Cytotechnology (2015) 67:689–697
DOI 10.1007/s10616-014-9778-0

JAACT SPECIAL ISSUE

Design of serum-free medium for suspension culture of CHO cells on the basis of general commercial media

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Received: 18 May 2014 / Accepted: 31 July 2014 / Published online: 23 August 2014
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Abstract The design of serum-free media for suspension culture of genetically engineered Chinese hamster ovary (CHO) cells using general commercial media as a basis was investigated. Subcultivation using a commercial serum-free medium containing insulin-like growth factor (IGF)-1 with or without FCS necessitated additives other than IGF-1 to compensate for the lack of FCS and improve cell growth. Suspension culture with media containing several combinations of growth factors suggested the effectiveness of addition of both IGF-1 and the lipid signaling molecule lysophosphatidic acid (LPA) for promoting cell growth. Subcultivation of CHO cells in suspension culture using the commercial serum-free medium EX-CELL^{TM}302, which contained an IGF-1 analog, supplemented with LPA resulted in gradually increasing specific growth rate comparable to the serum-containing medium and in almost the same high antibody production regardless of the number of generations. The culture with EX-CELL^{TM}302 supplemented with LPA in a jar fermentor with pH control at 6.9 showed an apparently higher cell growth rate than the cultures without pH control and with pH control at 6.8. The cell growth in the medium supplemented with aurintricarboxylic acid (ATA), which was much cheaper than IGF-1, in combination with LPA was synergistically promoted similarly to that in the medium supplemented with IGF-1 and LPA. In conclusion, the serum-free medium designed on the basis of general commercial media could support the growth of CHO cells and antibody production comparable to serum-containing medium in suspension culture. Moreover, the possibility of cost reduction by the substitution of IGF-1 with ATA was also shown.

Keywords Chinese hamster ovary (CHO) cells · Serum-free medium · Suspension culture

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Introduction

The production of genetically engineered proteins by animal cell culture is a common method of obtaining glycoproteins such as monoclonal antibodies for therapeutic purpose (Dingermann 2008). Biotechnology products for clinical use should be produced in serum-free media because animal-derived serum is expensive and is likely to contain adventitious agents that may impair human health. So, it is necessary to

find a serum-free medium appropriate for each cell line. For example, in current large-scale production of genetically engineered proteins, it is necessary to culture Chinese hamster ovary (CHO) cells, which primarily adhere to the surface of tissue culture flasks during their growth, in a suspension with stirring (Chu and Robinson 2001). This is the reason why serum-free medium should be developed aiming at suspension culture containing higher shear stress compared with static culture (van der Pol and Tramper 1998). Because much knowledge and suitable techniques are required to design serum-free media appropriate for the production of drugs, commercial suppliers provide serum-free media that are appropriate for the growth of typical cell lines and expression of proteins used for drug production (Schröder et al. 2004; Zhang et al. 2013). Even in such culture media, it is necessary to modify their components appropriate for each clone.

Insulin-like growth factor 1 (IGF-1) is a commonly used growth factor for CHO cells and is reported to have a strong antiapoptotic effect via IGF-1 receptors (Pak et al. 1996; Rasmussen et al. 1998). On the other hand, lysophosphatidic acid (LPA) is mitogenic for CHO cells via G protein-coupled receptors (GPCRs; Sakai et al. 1999). Thus, there may be a possibility that IGF-1 and LPA have a synergism. However, IGF-1 is expensive as a culture medium component. On the other hand, it was reported that the addition of antioxidant chelators such as aurintricarboxylic acid (ATA) prevents apoptosis (Yun et al. 2003), which tends to occur under serum-free conditions. Thus, it is expected that ATA could work together with LPA instead of IGF-1, because ATA is much cheaper than IGF-1.

In this study, a serum-free medium with LPA and IGF-1 was designed for CHO cells on the basis of a commercial serum-free medium. The use of ATA, instead of IGF-1, was also examined in this study.

Materials and methods

Cells

CHO cells genetically engineered to express genes encoding the human immunoglobulin G (IgG) antibody to a tumor-specific antigen were used. The cells were derived from stable cell line established by introducing a plasmid into a line of CHO cells lacking dihydrofolate reductase (DHFR) (DXB11; Urlaub and Chasin 1980) which were kindly provided by Dr. L. A. Chasin at Columbia University. The plasmid contained the neomycin resistance gene, the DHFR gene, and the heavy- and light-chain genes of IgG downstream of the SV40 promoter.

Culture media

Two types of commercially available serum-free medium for CHO cells supplemented with hypoxanthine (100 μM; Invitrogen, Carlsbad, CA, USA), thymidine (16 μM; Invitrogen), and glutamine (4 mM; Invitrogen) were used in cell culture experiments. EX-CELL^{TM} 302 (JRH Biosciences; currently, SAFC Biosciences, St. Louis, MO, USA) is a serum-free culture medium containing an IGF-1 analog (LONGR^3 IGF-1) and insulin. EX-CELL^{TM}325 (JRH Biosciences; currently, SAFC Biosciences) is a serum-free culture medium not containing protein components. Monooleoyl LPA (Avanti Polar Lipids, Inc., Alabaster, AL, USA) was dissolved in phosphate-buffered saline (PBS) at 1 mmol/L and added to the serum-free media in specific experiments. The IGF-1 analog LONGR^3 IGF-1 (50 ng/mL; GroPep Bioreagents, Adelaide, South Australia, Australia), recombinant human insulin (10 ng/mL; Sigma-Aldrich, St. Louis, MO, USA), and the epidermal growth factor (EGF) analog LONG EGF (25 ng/mL; GroPep Bioreagents) were added as growth factors in specific experiments.

Shake flask culture

Conical flasks with caps (#430183/250 mL and #430421/125 mL; Corning Inc., Corning, NY, USA) were used for shake flask culture. After the cells were seeded in 50 or 25 mL of the media in the flasks, the gas phase was replaced with air containing 5 % CO_2, and then the flasks were sealed with caps. The flasks were placed in a shaking incubator, which was not equipped with a CO_2 controller (INNOVA model 4300; New Brunswick Scientific Co., Inc., Edison, NJ, USA), that was operated at 125 rpm and 37 °C. When the shake flask culture was continued after the sampling of the media during the culture period, the gas phase was replaced with air containing 5 % CO_2 and the flasks were sealed. Culture experiments were performed twice and the same trends were confirmed.

Jar culture

A 3-L bioreactor (BIO MASTER D type; ABLE & Biott Co., Ltd., Tokyo, Japan) was used for the jar culture experiments. The culture was stirred at 60 rpm, during which the dissolved oxygen level was maintained at or above 90 mmHg by flowing air containing 5 % CO_2 into a Teflon tube. The temperature was maintained at 37 °C. The pH was controlled at predetermined values by adding 1 M HCl or 0.5 M $NaHCO_3$. Culture experiments were performed twice and the same trends were confirmed.

Analysis of cell number

Cell culture samples were diluted 40-fold by adding an electrolyte (ISOTON-II; Beckman Coulter Inc., Brea CA, USA), and cell density was measured using a Coulter counter (Z2; Beckman Coulter Inc.). Cell viability was determined on the basis of the number of viable cells and the total number of cells that were measured by erythrosine B staining using a counting chamber. The density of viable cells was calculated by multiplying the cell density measured using a Coulter counter by the cell viability. In the cell culture experiments using ATA, however, the density of viable cells was calculated on the basis of the numbers of viable and dead cells measured by trypan blue staining using a counting chamber.

Concentration of antibodies

The concentration of antibodies was determined by high-performance liquid chromatography (HPLC) using a protein G-immobilized column Poros G/20 (4.6 × 100 mm; PerSeptive Biosystems Inc., Framingham, MA, USA) with UV detection. Purified antibodies (2.43 mg/mL) were diluted with a fresh medium to 243, 121.5, and 60.75 μg/mL to prepare standard curves. When the concentration of the antibodies in a specimen was higher than 243 μg/mL, the specimen was diluted with a fresh medium. The concentration of the antibodies in the specimen was calculated using the regression lines that were prepared on the basis of the peak heights of the measured reference materials and that of the fresh medium (0 μg/mL) measured as a blank.

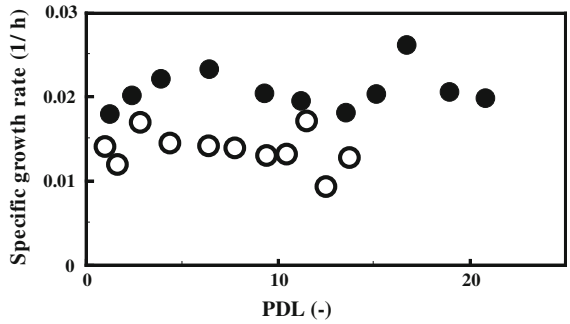

Fig. 1 Effect of FCS addition on cell growth in suspension culture using commercial serum-free medium containing IGF-1. CHO cells in a stock ampoule were directly inoculated and subcultivated in suspension in flasks with shaking with (*closed circle*) or without (*open circle*) the addition of 5 % FCS to the commercial serum-free medium EX-CELL™ 302 containing IGF-1. *PDL* population doubling level

Statistical analysis

In order to determine the statistical significance of the results, Student's t-test was applied. The differences between groups were considered significant at $p > 0.05$. The influence of four growth factors (IGF-1, EGF, insulin and LPA) with their two different levels on cell proliferation was studied using L16 orthogonal array design. The data obtained from these experiments were subjected to two-way factorial analysis of variance (ANOVA). The L16 orthogonal array design was developed and analyzed using SAS v8.2 software (SAS Institute, Cary, NC, USA). The significant factors on the cell proliferaion were considered at $p > 0.05$.

Results

Assessment of cell growth in commercial serum-free media

Frozen cells that were not adapted to grow in serum-free media were thawed and cultured in flasks with shaking using EX-CELL™ 302, a serum-free medium for CHO cells containing the IGF-1 analog, supplemented with or without 5 % FCS. The cells were subcultured every 2–4 days up to 11 times. Figure 1 shows the specific growth rate at each generation, starting from zero at the time of thawing.

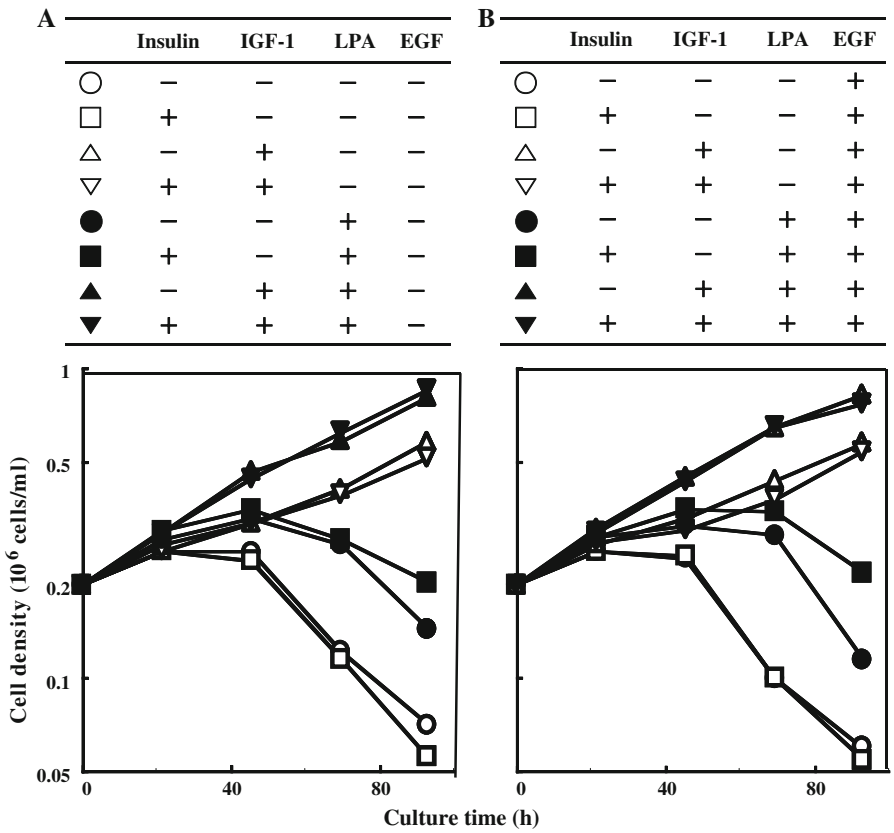

Fig. 2 Effects of growth factors on CHO cell growth. CHO cells were grown in suspension culture in flasks with shaking with addition of growth factors in several combinations shown in the above table on the basis of the serum-free medium EX-CELL™ 325 not containing IGF-1. The combinations were separated into groups A and B

The mean specific growth rate in each subculture in EX-CELL™ 302 supplemented with 5 % FCS was 0.021 h^{-1} (SD = 0.0019), which was significantly ($p < 0.05$) higher than that (0.014 h^{-1}, SD = 0.0015) in the serum-free medium. The cell viability did not decrease below 90 % either with or without the addition of serum (data not shown). The growth rate in the serum-free medium was significantly lower than that in the serum-supplemented medium, indicating the necessity of additives other than IGF-1 to obtain a sufficient growth rate.

Appropriate combination of additives to serum-free medium

The optimum combination of growth factors for stimulating the proliferation of CHO cells was examined to realize cell growth that is compatible with that in the serum-containing media. The possible growth factors were supposed to be the ligands that activate tyrosine-kinase-type receptors (i.e., IGF-1, EGF, and insulin) and LPA, the ligand for GPCRs that likely increase the intensities of signals from the activated receptors through crosstalk. EX-CELL™325, which is a serum-free medium without IGF-1 and does not support cell growth by itself, was supplemented with those growth factors in accordance with the protocol for L16 orthogonal arrays and cultured in flasks with shaking. Figure 2 shows the viable cell density in shake flask cultures.

The density of viable cells was the same up to the second day of cultivation in the cultures in which the downstream signaling pathway of IGF-1 receptors or GPCRs were activated independently; however, thereafter, cell growth stopped and the density of viable cells decreased in the cultures in which the signaling pathway of GPCRs alone were activated. On the other hand, in the cultures in which the signaling pathway of IGF-1 receptors alone were activated, the density of viable cells continued to increase after the third day. Moreover, cell growth was further promoted in the cultures in which the signaling pathways of both receptors were activated concurrently compared with the cultures in which the signaling pathways were activated

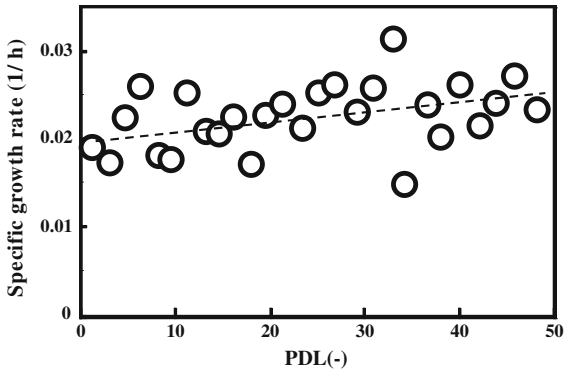

Fig. 3 Specific growth rates during the subcultivation with serum-free medium containing LPA and IGF-1. CHO cells were subcultivated 27 times in shake flask culture using the serum-free medium EX-CELL^{TM}302 containing the IGF-1 analog supplemented with LPA. *PDL* population doubling level

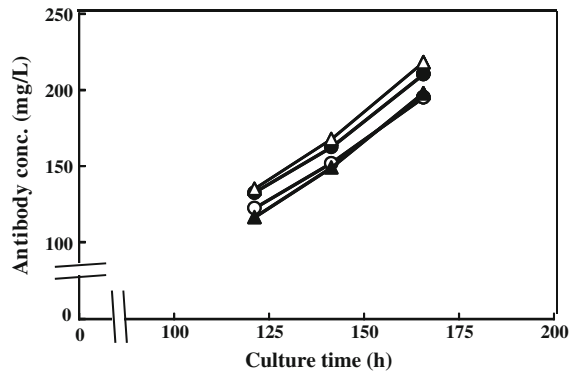

Fig. 4 Antibody production by subcultivated cells in serum-free medium containing LPA and IGF-1. Culture for 7 days was performed for antibody production using stored cells at six (*open circles*), 21 (*closed circles*), 38 (*open triangles*), and 54 PDLs (*closed triangles*) during the subcultivation shown in Fig. 3. Each point is the average of three experiments

independently. Namely, the growth of CHO cells was promoted by the downstream signals of IGF-1 receptors in combination with those of GPCRs at the early stage of culture, while cell growth was maintained by the signals of IGF-1 receptors from the third day onward. From the above findings, the addition of both the IGF-1 analog and LPA seemed to be effective in promoting the growth of CHO cells. Moreover, the experimental design and statistical analysis (ANOVA) of these data on day 4 shown in Fig. 2 revealed that LPA [$F(1,10) = 99.0$, $p < 0.05$] and IGF-1 [$F(1,10) = 929.5, p < 0.05$] had a marked effect on cell growth and there was a significant interaction between those two components [$F(1,10) = 16.0, p < 0.05$].

Assessment of cell growth and productivity intended for commercial production

Because the growth of CHO cells was promoted by their stimulation with both IGF-1 and LPA, cell growth and productivity in the presence of both of the growth factors were assessed by shake flask culture using the serum-free medium EX-CELL^{TM}302 containing the IGF-1 analog supplemented with LPA. The cells were subcultured up to 27 times to estimate the performance of the cells in the commercial production using the newly designed serum-free medium. Figure 3 shows the specific growth rate at each population doubling level (PDL), which reached 49 at the final subculture.

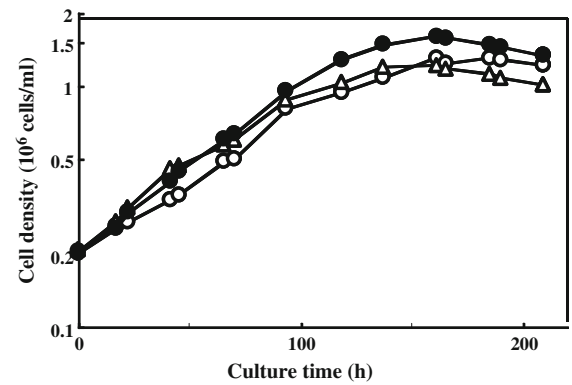

Fig. 5 Effect of pH control on cell growth in serum-free medium. Cell culture in the serum-free medium EX-CELL^{TM}302 supplemented with LPA was performed in a jar fermentor without (*open triangles*) or with pH control at 6.8 (*open circles*) and 6.9 (*closed circles*)

The specific growth rate (μ) gradually increased with increasing number of subcultures in EX-CELL^{TM}302 supplemented with LPA, with the mean specific growth rate in each subculture being 0.022 h^{-1}. This was equivalent to that in EX-CELL^{TM}302 supplemented with 5 % FCS (0.021 h^{-1}, Fig. 1). This result indicated that the stimulation with both IGF-1 and LPA had the growth-promoting effect equivalent to that by the addition of serum.

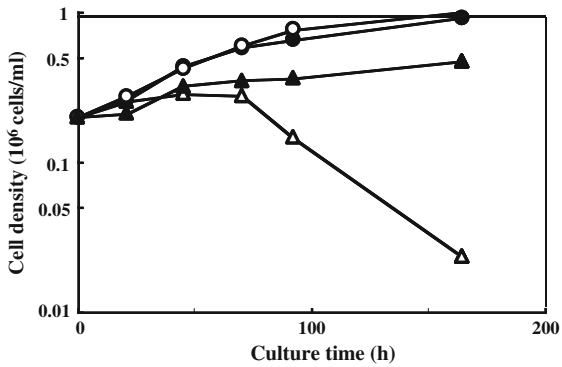

Fig. 6 Effect of combined addition of ATA with LPA on cell growth. CHO cells were cultivated in flasks for 7 days using the serum-free medium EX-CELL^{TM}325 supplemented with 10 μM LPA (*open triangles*), 200 μM ATA (*closed triangles*), 50 μg/L IGF-1 and 10 μM LPA (*open circles*), and 200 μM ATA and 10 μM LPA (*closed circles*)

The cells frozen and stored at 0, 16, 33, and 49 PDLs were recovered and subcultured with flask shaking for 7 days (+5 to 6 PDLs) using EX-CELL^{TM}302 supplemented with LPA, during which antibody productivity was determined. The cells cultured in EX-CELL^{TM}302 showed almost the same antibody production, which reached 200 mg/L on the seventh day of the culture, regardless of the number of PDLs (Fig. 4). Also, this productivity was comparable to that of cells cultured in the serum-containing medium (223 mg/L, 17 PDLs, average of two cultures) and apparently higher than the that of cells cultured in EX-CELL^{TM}302 containing not LPA but IGF-1 (144 mg/L, 11 PDLs; data not shown).

Effects of pH on cell growth

To determine the effects of pH on cell growth using the serum-free medium, cells were cultivated in the serum-free medium EX-CELL^{TM}302 supplemented with LPA in a jar fermentor with accurate control of culture pH at 6.8 and 6.9 or without pH control (Fig. 5). The specific growth rate of cultures without pH control or with pH control at 6.8 rapidly decreased from the fourth day onward. On the other hand, the specific growth rate after the fourth day was improved by controlling pH at 6.9 and the maximum cell density reached 1.6×10^6 cells/mL on the seventh day. These results indicated that the accurate control of pH is necessary for promoting cell growth in the serum-free medium.

Use of ATA as alternative to IGF-1

Because the recombinant IGF-1 analog is expensive, ATA was tested as a substitute of IGF-1. CHO cells were cultivated with shaking flask for 7 days using the serum-free medium EX-CELL^{TM}325 with the addition of only ATA and the addition of both IGF-1 (or ATA) with LPA (Fig. 6). There was only slight growth in the addition of only ATA. On the other hand, the cell growth in the medium supplemented with ATA in combination with 10 μmol/L LPA was synergistically promoted similarly to that in the medium supplemented with LONG R^3 IGF-1 and LPA.

Discussion

The media used in this study are commercially available serum-free media for CHO cells, which can be used for drug manufacturing. They are useful for drug manufacturers because the components of such media are optimized to maximize the growth of CHO cells and the production of genetically engineered proteins. Moreover, they are developed according to the strict safety requirements imposed by regulatory authorities such as the FDA for the culture medium materials; for example, animal-derived materials should be excluded as much as possible (http://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/SAFC/Bulletin/t080.pdf). Both the serum-free media used in this study, EX-CELL^{TM}302 and EX-CELL^{TM}325 (Sigma-Aldrich), include soy hydrolysate. Moreover, they are designed to be suitable for suspension culture that can be easily scaled up by adding Pluronic F-68. In addition, EX-CELL^{TM}302 contains growth factors (IGF-1 analog and insulin), but EX-CELL^{TM}325 does not. To culture the cells that are not adapted to grow in serum-free media under serum-free conditions, components with a strong growth-promoting effect or antiapoptotic effect are required. Therefore, it is preferable to use EX-CELL^{TM}302 that contains the IGF-1 analog and insulin.

However, the concentration of polypeptides in EX-CELL^{TM}302 is not disclosed by the manufacturer. Considering the possibility that the added insulin affects not only insulin receptors but also IGF-1 receptors depending on the concentration of insulin added, EX-CELL^{TM}302 is not suitable for the analysis

of the effects of growth factors. On the other hand, the advantage of EX-CELL^{TM}325 is that it is generally cheaper than EX-CELL^{TM}302 because it is a protein-free medium without expensive polypeptide components. CHO cells required adaptation to grow in EX-CELL^{TM}325 (data not shown). EX-CELL^{TM}325, therefore, was suitable for the analysis of growth factors. The effects of the growth factors were clearly determined because the cells used in this study did not grow in this medium without the growth factors (Fig. 3).

IGF-1 is a polypeptide growth factor and has been reported to be effective in promoting the growth of CHO cells. Pak et al. (1996) generated genetically engineered cells expressing IGF-1 and transferrin and found that these cells grew in protein-free media. Moreover, Rasmussen et al. (1998) obtained DXB11-derived cells, named Veggie-CHO, which grew in protein-free media after adaptation of the cells for 3 months. They subcultured the cells nearly 100 times while gradually decreasing the serum concentration from 7 % to adapt the cells to grow in serum-free media supplemented with IGF-1 and transferrin. Although the cells adapted to grow in the serum-free media (SF-CHO) lost their dependence on transferrin for growth, IGF-1 was reported to be an essential growth factor for the cells to grow under serum-free conditions. In our present study, IGF-1 was confirmed to be effective in promoting the growth of CHO cells under serum-free conditions. However, because cell growth was further promoted when serum and IGF-1 were both added to the media (Fig. 1), serum seemed to contain some components other than IGF-1 that promoted cell growth.

In addition to polypeptide growth factors such as IGF-1, phospholipid growth factors (PLGFs) have been found to show growth-factor-like properties (Goetzl and An 1998). LPA and sphingosine-1-phosphate (S1P) have been reported to promote the growth of many types of cell (Tigyi et al. 2000). LPA is one of the PLGFs most commonly detected in serum at a concentration of approximately 2–20 μM (Moolenaar 1996). At least six subtypes of human and mouse GPCRs have been identified as LPA-specific receptors (Houben and Moolenaar 2011). DNA synthesis and cell division were stimulated when LPA was added to the culture of resting-stage fibroblasts in the absence of polypeptide growth factors (van Corven et al. 1989, 1992). CHO cells were also found to express the receptors for LPA (Holdsworth et al. 2005), and the growth of CHO cells was promoted in the protein-free α-minimum essential medium (MEM), supplemented with 1-oleoyl LPA (Sakai et al. 1999). In our present study, however, the stimulation with LPA alone induced a temporary cell growth, not a continuous growth (Fig. 2). The observations that cell growth induced by LPA alone was temporary and that the cell growth rate decreased with decreasing pH were considered related to the stability of LPA under our culture conditions, because the precise control of culture pH at 6.9 was especially important for cell growth, and because cultures with pH control at 6.9 showed cell density that improved to 130 and 122 % compared with cultures with no pH control and with pH control at 6.8 (Fig. 5), respectively. On this assumption, an additional amount of LPA was added to the medium in which pH was controlled at 6.8, which, however, showed no effects (data not shown). This finding indicated that the temporary effects of LPA were not related to the stability of LPA.

Similarly to LPA, endothelin, whose receptor is GPCR, did not activate the DNA synthesis of Swiss 3T3 cells by itself; however, it synergistically enhanced the DNA synthesis of polypeptide growth factors such as EGF (Brown and Littlewood 1989). It was reported that the synergy between endothelin, insulin, and IGF-1 was limited. On the other hand, the stimulation of bombesin and vasopressin synergistically promoted cell proliferation in the presence of both insulin and IGF-1 (Rozengurt 1986). In this study, the stimulation with insulin, IGF-1, and EGF, which are the ligands of tyrosine kinase-type receptors, in the presence of LPA was expected to promote the proliferation of CHO cells leading to cell growth. The growth-promoting effect of each combination of the growth factors on CHO cells was examined in accordance with the experimental design. The results indicated that cell growth was promoted by stimulation with both IGF-1 and LPA. When the growth-promoting effect was assessed by subculture experiments, the observed cell growth was equivalent to that in the medium supplemented with serum and IGF-1.

It has been reported that the mitogen activities of GPCRs are related to their crosstalk with receptor tyrosine kinases and focal adhesion complexes via G-proteins and the activation of extracellular signal-regulated kinase (ERK)/mitogen-activated protein

kinase (MAPK) pathways by GPCR-β arrestin complexes that are G-protein-independent (Luttrell et al. 1999; Luttrell 2005). The GPCR-β arrestin complexes are formed when GPCRs move into cells to decrease the sensitivity of GPCRs to ligands (desensitization) and to act as signal transducers, activating the effector molecules in signaling pathways such as ERK1/2 (Luttrell and Gesty-Palmer 2010). The temporary, not continuous, growth-promoting effect of the stimulation with LPA alone in this study seemed to be the result of the desensitization of LPA receptors. Moreover, the activation of survival signaling pathway seemed to be weak because the number of viable cells decreased owing to apoptosis. On the other hand, insulin and IGF-1 stimulate not only cell proliferation via ERK/MAPK signaling pathways but also cell survival via phosphatidylinositol-3-kinase (PI3K)/Akt signaling pathways. In this study, neither the growth-promoting effect of insulin alone nor the synergy between insulin and LPA was observed at physiological concentrations, at which IGF-1 receptors were not activated but insulin receptors alone were activated, i.e., 10 ng/mL and 10^{-9} mol/L (Johansson and Arnqvist 2006). This finding indicated that not the signals from insulin receptors but those from IGF-1 receptors are essential for the growth of CHO cells. In addition, cell growth was synergistically promoted by the activation of GPCRs by LPA in addition to the activation of the downstream signaling transducers of IGF-1 receptors. The signals from both receptors seemed to synergistically activate ERK/MAPK growth-signaling pathways.

It has been reported that ATA has the antiapoptotic effect on CHO cells and many other cell lines and acts as a growth promoter similarly to insulin in CHO cells (Liu et al. 2001). Beery et al. assumed that the antiapoptotic effect of ATA was attributed to its activity on the cell surface, because ATA is a polyanion, which cannot permeate through the cell membrane. Using the human breast cancer cells MCF-7, they found that ATA stimulated cell growth via ERK/MAPK signaling pathways and cell survival via PI3K/Akt signaling pathways via the phosphorylation of IGF-1 receptors (Beery et al. 2001). In this study, the cells were cultured in the medium supplemented with ATA alone to examine the possibility that ATA promotes the growth of CHO cells as an alternative to IGF-1; however, the growth-promoting effect of ATA was insufficient (Fig. 6). Although the molecular mechanism underlying the activation of IGF-1 receptors by ATA has not been sufficiently clarified, ATA is considered to activate the downstream signaling transducers of IGF-1 receptors similarly to IGF-1. Hence, it was assumed that the growth rate of the cells stimulated with both ATA and LPA would be equivalent to that of the cells stimulated with both IGF-1 and LPA. As expected, the cell growth rates in both experiments were similar. Namely, CHO cells were able to grow without requiring adaptation in EX-CELL^{TM}325, a protein-free medium without expensive polypeptide components, by supplementing with inexpensive ATA and LPA.

Conclusions

The serum-free medium with the addition of both IGF-1 and LPA, which was designed on the basis of a commercial serum-free medium, could support the growth of CHO cells and antibody production comparable to serum-containing medium in suspension culture. Moreover, the possibility of cost reduction by the substitution of IGF-1 with ATA was also shown.

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