Change of Insulin-like Growth Factor Gene Expression in Chinese Hamster Ovary Cells Cultured in Serum-free Media

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Abstract Although the sera used in animal cell culture media provide the macromolecules, nutrients, hormones, and growth factors necessary to support cell growth, it could also be an obstacle to the production of recombinant proteins in animal cell culture systems used in many sectors of the biotechnology industry. For this reason, many research groups, including our laboratory, have been trying to develop serum-free media (SFM) or serum-supplemented media (SSM) for special or multi-purpose cell lines. The Chinese hamster ovary (CHO) cell, for example, is frequently used to produce proteins and is especially valuable in the large-scale production of pharmaceutically important proteins, yet information about its genome is lacking. Also, SFMs have only been evaluated by comparing growth patterns for cells grown in SFMs with those grown in SSM or by measuring the titer of the target protein obtained from cells grown in each type of medium. These are not reliable methods of obtaining the type of information needed to determine whether an SFM should be replaced with an SSM. We carried out a cDNA microarray analysis to evaluate MED-3, an SFM developed in our laboratory, as a CHO culture medium. When CHO cells were cultured in MED-3 instead of an SSM, several genes associated with cell growth were down-regulated, although this change diminished over time. We found that the insulin-like growth factor (IGF) gene was representative of the proteins that were down-regulated in cells cultured in MED-3. When several key supplements – including insulin, transferrin, ethanolamine, and selenium – were removed from MED-3, the *IGF* expression was consistently down-regulated and cell growth decreased proportionately. Based on these results, we concluded that when an SFM is used as a culture medium, it is important to supplement it with substances that can help the cells maintain a high level of *IGF* expression. The data presented in this study, therefore, might provide useful information for the design and development of SFM or SSM, as well as for the design of genome-based studies of CHO cells to determine how they can be used optimally for protein production in pharmaceutical and biomedical research.

Keywords: IGF, gene expression, Chinese hamster ovary cells, serum-free media

INTRODUCTION

Animal sera in animal cell culture media provide a broad range of macromolecules, including lipid carrier proteins, growth and adhesion factors, low molecular-weight nutrients, hormones, and growth factors [1]. Fetal bovine serum (FBS), the animal serum used most frequently in culture media, may contain many undefined components that could be obstacles to the production of medically useful proteins in animal cell culture systems [2,3]. For this reason, many investigators have been trying to develop serum-free media (SFM) or serum-supplemented media (SSM) for special or multipurpose cell lines.

The inclusion of pure recombinant growth factors to produce an SFM is highly recommended as a means of maintaining the highest quality standards in many sectors of biotechnology and pharmaceutical research. One of the most widely used recombinant growth factors is insulin-like growth factor (IGF) [17]. Originally discovered in serum, IGF is a member of a family of peptides that plays an important role in mammalian growth and development [16,18-20]. The IGFs are considered key growth factors in industrial cell cultures; their removal from serum can reduce the cell growth-promoting activity of a culture by as much as 90%. Almost all cells have type I IGF receptors, which mediate the biological activity of IGFs, which includes the regulation of cell proliferation or arrest by increasing or decreasing the synthesis of protein complexes that regulate the cell cycle through tyrosine kinase receptor-mediated signaling pathways [10]. Activation and repression of cell-cycle regulating protein complexes, in turn, regulates the recruitment of cells in the G0 or G1 phase of the cell cycle and their progres-

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sion through G1/S and G2/M. In most mammalian cells, the initial event triggered by mitogens to promote the progression of the cell to the G1 phase of the cell cycle is the induction of the D-type cyclin activity [21].

Established mammalian cell lines are invaluable research tools, providing *in vitro* models for a wide range of biological investigations. The Chinese hamster ovary (CHO) cell line [14] is among the most widely used cell lines in published studies. CHO cells, in addition to their importance in research, are the most utilized hosts for the large-scale production of pharmaceutically important proteins, accounting for the production of billions of dollars worth of therapeutic protein products annually [14,15]. Despite their value in biomedical research and industrial pharmaceutical protein production, genome-based information on CHO cells has not been investigated completely.

Unlike the genome (the profile of gene expression), the transcriptome is highly dynamic, changing rapidly in response to perturbations in cell culture conditions. By monitoring the genes that are transcribed from genomic DNA and differences in gene expression, we can obtain the critical biological information necessary to understand the detailed molecular mechanisms that are responsible for the development of complex cellular phenotypes and functions [4]. Since DNA microarray technology was first introduced by Schena *et al.* in 1995 [5], this high-throughput technology has become a common procedure in biological and biotechnological research [7,22].

Until now, SFM development has been evaluated simply by comparing growth patterns for cells grown in SFM versus SSM or by measuring the titer of target proteins produced in each type of medium [3]. However, these are not reliable methods for obtaining enough information about the SFM to compare it with the SSM. In the present study, we carried out a cDNA microarray analysis to evaluate MED-3, an SFM developed in our laboratory, in terms of its effect on SFM-adapted and immunoglobulin IgG-cloned CHO cells. We analyzed the gene expression patterns and identified genes that are expressed differentially in CHO cells cultured in MED-3 compared with those cultured in SSM. The data presented in this study might provide information that will prove useful for designing and developing an SFM or an SSM and for the genome-based study of CHO cells to enhance their use in pharmaceutical and biomedical research.

MATERIALS AND METHODS

CHO Cell Cultures

SFM-adapted and IgG-cloned CHO cells were cultured in a monolayer in minimal essential medium-α (MEM-α, Invitrogen, Carlsbad, CA, USA) supplemented with 5% dialyzed fetal bovine serum (dFBS, Invitrogen), 0.22% sodium bicarbonate, and 320 nM methotrexate (MTX, Sigma, St. Louis, MO, USA) at 37°C in a humidified atmosphere of 5% CO_2. The cell density in the culture was maintained between 1×10^5 and 5×10^5 cells/mL. The SFM used in this study, called MED-3, was prepared in our laboratory using Dulbecco modified Eagle medium (DMEM)/F12 (1:1) supplemented with vitamins, amino acids, lipids, and fatty acids, hydrocortisone, surfactants, and other organic and inorganic compounds. The medium also contained 10 mg insulin, 5 mg transferrin, 2 mg ethanolamine, 5 μg selenium (sodium selenite), and 10 μg insulin-like growth factor (Long R3 IGF-1) per liter.

Cell-cycle Determination Using FACS-based Analysis

Two days before the cell cycles were synchronized, the cells were plated at a density of 5×10^5 cells/mL then removed from the maintenance culture and washed with MEM-α. Their cell cycles were synchronized by incubation in MEM-α containing 0.5% dFBS for 48 h at 37°C. After incubation, 80 to 85% of the cells were in the G0/G1 phase. The cells were then washed twice with phosphate-buffered saline (PBS) and then grown in MEM-α (5% dFBS) or MED-3. At an appropriate time, the cells were harvested using trypsin-ethylenediamine tetraacetric acid (Invitrogen), washed twice in PBS, and preserved in 70% ethanol buffered with PBS at 4°C. To determine the DNA content of the cells, we washed the preserved cells once with PBS containing 1% FBS and stained the DNA with propidium iodide (PI, 1 mg/mL; Sigma) in the presence of ribonuclease I (RNase, 10 mg/mL, Sigma). A flow cytometry analysis of 10,000 cells was then carried out using a Becton Dickinson FACScalibur cytometer (Franklin Lakes, NJ, USA).

DNA Microarray Analysis

DNA microarray hybridization was used to detect changes in gene expression in CHO cells cultured in MEM-α compared with MED-3. After a 48-h synchronization period, the CHO cells were cultured in MEM-α (5% dFBS) or MED-3 for a given length of time and then harvested. Total RNA was extracted from each sample using a Trizol kit (Invitrogen) according to the manufacturer's instructions. RNA was dissolved in 20 μL of RNase-free H_2O at a temperature of 55°C for 5 min to increase its solubility. The purity of the RNA was verified by means of the absorption ratio at $OD_{260nm/280nm}$. The DNA microarray used in this work contained 5,592 mouse genes (TwinChip™ Mouse-6K, Digital Genomics Inc., Seoul, Korea). The cDNA made from mRNA isolated from CHO cells cultured in MED-3 was labeled with the fluorescent dye Cy5, while the cDNA from CHO cells cultured in MEM-α was labeled with another fluorescent dye, Cy3. The color images of the hybridization results were created by merging the green images of Cy3 fluorescence and the red images of Cy5 fluorescence.

Reverse Transcription and Real-time Polymerase Chain Reaction Analysis

A 5-μg sample of total RNA in the sample preparation underwent reverse transcription (RT) through its exposure to 100 U of MML-V reverse transcriptase (Bioneer, Daejeon, Korea) and 50-pmol dT_{16} primers (Bioneer), which

Table 1. Primer used for RT-PCR analysis

Unigene	Gene		Primer sequence
Mm2770	*IGF*	Sense	TGGATGCTCTTCAGTTCGTG
		Antisense	GTCTTGGGCATGTCAGTGTG
Mm7141	*PCNA*	Sense	CCACATTGGAGATGCTGTTG
		Antisense	CAGTGGAGTGGCTTTTGTGA
Mm5289	*GAPDH*	Sense	CCATGGAGAAGGCTGGGG
		Antisense	CAAAGTTGTCATGGATGACC

RT-PCR, reverse transcriptase-polymerase chain reaction.

were used according to the manufacturer's instructions. To analyze the expression of *IGF*, we designed specific primers (Table 1) and used iCycler iQ Real-Time Polymerase Chain Reaction (PCR) detection system (Bio-Rad, Hercules, CA, USA). The following real-time PCR protocol was used: denaturation (95°C for 15 min); 3-segment amplification and quantification (denaturation at 95°C for 50 s, annealing at 56°C for 20 s, and extension at 72°C for 20 s), which was repeated 45 times; and melting curve development (60~95°C with a heating rate of 0.1°C/s and continuous monitoring of fluorescence), and finally a cooling program, in which the temperature was reduced to 4°C.

RESULTS AND DISCUSSION

Analysis of Cell-cycle Patterns in CHO Cells

The SFM-adapted and IgG-cloned CHO cells were maintained as a monolayer (density: 1-5 × 10^5 cells/mL) in MEM-α supplemented with dFBS (5%), sodium bicarbonate (0.22%), and MTX (320 nM). MED-3 was prepared using DMEM/F12 supplemented with growth factors, vitamins, amino acids, lipids and fatty acids, and surfactants. We initially attempted to determine whether there were any changes in the growth pattern for CHO cells grown in MEM-α compared with MED-3. As shown in Fig. 1, there was no great difference in the CHO cell growth pattern in MEM-α versus MED-3 over a 7-day period, which suggests that an MED-3 has the potential to function as an SSM. We evaluated this further and identify potential biological changes in CHO cells cultured in MED-3 compared with MEM-α.

We first evaluated the effects of MED-3 on CHO cell growth by comparing changes in the cell cycle when CHO cells were cultured in MED-3. CHO cell cycles were synchronized by culturing them in MEM-α supplemented with 0.5% dFBS for 48 h. We found that 80% of the cells were in the G0/G1 phase of the cell cycle after 48 h of "starvation" (Fig. 2). When the cells were restimulated with MEM-α, the cell cycle progressed from the G0/G1 phase to the S phase; 40% of the cells were in the S phase after 15 h (Fig. 2A). By contrast, when the cells were restimulated with MED-3, it took 18 h for the cell cycle to progress from the G0/G1 phase to the S phase (Fig. 2B). These data suggest that an SFM might

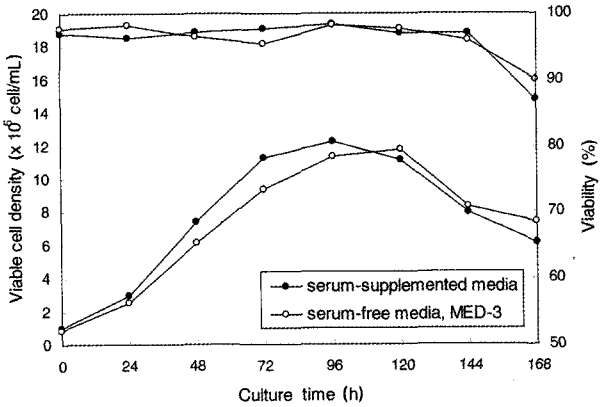

Fig. 1. Time-dependent growth pattern for CHO cells cultured in an SSM or SFM. The CHO cells were cultured in either an SSM (MEM-α) or an SFM (MED-3 – an SFM developed in our laboratory) for 1 to 7 days. Cell density and cell viability were determined by cell counting after tryphan blue staining.

be equivalent to an SSM in terms of its effects on CHO cells in culture, at least in terms of maintaining cellular growth.

Analysis of Gene Expression Patterns of the CHO Cells

We next examined the gene expression patterns in CHO cells cultured in MED-3 compared with those in CHO cells cultured in SSM. The cell cycles were synchronized for 48 h then restimulated in MEM-α or MED-3 for 6~24 h. The differences in gene expression between the 2 media were analyzed using a cDNA microarray. This genome-wide tool has been used frequently in many labs, including ours [4,6], to assess expression profiles for whole transcripts and to identify known and unknown genes in the cell. The cDNA microarray analysis was carried out using a mouse cDNA microarray containing 5,592 mouse genes. Although little information about gene sequences in the CHO is publicly available and little is known about the relationship between genomic sequences in CHOs and mice, Wlaschin *et al.* [8] recently isolated more than 4,600 expressed sequence tags (ESTs) in CHO cells and reported that a comparative analysis of CHO EST sequences with known gene sequences of other species suggested that CHO sequences are generally very similar to those in mice.

As shown in Table 2, the number of up-regulated genes in CHO cells that had been restimulated for 6 h in SSM following a 48-h cell-cycle synchronization session (as a control) more than doubled compared with the number in CHO cells that were re-stimulated in MED-3. The most highly up-regulated transcripts in the mouse array were *PFKM*, a gene involved in the expression of phosphofructokinase (PFK) in muscle (5.4-fold increase), and *RAB6*, a gene involved in the expression of Ras-associated protein 6 (4.6-fold increase). In isolated mussel digestive gland cells, IGF1 activates tyrosine kinase receptors, which induces the activity of the key glycolytic enzymes PFK and pyruvate kinase [10]. It has been re-

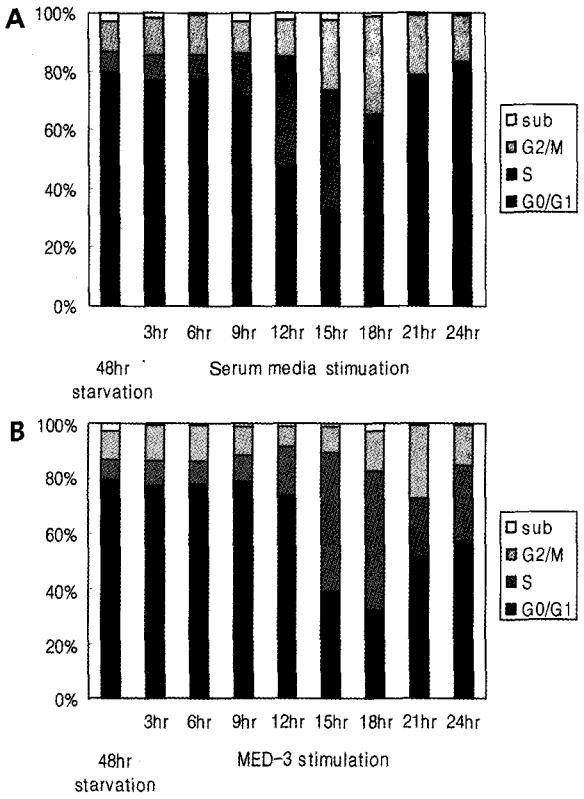

Fig. 2. Percentage of CHO cells in the cell cycle of G0/G1, S, and G2/M phase. The CHO cell cycles were synchronized by culturing these cells in MEM-α supplemented with 0.5% dFBS for 48 h, then re-stimulating them in this SSM (A) or in an SFM (MED-3) (B). The percentage of CHO cells in the G0/G1, S, and G2/M phase of the cell cycle was then determined by FACS-based analysis.

ported that members of the RAB protein family may be involved in the induction of glucose transport and cell growth and may play a role in regulating the kinetics of endocytosis [11]. These findings suggest that insulin- or IGF1-related intracellular and molecular signaling events are triggered in CHO cells that are re-stimulated in cultures containing SSM after 48 h of cell-cycle synchronization. Interestingly, this hypothesis was based directly on the observation that the genes for cyclin D1 and IGF1 were also moderately up-regulated (2.6- and 2.1-fold, respectively) in CHO cells that had been re-stimulated in SSM for 6 h (Table 2). These findings correlate with observations reported by others that mitogenic (including IGF1-triggered) signal transduction promotes the up-regulation of cyclin D1 and cell proliferation in oligodendrocyte progenitor cells [9] and cancer cell lines [12,13].

Analysis of *IGF* Transcript Expression Patterns

When the restimulation time was increased from 6 to 12 h for CHO cells cultured with either MEM-α or MED-3 following a 48-h cell-cycle synchronization period, the number of up-regulated genes increased to 53, whereas the number of down-regulated genes in CHO

Table 2. Genes up-regulated in CHO cells cultured in an SSM* compared with an SFM†,‡

Gene	Description	Change§
PFKM	phosphofructokinase, muscle	5.4
RAB6	RAB6, member RAS oncogene family	4.6
NUMb	numb gene homolog (Drosophila)	3.3
TAPBP	TAP binding protein	2.7
CCND1	cyclin D1	2.6
TCTE3	t-complex-associated testis expressed 3	2.5
SMT3IP1	smt3-specific isopeptidase 1	2.3
ART5	ADP-ribosyltransferase 5	2.2
SLC20A1	solute carrier family 20, member 1	2.2
EXT1	exostoses (multiple) 1	2.1
IGF1	insulin-like growth factor 1	2.1
DAG1	Dystroglycan	2.1
ANK1	ankyrin 1, erythroid	2.1

*MEM-α served as the SSM in this study.
†MED-3 served as the SFM in this study.
‡Re-stimulation time after 48-h period of cell-cycle synchronization was 6 h.
§SSM value is expressed as a multiple of the SFM value.
CHO, Chinese hamster ovary; SSM, serum-supplemented medium; SFM, serum-free medium.

Table 3. Number of genes up- or down-regulated in CHO cells cultured in an SSM* compared with an SFM†

Re-stimulation time‡	Upregulated genes	Down-regulated genes
12 h	53	23
18 h	16	8

*MEM-α served as the SSM in this study.
†MED-3 served as the SFM in this study.
‡Re-stimulation time after 48-h period of cell-cycle synchronization was 6 h.
CHO, Chinese hamster ovary; SSM, serum-supplemented medium; SFM, serum-free medium.

cells re-stimulated with MEM-α was 23 (Table 3). Among the up-regulated genes were *IGF* and the gene for IGF binding protein 1 (*IGFBP1*), which increased by 3.2- and 3.6-fold, respectively (data not shown). After 18 h of restimulation in MEM-α or MED-3, the number of up- and down-regulated genes decreased to 16 and 8, respectively, and *IGFBP1* – but not *IGF* – was up-regulated 3.1-fold (data not shown).

We confirmed the results of cDNA microarray analysis by quantifying and profiling the *IGF* transcript in CHO cells. These cells were restimulated in either MEM-α or MED-3 (as a control) for various periods of time (6, 12, 18, and 24 h) following a 48-h cell-cycle synchronization period. The amount of *IGF* transcript in each sample was then quantified and profiled using real-time PCR analysis

Table 4. Change in rate of expression for *IGF* and *PCNA* transcripts in CHO cells cultured in an SSM* compared with an SFM†,‡

Re-stimulation time§ (h)	Gene		
	IGF-1	*PCNA*	*GAPDH*‖
6	2.1	1.2	1.1
12	4.3	1.6	1.7
18	3.8	1.8	1.5
24	2.5	1.5	1.6

*MEM-α served as the SSM in this study.
†MED-3 served as the SFM in this study.
‡Real-time PCR analysis was carried out.
§Re-stimulation time after a 48-h cell-cycle synchronization period.
‖GAPDH mRNA was used as a positive control.
CHO, Chinese hamster ovary; SSM, serum-supplemented medium; SFM, serum-free medium; IGF, insulin growth factor; PCNA, proliferating cell nuclear antigen; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Table 5. Effect of media composition on *IGF* mRNA expression and cell density*

No.†	Component removed	*IGF* mRNA‡	Cell density‡
1	None (MED-3)	1.00	1.00
2	Insulin	0.54	0.87
3	Transferrin	0.62	0.84
4	Ethanolamine	0.68	0.88
5	Selenium	0.64	0.96
6	ITES	0.48	0.72
7	IGF	0.42	0.82
8	ITES + IGF	0.36	0.73

*The data in this table represents the findings in CHO cells cultured in an SFM (MED-3) that obtained by real-time PCR analysis (see Fig. 3).
†The media removed at least one of the supplements listed in this column.
‡Results when each supplement was removed from MED-3 for 48 h.
IGF, insulin growth factor; PCR, polymerase chain reaction.

(Table 4). Analysis of the expression patterns for *PCNA* and *GAPDH* transcripts was included to serve as a control. As shown in Table 4, IGF gene expression in the cells that were re-stimulated in MEM-α increased 2.1-fold after 6 h in culture and 4.3-fold after 12 h in culture, then decreased slowly after 18 and 24 h of restimulation in MEM-α (3.8- and 2.5-fold, respectively) while *PCNA* and *GAPDH* expression remained at basal levels in the same sample. Taken together, these observations indicate that an SSM has a greater potential than an SFM to stimulate serum-starved CHO cells by inducing an increase in the expression of *IGF* and *IGFBP1*.

Effect of Media Components on *IGF* Gene Expression

The effect of some MED-3 culture supplements – *e.g.*, insulin, transferrin, ethanolamine, and selenium – on the expression of *IGF* was evaluated by monitoring changes in the expression of *IGF* using real-time PCR when these components were removed from MED-3 one by one (Table 5, Fig. 3). When these supplements were removed, the expression of *GAPDH* – a housekeeping gene – did not change (data not shown), but the expression of *IGF* was consistently down-regulated and cell growth decreased proportionally (Table 5 and Fig. 3C). The removal of IGF from MED-3 resulted in a particularly dramatic down-regulation of the *IGF* transcript expression and cell growth. These findings indicate that when an SFM is designed, it might be important to select supplements that will support a high level of IGF expression in cultured cells.

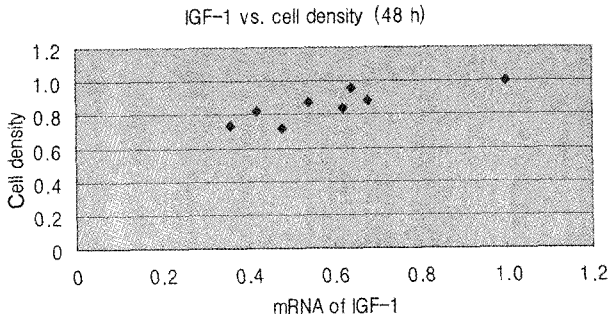

Fig. 3. Effect of media composition on the expression of *IGF* mRNA. The CHO cells were cultured in an SFM (MED-3) for 48 h. One or more supplement was removed from the SFM as described in Table 5. A real-time PCR analysis was then carried out to determine the amount of *IGF* mRNA. The relationship between *IGF* mRNA and cell density was determined. The numbers are as described in Table 5.

The data presented in this study might provide useful information, not only for designing and developing SFM or SSM, but also for studying the transcriptome in CHO cells, which may spur the use of these cells to identify and evaluate a greater variety of proteins in pharmaceutical and biomedical research.

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