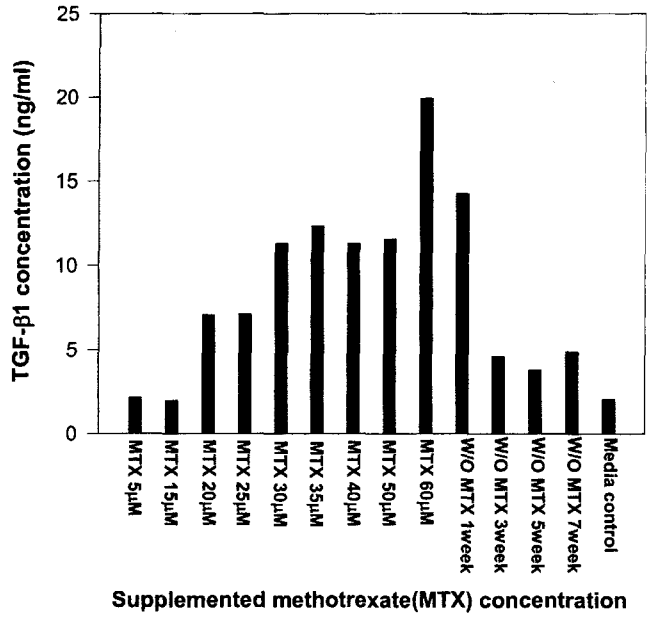

Fig. 2. Effect of methotrexate on TGF-β1 production by AP-1 cells during a high-productive cell line selection process ("W/O MTX 1 week" implies that the culture was incubated for 1 week without an MTX supplement in the culture medium).

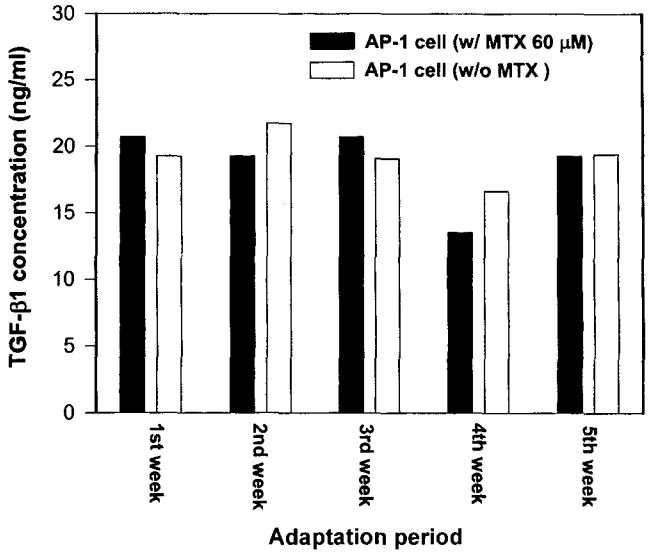

Fig. 3. TGF-β1 production of AP-1 cells in the absence of methotrexate in culture medium (the AP-1 cells were adapted for 3 months in a medium supplemented with 60 μM of methotrexate).

was selected as a high producer (named AP-1) and subjected to further gene amplification experiments using various concentrations of MTX. Thus, the recombinant CHO cells were gradually adapted to progressively higher concentrations of MTX (from 0.1 μM to 60 μM) supplemented in the cell culture medium. Approximately 2 weeks were required for the cells to adapt to each increased MTX concentration and form a complete monolayer in the cell culture dish. Figure 2 shows the effect of the MTX treatment on TGF-β1 production by the AP-1 cells. A significant increment in the TGF-β1 concentration was observed as MTX concentration was gradually increased. It was remarkable that, when the selected cells acquired a resistance to 60 μM of MTX, their TGF-β1 production increased up to 20 ng/ml, about a ten-fold increase from that of the resistant cell line screened at 5 μM of MTX. At higher concentrations of MTX, the resistant cells appeared to amplify more copies of the DHFR gene in order to struggle against the cytotoxic drug, MTX, along with copies of the TGF-β1 cDNA adjacent to the DHFR gene. It has been known that, when the amplification unit is large, the amplification of the DHFR gene generally results in the co-amplification of the gene to be expressed [2, 23]. However, when the resistant cells were exposed to MTX concentrations above 60 μM, they were still able to survive under these harsh environments, however, there was no further increase in TGF-β1 biosynthetic ability (data not shown), implying an inherent restriction in the extent of gene amplification in the DHFR-MTX system.

To investigate the stability of the recombinant AP-1 cells, the 60 μM MTX-adapted cells were further incubated without additional MTX treatment. It was observed that the TGF-β1 production was sustained (16–19 ng/ml) during the first week without MTX, however, a significant reduction was observed in the producers' TGF-β1 biosynthetic ability after a lapse of 3 weeks (Fig. 2), implying a rapid loss of the transfected TGF-β1 cDNA when the cells were no longer exposed to selection pressure, such as MTX treatment. It is possible that the 2 weeks of adaptation at each MTX concentration used in the current study was too short to obtain genetically stabilized recombinant cells.

To further investigate the lack of stability, as mentioned above, the adaption period of the AP-1 cells was extended to 3 months in the presence of 60 μM MTX. After such a long adaptation, the TGF-β1 productivity was compared every week for two cases where the cells were incubated for a further 5 weeks either with or without 60 μM MTX supplement (Fig. 3). Interestingly, in contrast to the results obtained in Fig. 2, the cells without further exposure to MTX for 5 weeks exhibited almost the same levels of TGF-β1 production (approximately 20 ng/ml) as the cells treated continuously with MTX. This result demonstrates that the genetic stability of the transfected cDNA increased progressively relative to the extension of the adaptation time of the recombinant cells in the selective medium with the cytotoxic selective agent, MTX. In addition to the TGF-β1 productivity, the morphology of the transfected cells was distinctively altered (shown in Fig. 4): During the early stage of the MTX treatment, the transfected cells grew larger than the nontransfectant and exhibited low cell density of

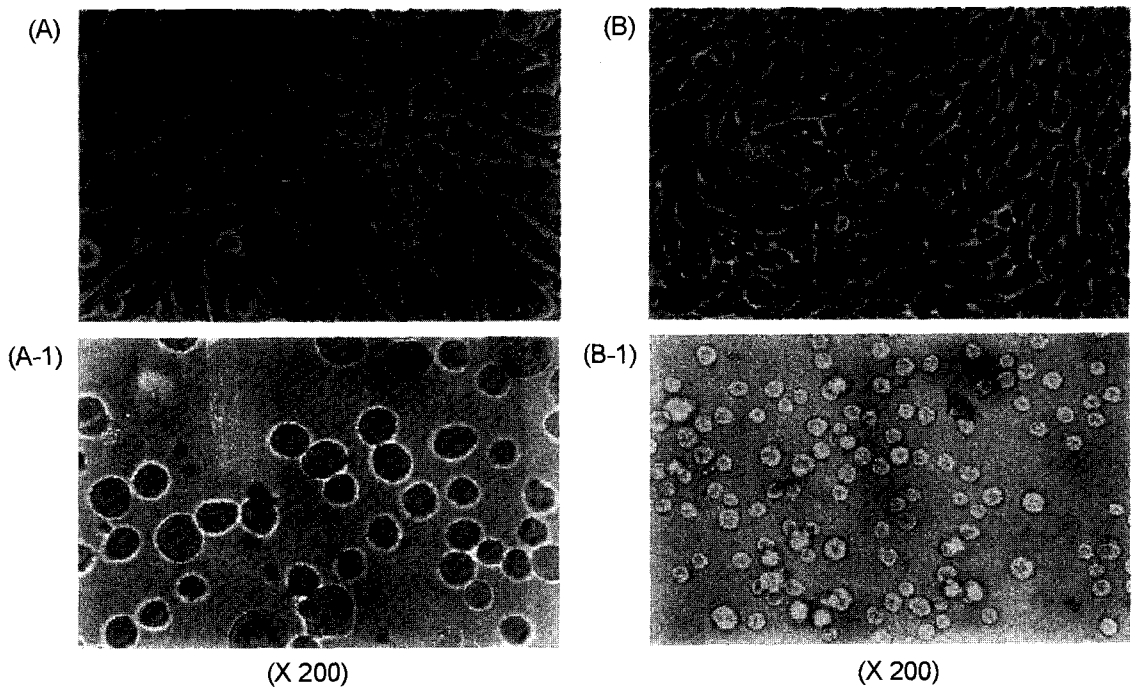

Fig. 4. Comparison of cell morphology between transfected and nontransfected cells. Transfected CHO cells; A: anchorage dependent cells; A-1: trypsinized cells. Nontransfected CHO cells; B: anchorage dependent cells; B-1: trypsinized cells

about 1×10^5 cells/ml after 5 days of culture. However, as the transfected cells became more resistant to MTX, the maximum cell density reached about 3×10^5 cells/ml.

Selection of High-Yielding Cell Lines Through Monoclonal Screening

The high-yielding cell lines showing resistance to 60 μM of MTX in the above experiment were further incubated in the presence of the same MTX level, then monoclonal screening was performed to select the higher TGF-β1-producing cell lines. A serial dilution method was adopted to obtain just one cell per well in a 96-well microculture plate supplemented with 250 μl of the cell culture medium. After the formation of complete monolayers, the selected high-yielding cells were again transferred to a mid-sized cell culture dish (35×10 mm), and then to an even larger-sized dish (60×15 mm) for further screening (refer to Materials and Methods). The monoclones incubated at each stage were compared in terms of their TGF-β1 productivity and cell concentration.

As represented in the histogram in Fig. 5, various amounts of TGF-β1 were produced in the recombinant

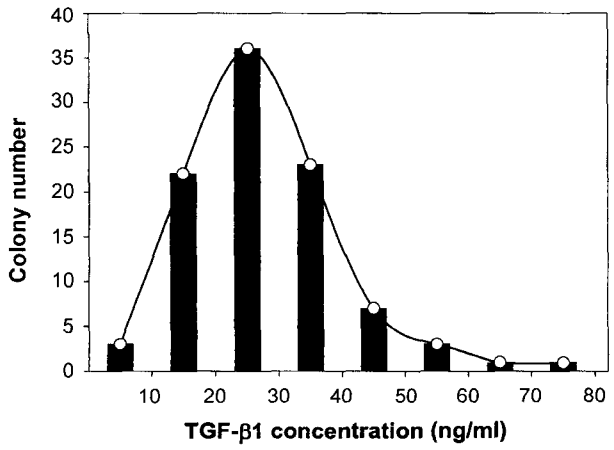

Fig. 5. Histogram for amount of TGF-β1 produced by screened CHO cells incubated in 96-well culture plates.

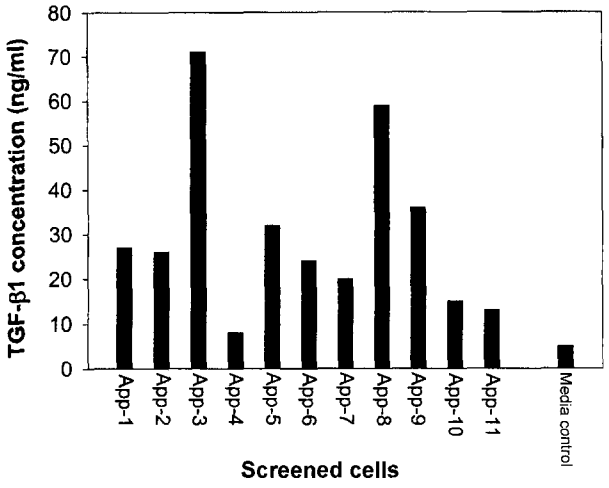

Fig. 6. TGF-β1 production of screened CHO cells (11 kinds of App-series cell lines) incubated in 24-well culture plates.

CHO cells incubated in a 96-well microculture plate at 37°C for 5 days in a humidified CO_2 incubator. Only 2% of the total screened cells were considered to be high-yielding producers with TGF-β1 productivity within a range of 60–80 ng/ml. However, a large portion of the screened cells (approximately 84%) produced TGF-β1 ranging from 10 to 40 ng/ml, implying that monoclonal selection should still be carried out for efficient screening even after the transfection and amplification of the transfected gene.

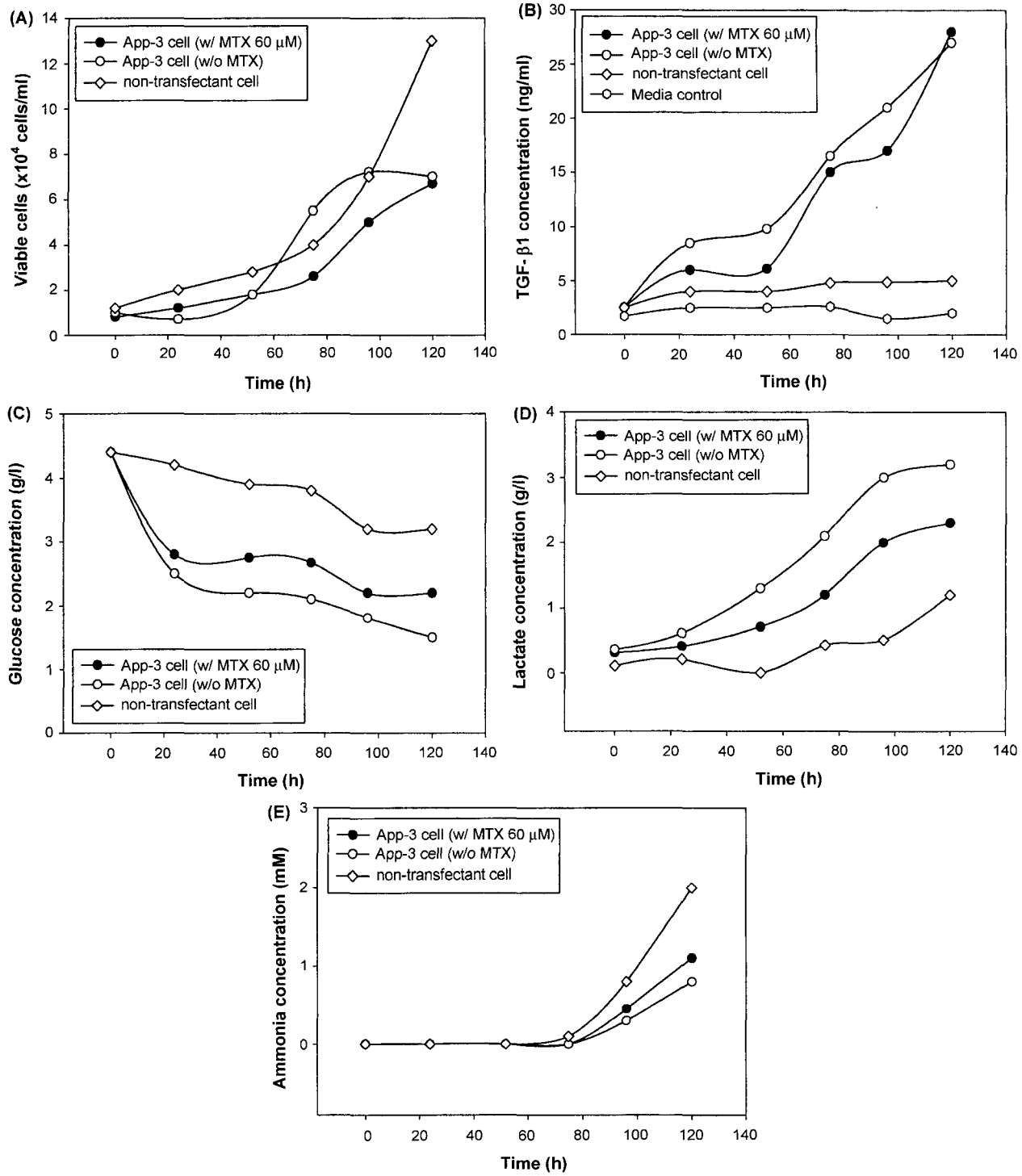

Fig. 7. Comparison of time-course profiles of TGF-β1, ammonia, and lactate production, and glucose consumption between selected App-3 cell line (in the presence and absence of 60 μM methotrexate) and nontransfectant as control.

Forty two high-yielding cell lines selected in the above experiment were again transferred to a 24-well culture plate (35×10 mm dish) for further screening, and 11 high-producers out of them were finally selected (named as App-1 to App-11). After monolayers were completely formed in the 24-well culture plate, the individual cell lines were compared in terms of their TGF-β1 biosynthetic ability (Fig. 6). Notably, very similar results to those observed in the 96-well culture plate (Fig. 5) were obtained: the TGF-β1 production for two cell lines was within a range of 60–80 ng/ml, whereas the remaining 9 cell lines produced from 10 to 30 ng/ml.

Investigation of Physiological Characteristics in Recombinant CHO Cell Cultures

From the above experiments, the high-productive cell lines, App-3, App-5, and App-8, were finally chosen for investigation of their physiological characteristics in cell cultures with a large-sized culture dish (60×15 mm). Initially, the App-3 cell line was compared with the nontransfectant (Appl cell line) of the TGF-β1 cDNA in terms of cell growth, TGF-β1 productivity, sugar uptake, and ammonia formation in the presence or absence of MTX.

The results obtained from 5-day cell cultures are shown in Fig. 7. A significant increase (about 82%) in the TGF-β1 production by App-3 was observed compared with the Appl cell line, despite a 46% lower cell density [Figs. 7(A) and 7(B)]. It was remarkable that, in the case of App-3 cells, the TGF-β1 biosynthetic ability was almost identical, regardless of the MTX supplement in the cell culture medium [Fig. 7(B)], thus demonstrating the stability of the recombination of the transfected TGF-β1 cDNA. As for ammonia, biosynthesized as a toxic byproduct during cell cultures, the App-3 with MTX produced less than 1.0 mM of ammonia, a significantly lower quantity than that produced by the Appl cells [Fig. 7(E)]. However, the amount of lactate produced, another toxic byproduct, was approximately 2-fold higher in the App-3 with MTX and about 3-fold higher without MTX than the nontransfectant, Appl [Fig. 7(D)]. Accordingly, it would appear that although App-3 did not consume glutamine effectively, which is generally used as an efficient carbon and energy source in a mammalian cell culture, it did utilize glucose more favorably. This was also confirmed by the rapid uptake of glucose by App-3 [Fig. 7(C)]. Interestingly, physiological phenomena similar to the above were also observed in other transfected cell lines developed by the current authors (data not shown). In Fig. 7(C), regardless of the addition of MTX, App-3 was observed to consume glucose at least two times more rapidly than the nontransfectant, Appl. The fast utilization of glucose and large lactate yield by this transfected cell line demonstrated that a large portion of the glucose consumed was not used for cellular biosynthesis, but rather converted to an unfavorable byproduct, lactate, thereby inhibiting the sustenance of the transfected cell growth. This inference is also supported by the significantly lower cell density and higher lactate production by App-3, as already explained.

Table 1 summarizes the cultivation results, including the maximum specific cell growth rate (μ_m), specific TGF-β1 production rate (q_p), and specific sugar uptake rate (q_s) for the following cells: App-3, App-5, and App-3, which exhibited high TGF-β1 production during the monoclonal screening processes, the initially transfected AP-1 cell line (clone A4), and the nontransfectant of TGF-β1 cDNA as the control. As explained above, the culture conditions for the four kinds of cell lines (App-5, App-8, AP-1, and the nontransfectant), were identical to those used for App-3. The profiles of TGF-β1, ammonia and lactate productions, and glucose consumption were also investigated for each cell line, as previously shown in Fig. 7 for the App-3 cell line. However, only the final results are summarized in Table 1 for easy comparison.

In the presence of 60 μM MTX, the q_p of all the App-series cells increased significantly in comparison with the nontransfectant, in spite of their lower specific growth rate.

Table 1. Specific growth rate (μ), specific production rate (q_p), and specific sugar uptake rate (q_s) of screened cell lines and non-transfectant cells (60×15 mm culture dish)

Cell line	Maximum specific growth rate in exponential phase (μ) (h^{-1})	Specific TGF-β1 production rate (q_p) (ng/10^4 cells/h)	Specific sugar uptake rate (q_s) (g/10^4 cells/h)
App-3 cell (w/ MTX 60 μM)	0.018	0.032	0.00213
App-3 cell (w/o MTX 60 μM)	0.02	0.031	0.00271
App-5 cell (w/ MTX 60 μM)	0.013	0.015	0.00252
App-5 cell (w/o MTX 60 μM)	0.02	0.015	0.00235
App-8 cell (w/ MTX 60 μM)	0.012	0.03	0.00164
App-8 cell (w/o MTX 60 μM)	0.022	0.019	0.00222
AP-1 cell (w/ MTX 60 μM)	0.022	0.008	0.00122
AP-1 cell (w/o MTX 60 μM)	0.02	0.0067	0.00081
nontransfectant cell	0.019	0.002	0.001

In particular, the q_p values for both the App-3 and App-8 cells were very high, amounting to 0.032 and 0.03 ng/10^4 cells/h, respectively, which were almost 4-fold higher than that of the AP-1 cell line (0.008 ng/10^4 cells/h). In the case of the specific sugar uptake rate (q_s), 1.7-fold and 1.3-fold increment was observed for the App-3 and App-8 cells, respectively. In contrast, the App-5 cells showed remarkably stable biosynthesis of TGF-β1 in both the presence and absence of MTX, as already demonstrated in the cultures of the App-3 cell line. In the case of the nontransfectant, the specific TGF-β1 production rate (q_p) significantly declined in spite of normal cellular growth, showing the smallest amount of q_p (0.002 ng/10^4 cells/h) among all the cells investigated. Accordingly, it was concluded that both monoclonal selection and continuous investigation of the physiological characteristics should be performed, even after the transfection and amplification of the transfected gene, for the successful screening of high TGF-β1 producers.

However, it should also be mentioned that the high-productive cells did not grow in the late exponential phase, in spite of the maximum growth rate being almost identical to that of the nontransfectant during the mid-exponential phase, thereby leading to a lower final cell concentration by day 5 [Fig. 7(A)]. To maximize the volumetric production rate (Q_p) ($Q_p=q_p \cdot X$, μg/l/h) which is the most important parameter in a cell culture, it is essential to obtain the maximum amount of cells (X) and to improve the specific productivity (q_p) of a recombinant protein. Accordingly, bioreactor operating strategies using a fed-bach culture, which is generally regarded as an efficient culture method, are currently being investigated to make the most of the producer's physiology, thereby leading to the maximum volumetric productivity.

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