Studies on chemically defined platform media for CHO cell fed-batch culture process

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Chapter 1 Introduction

1.1 Therapeutic antibody production by using Chinese hamster ovary cells

Monoclonal antibodies (mAbs) and Fc fusion proteins have become major drug modalities for the treatment of a wide range of diseases, especially in the areas of autoimmune/inflammatory disorders and oncology (Aggarwal 2014; Philippidis 2017). Six of top 10 global prescription drugs in 2016 were mAbs and Fc fusion protein (Table 1).

Table 1 Top 10 global prescription drugs in 2016 in terms of sales (Philippidis 2017)

Rank	Brand name of product	Modality	Sales (US $BN)	Indication
1	Humira	mAb	16.078	rheumatoid arthritis
2	Harvoni	small molecule	9.081	hepatitis C virus
3	Enbrel	Fc fusion protein	8.874	rheumatoid arthritis
4	Rituxan	mAb	8.583	non-Hodgkin's lymphoma
5	Remicade	mAb	7.829	rheumatoid arthritis
6	Revimid	Small molecule	6.974	multiple myeloma
7	Avastin	mAb	6.752	colorectal cancer
8	Herceptin	mAb	6.751	breast cancer
9	Lantus	insulin analog	6.054	diabetes
10	Prevnar13	vaccine	5.781	prevention of pneumococcal pneumonia

Because mAb therapies usually require large doses over a long period of time, a large amount of mAb must be produced to meet the demands of clinical development and the commercial market. To meet the strong demand, significant efforts have been made by the biopharmaceutical industry to develop a high-yield cell culture process. Chinese hamster ovary (CHO) cell lines have been used most widely to produce mAbs, and there have been significant improvements in the CHO cell fed-batch culture process over the last few decades (Zhu 2012). Fed-batch cultures are used widely to produce therapeutic mAbs. The advantages of a fed-batch culture over other culture processes, such as batch, perfusion, and continuous cultures, are its higher product concentration, ease of operation, and faster development. Typical product concentrations of fed-batch processes were in the range of 0.1–1 g/L in the 1990s, and then they improved to 1–10 g/L in the 2000s (Huang et al. 2010; Li et al. 2010; Wurm 2004). However, the advantages of the current high-yield fed-batch cultures over batch cultures have not been reported. Thus, the effect of the feed on the product concentration is not commonly recognized in the current high-yield fed-batch cultures, although previous study have reported that fed-batch cultures have yielded product titers that are 7.6-fold greater in average than those of batch cultures (Sauer et al. 2000).

1.2 Platform approach for therapeutic monoclonal antibody production

Faster development of a high-yield cell culture process has also been an area of focus among biopharmaceutical companies because of the increasing number of therapeutic mAb candidates and to shorten the period needed to reach Phase 1 clinical trials. For example, one researcher and six to twelve months effort is generally needed at minimum to develop a high-yield cell culture process for each therapeutic mAb candidate. In regards to required amount of mAb, hundreds gram to 1 kg of purified mAb is generally required for formulation development, stability study of drug substance and drug product, drug product production process development, preclinical study, and phase 1 clinical study. The platform approach is a practical solution to enable faster development of therapeutic mAb candidate production processes. A high yield platform fed-batch culture process that can be utilized for multiple mAb producing cell lines will eliminate a six to twelve month process development period, multiple production cell culture batches and then will finally enable faster Phase 1 clinical trial. The overview of background and purpose of this study is shown in Figure 1.

Platform fed-batch cell culture process generally consists of company-specific host cell line, expression vector, and cell culture medium and feed media. Platform cell culture medium and feed media are used based on the assumption that production cell lines derived from a common host cell line with a common expression vector will have

similar nutritional requirements. However, no studies have examined the development of platform fed-batch culture medium and feed media to date.

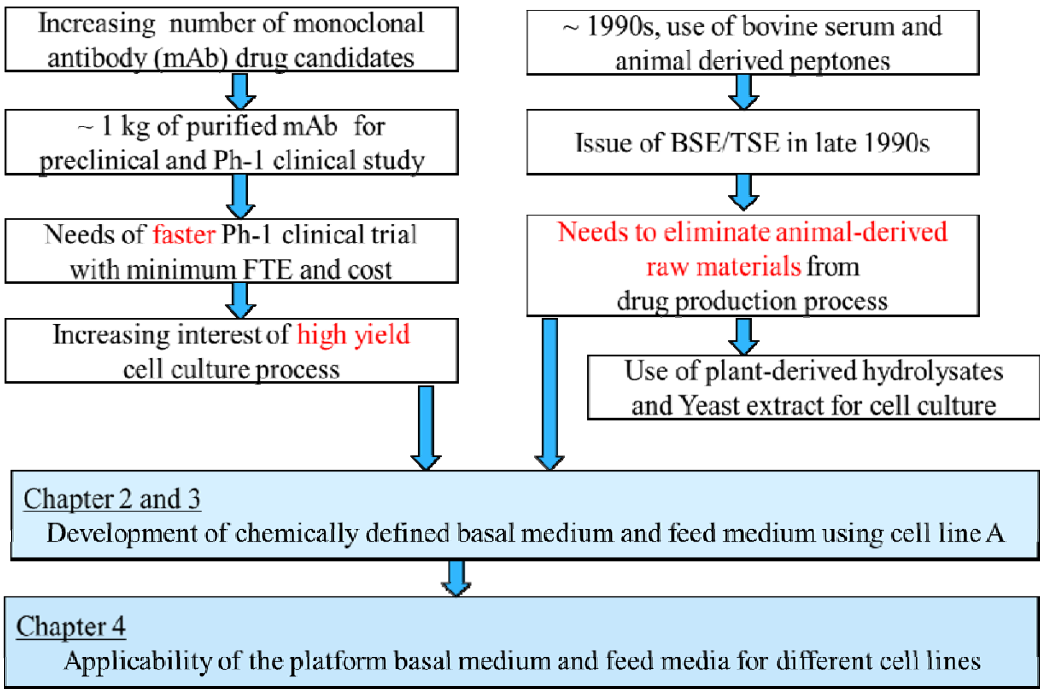

Fig. 1 Overview of background and purpose of this study

1.3 Development of chemically defined media and choline chloride as an essential component

Fetal bovine serum and animal-derived hydrolysates were widely used in cell culture media until the 1990s to produce therapeutic recombinant proteins, including mAbs. However, to eliminate the risk of contamination with adventitious agents, such as viruses and transmittable spongiform encephalopathy (TSE), serum-free and

animal-derived hydrolysate-free media were developed extensively and became increasingly common in the 2000s. To replace animal-derived hydrolysates, plant-derived hydrolysates and yeast extracts have been widely used in biopharmaceutical production processes since the 2000s because hydrolysates can improve cell growth and product yield in serum-free processes (Burteau et al. 2003; Sung et al. 2004). However, there are a few drawbacks in use of those hydrolysates for biopharmaceutical production. First of all, lot-to-lot variation is well known among hydrolysates (Luo et al. 2007). Therefore, prescreening of hydrolysate lots is typically required by using small scale performance testing to eliminate low-performance hydrolysate lots. Secondly, the nutrient composition of hydrolysates is not balanced with cellular requirement. So various components, such as ash and salts, that come from hydrolysates are excessive supply in the cell culture medium. In contrast to hydrolysate-containing medium, all components in chemically defined medium can be balanced with cellular requirement. In addition, use of purified components leaves chemically defined media free of lot-to-lot variation. In the 2000s, there was a substantial increase in the understanding of the nutritional requirements of CHO cells. Eventually, the development of chemically defined media and feed formulations, in combination with the adaptation of CHO cells to chemically defined media, led to mAb

titers as high as 10 g/L (Huang et al. 2010; Lu et al. 2013). The overview of historical background for development of chemically defined fed-batch basal medium and feed media is shown in Figure 2.

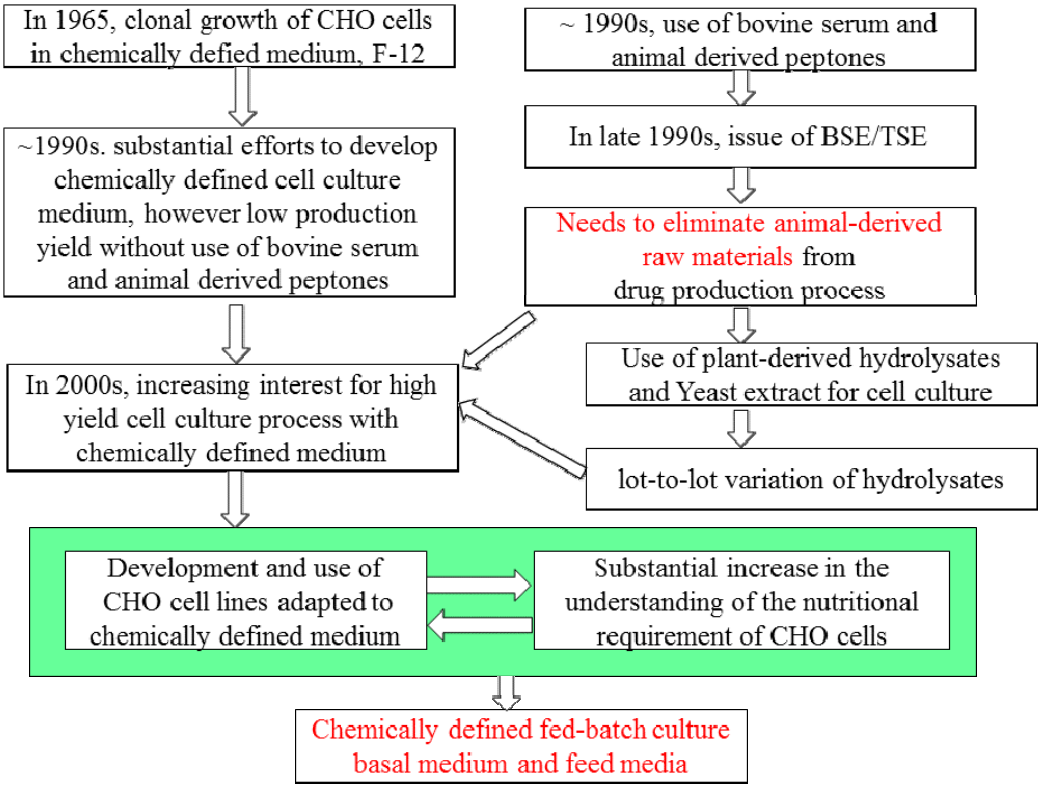

Fig. 2 Overview of historical background for development of chemically defined fed-batch basal medium and feed media

Chemically defined media for mammalian cells have been of great interest among investigators since the 1960s. Ham (1965) first reported clonal growth of CHO cells in a chemically defined synthetic medium, F-12. Since then, the components of F-12 have been used in chemically defined media such as DMEM/F-12, which is the most widely

used medium for serum-free cultures (Jayme et al. 1997). Choline chloride is a component of F12 medium. Choline was first identified in ox bile in 1862 (Strecker 1862). The nutritional importance of choline was first reported in 1932, when it was shown that a dietary choline deficiency caused a fatty liver in rodents (Best and Huntsman 1932). The major metabolic use for choline is as a precursor in the biosynthesis of phosphatidylcholine and sphingomyelin, two phospholipids that are components of biological membranes (Zeisel 2006). Choline is also a major source for methyl groups via its metabolite, betaine, which participates in S-adenosylmethionine synthesis pathways. It was reported previously that choline deprivation limited cell growth and induced cell death in a hybridoma cell culture (Ishaque and Al-Rubeai 2002). Fortifying medium with choline chloride in CHO cell batch cultures enhanced cell growth and mAb production (Kim et al. 2005). Despite the aforementioned importance of choline in mammalian cells, little attention has been paid to the optimal choline ratio in cell culture media, and no studies have reported the effect of choline limitation on the quality of recombinant proteins and mAbs.

1.4 Overview of the present study

The overview of the present study is shown in Figure 3. In chapter 2, a chemically defined basal medium and feed media were developed using a single CHO cell line.

During the media development, choline was found to be one of the potential limiting nutrients in the fed-batch culture. In chapter 3, to determine whether choline was a limiting nutrient in the fed-batch culture, 2-fold and 4-fold choline-enriched feed media were prepared and evaluated in fed-batch cultures using cell line A. Then the author found that a choline limitation during the fed-batch culture caused a lower cell viability, a lower mAb titer, a higher mAb aggregate content, and a higher mannose-5 content. Thus, the choline to glucose ratio in the feeds was optimized. The optimized medium and feeds yielded a 6.4 g/L mAb titer, which is 12-fold higher than that of the batch culture using a commercially available, chemically defined CD CHO medium. In chapter 4, applicability of the developed basal medium and feed media were tested on three different cell lines.

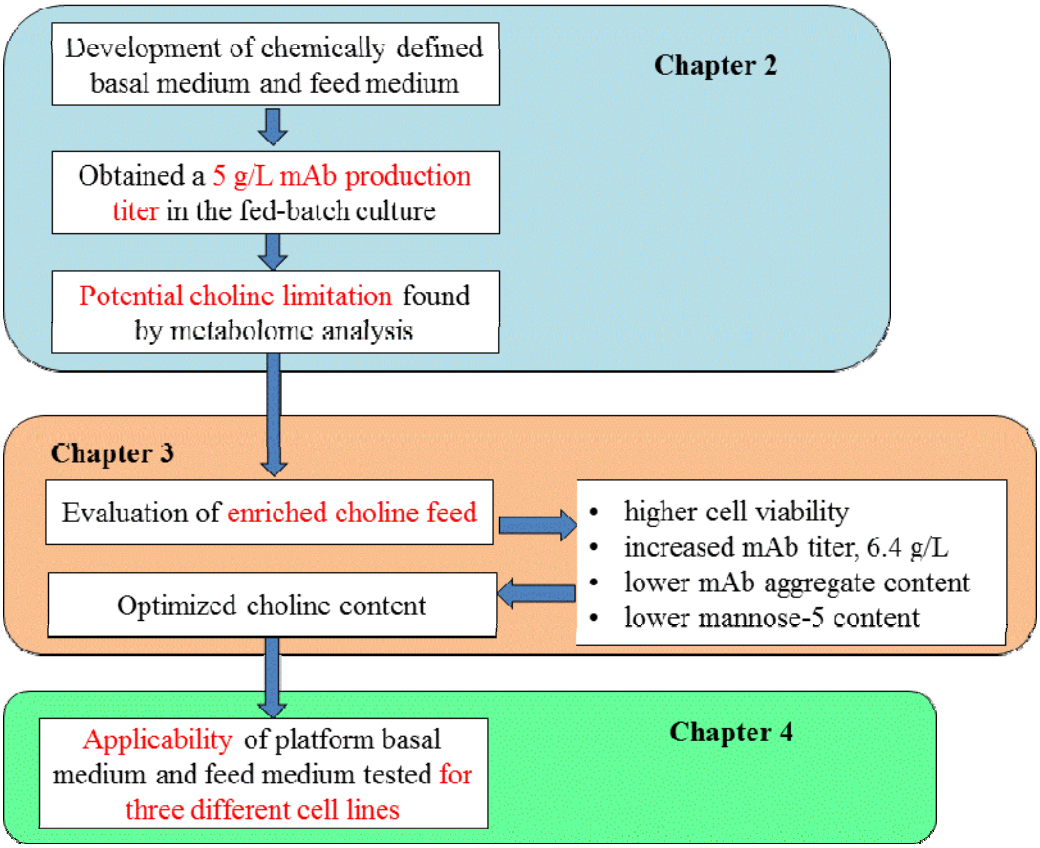

Fig. 3 Overview of the present study

Chapter 2 Development of chemically defined basal medium and feed media

2.1 Introduction

Serum-free chemically defined media for mammalian cells have been of great interest among investigators since Ham (1965) first reported clonal growth of CHO cells in a chemically defined synthetic medium named F-12. Chen et al. (2000) reported development of a chemically defined protein free medium which consisted of DMEM/F12 medium supplemented with amino acids, ascorbic acid, ferric sulfate, ethanol amine, putrescine, sodium pyruvate aurintricarboxylic acid (ATA), pluronic F68 and vitamin K_3. On this medium the viable cell density of CHO cells reached 1.92×10^6 cells/mL in batch culture. ATA is a mild chelate, which has the function for the replacement of transferrin in cell culture (Bertheussen 1993). During the last decade chemically defined media became commercially available from all major mammalian cell culture medium manufacturers. Investigators at several biopharmaceutical companies reported development of chemically defined fed-batch processes (Ma et al. 2009; Huang et al. 2010; Yu et al. 2011). However the composition of the various chemically defined media that are used in biopharmaceutical manufacturing has not been disclosed.

Fed-batch culture is most widely used for the production of mAbs. The major

advantage of fed-batch culture over other culture operations, such as batch, perfusion and continuous cultures, is that it enables a higher product concentration. The final product concentration in fed-batch culture is generally determined by the viable cell density, viable culture duration and specific production rate of the product. Therefore basal medium and feed media for fed-batch culture were developed to increase maximum viable cell density and prolong viable culture duration (Bibila et al. 1995; Ma et al. 2009; Huang et al. 2010). The viable cell density and viable culture duration have been increased by feeding limiting nutrients, such as glucose, amino acids, vitamins, and minerals (Bibila et al. 1994; Xie et al. 1996; deZengotita et al. 2000; Kuwae et al. 2005). The depletion of key nutrients, such as amino acids, vitamins, or trace elements can terminate cell growth and also decrease cell viability and recombinant protein production. However, media which is overly enriched may also inhibit cell growth, due to component concentration dependent toxicity effect and high culture osmolarity. Actually, there is a substantial negative effect on cell growth at 400 mOsm or above (Bibila et al. 1994). Bibila et al. also reported that feeding of the concentrated feed solution, which contained all medium components at 10-fold their basal medium levels except sodium chloride, potassium chloride, and sodium bicarbonate, resulted in 7-fold increase in the final mAb titer (1 g/L) compared to batch culture. Sodium chloride,

potassium chloride and sodium bicarbonate were not included in the concentrated feed solution to minimize osmolality increase during the fed-batch culture. The technique of the 10-fold concentrated feed medium was useful to quickly improve antibody titer in the early stage fed-batch culture development. However the composition of the 10-fold concentrated feed medium did not meet cellular nutrient requirement, so a reiterative process of spent media analysis, single-component titration and improvement of feed composition is needed to further increase final antibody titer.

As well as media development, feeding strategy is also important not only for fed-batch process control but also for media development. Dynamic feeding has been utilized as a method to adapt a process to the real time nutrient requirement of cells. Zhou et al. (1997) successfully used the integral of viable cell concentration over time (IVC) to determine feeding amount and also used on-line oxygen uptake rate measurement to aid empirically the adjustment of the feeding time points and amounts by inferring time points of nutrient depletion. Huang et al. (2010) also successfully utilized IVC to determine feed amount and demonstrated to develop an optimized fed-batch process with mAb titers above 10 g/L. The author (Kuwae et al. 2005) previously achieved a stoichiometrically balanced feed by using glucose as an indicator. Lu et al. (2013) evaluated both integral capacitance to estimate cell growth and glucose

concentration to determine amount of the feed and then found glucose to be the best feed indicator. In this study, the author again selected glucose as an indicator to determine the feed rate of all other nutrients because glucose can be measured easily and accurately off-line and on-line.

In this chapter, a chemically defined basal medium and feed media were developed using a single CHO cell line. The composition of the feed media were balanced stoichiometrically using glucose as an indicator through spent media analysis and titration of the components.

2.2 Materials and methods

Cell lines and culture medium

Recombinant CHO cell line A expressing mAb A were used. Cell line A was established using CHOK1SV cells as the host cell and the GS Gene Expression System provided by Lonza Biologics (Slough, Berkshire, UK). mAb A is humanized IgG. The cells were passaged in CD CHO medium (Life Technologies, Carlsbad, CA, USA) containing methionine sulfoximine for selective pressure.

Chemically defined basal medium was developed for the fed-batch process by modifying the CD CHO medium with Takeda's proprietary supplements. Protein-free, chemically defined feed media developed in-house were used for the fed-batch culture. These feed media contain many components, such as glucose, amino acids, nucleosides, vitamins, and minerals.

Culture conditions

A batch suspension culture was conducted in a 250-mL Erlenmeyer flask (Corning Corp., Corning, NY, USA) on an incubator shaker SCS-20RG-2 (Sanki Seiki, Osaka, Japan), with shaking at 140 rpm, at 37 °C, 8% CO_2, and 90% humidity to avoid evaporation.

Fed-batch production cultures were conducted using 15-L glass bioreactors

equipped with an ABLE Pack controller (ABLE Corp., Tokyo, Japan). Agitation was maintained at 110 rpm in the 15-L bioreactor. The temperature was maintained at 37 °C using a heating blanket. The pH was controlled by CO_2 sparging and by adding 7.5% sodium bicarbonate. Dissolved oxygen was controlled at 20% air saturation using an air overlay and oxygen sparging. Starting from 93 h of culture, feed medium containing glucose was added continuously into the bioreactors to maintain the glucose concentration at 2 g/L. The feed rates of all the feed media were determined in proportion to the feed rate of glucose.

Routine bioreactors offline measurements

Offline dissolved oxygen, dissolved CO_2, and pH were measured daily with a RAPIDLab 348 blood gas analyzer (Siemens Healthcare Diagnostics, Frimley, UK). Cell viability was measured by trypan blue exclusion using a Vi-CELL XR cell counter (Beckman Coulter, Brea, CA, USA). Cell density was determined with a Z2 Coulter counter (Beckman Coulter). Viable cell density was calculated from the cell density and the cell viability. A portion of the broth from each daily sampling was centrifuged to obtain the supernatant for metabolite and product titer measurements. Glucose and lactate concentrations were determined with a BF-5 bioanalyzer (Oji Keisoku Kiki,

Hyogo, Japan). Osmolality was measured with an Osmostat OM-6040 (Arklay, Kyoto, Japan).

The mAb titer was measured using a high-performance liquid chromatography (HPLC) system (Waters, Milford, MA, USA) with a UV detector and a POROS Protein A affinity column (Applied Biosystems, Foster City, CA, USA).

Metabolomics assay

Both cell and spent medium samples were collected for a metabolomics analysis. Cell culture broth containing 1×10^7 viable cells was removed from the bioreactor and centrifuged at 1,000 × *g* for 3 min in a refrigerated centrifuge RL-130 (TOMY, Tokyo, Japan). The supernatant was collected as spent medium and stored at −80 °C. The cell pellet was suspended and washed twice using cold phosphate-buffered saline. After two washes, the cell pellet was frozen in liquid nitrogen and stored at −80 °C. The cell pellet and spent medium samples were sent on dry ice to Metabolon (Durham, NC, USA). The metabolomics analysis was conducted at Metabolon using gas chromatography– and liquid chromatography–mass spectrometry (Lawton et al, 2008).

2.3 Results

Chemically defined platform basal medium and feed media were developed using a single cell line, cell line A, expressing mAb A. Cell line A was selected for the platform media development because of its high peak-cell density and mAb production yield in a batch culture. The cell line showed a peak viable cell density of 5.9×10^6 cells/mL and a final mAb titer of 510 mg/L in CD CHO medium (Life Technologies) at 37 °C in a shake-flask batch culture (Fig. 4).

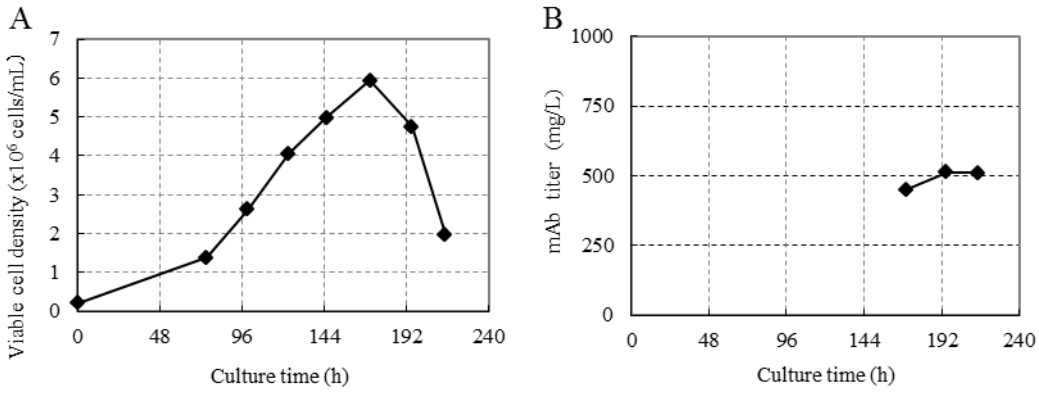

Fig. 4 Batch culture of cell line A in CD CHO medium. Cells were cultured at 37 °C in a shake-flask. A, cell growth; and B, the mAb titer

The first fed-batch culture was conducted using a set of in-house prototype feed media. During the feed media development, the compositions of amino acids and minerals in the platform feed media were balanced stoichiometrically as described by Kuwae et al. (2005), using glucose as an indicator of cell metabolism. Briefly, the feed rate of glucose was determined based on the glucose consumption rate so that the glucose concentration was maintained at 2 g/L via continuous feeding or above 1 g/L via daily bolus feeding during the fed-batch culture. The feed rates of all the feed media were determined in proportion to the feed rate of glucose. After the fed-batch culture, the spent media were analyzed to determine whether the concentrations of amino acids and minerals were limiting or excessive for cell growth. In this study, a limiting nutrient was defined as a nutrient that decreased to zero mmol/L in the spent medium during the fed-batch culture. An excessive nutrient was defined as a nutrient that increased to above the initial concentration in the spent medium during the fed-batch culture. When limiting or excess components were found, the compositions of those nutrients in the feed media were balanced stoichiometrically against glucose to meet cellular requirement. Meanwhile, the ratios of other nutrients, such as vitamins and amines, relative to glucose in the feed media were determined through titration in a group of nutrients in a fed-batch culture because of the lack of an in-house capability to

quantitatively analyze them in the spent media. Formulations of the feed media were designed to prevent precipitation and discoloration of the feed components so that the feed media could be stored at room temperature for at least 1 month. To this end, some nutrients were eliminated from the feed media and then enriched in the basal medium as needed. Thus, the basal medium for the fed-batch culture was developed in parallel with the feed media to maximize the mAb production titer.

To evaluate the performance of the platform basal medium and feed media, a fed-batch culture of cell line A was performed using a 10-L bioreactor in triplicate as described in the Materials and Methods. The cells were seeded at 2.5×10^5 cells/mL, and they reached a peak viable cell density of 1.7×10^7 cells/mL at 237 h of cultivation (Fig. 5A). The feed medium containing glucose was fed continuously after 93 h of cultivation to maintain the glucose concentration at 2 g/L (Fig. 5D). The cell viability was maintained above 80% until 357 h of cultivation (Fig. 5B). The mAb titer reached 5 g/L at 357 h of cultivation (Fig. 5C). Thus, the first platform basal medium and feed media increased the mAb production titer 10-fold compared with the batch culture in CD CHO medium in which the final MAb titer was 510 mg/L. The lactate profile showed that lactate metabolism changed from the production phase to the consumption phase at 122 h of cultivation (Fig. 5E). To further improve the basal medium and feed

media, a metabolome analysis was performed on the cell lysate and the spent media of the fed-batch culture. Among the metabolites analyzed, choline was found to be one of the potential limiting nutrients in the fed-batch culture. The choline concentration in the spent medium declined to almost zero at 213 h of cultivation (Fig. 5F), while the feed medium containing choline was fed continuously after 93 h of cultivation. The timepoint of choline depletion nearly coincided with the timepoint of the peak viable cell density and the onset of the decrease in cell viability.

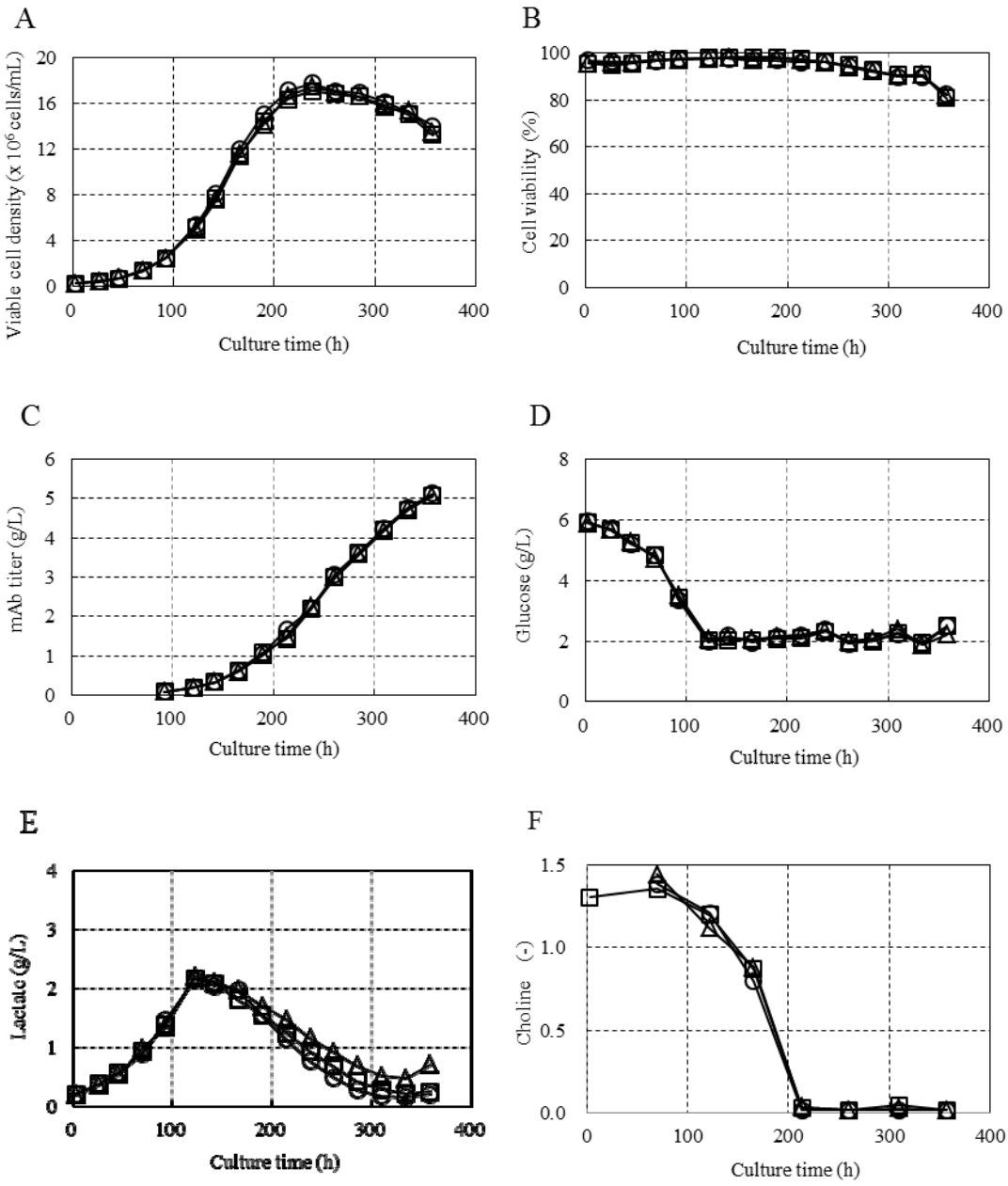

Fig. 5 Fed-batch culture of cell line A. Cells were seeded at 2.5×10^5 cells/mL in a 10-L bioreactor and cultured at 37 °C in triplicate. Feed medium containing glucose was fed continuously. A, cell growth; B, cell viability; C, the mAb titer; D, the glucose concentration in the spent medium; E, the lactate concentration in the spent medium; and F, the choline concentration in the spent medium.

2.4 Discussion

One of the advantages of chemically defined medium is that it enables the rational development of a stoichiometrically balanced feed to avoid both limiting and excess nutrients during a fed-batch culture. In this study, the composition of the feed media were balanced stoichiometrically using glucose as an indicator of cell metabolism through spent media analysis and titration of the components. The chemically defined basal medium and feed media developed increased the peak viable cell density 2.9 fold from 5.9×10^6 cells/mL to 17×10^6 cells/mL, prolonged the viable culture duration 1.9 fold from 8 days to 15 days, and increased the final mAb titer 10 fold from 0.5 g/L to 5 g/L compared to the batch culture on CD CHO medium. A 10-fold mAb titer increase from a batch culture has not yet been reported as far as the author knows. It was confirmed that glucose worked well as an indicator of cell metabolism to control feed rate of the all feed components in our chemically defined fed-batch process (data not shown) as reported previously (Kuwae et al. 2005; Lu et al. 2013).

In the fed-batch culture using the developed basal medium and feed media, a metabolic shift from lactate production to lactate consumption was observed. This type of lactate metabolic shift was previously reported as a desirable phenotype and typically observed at high-titer fed-batch processes (Kuwae et al. 2005; Luo et al. 2012; Ma et al.

2009, Zhou et al. 1997). On the other hand, the undesirable lactate metabolic phenotype is characterized by a high level of lactate concentration and lactate accumulation throughout the entire fed-batch culture run with a lower mAb titer. It is also reported that a lower copper concentration in the culture medium caused the undesirable lactate metabolic phenotype and the lower mAb titer compared to the higher copper concentration culture (Qian et al. 2011; Luo et al. 2012). The result obtained in this study suggests that copper concentration is not limiting in our fed-batch process.

A metabolome analysis of the spent media from the fed-batch culture identified choline as a potential limiting nutrient. In the following chapter, chapter 3, effect of choline on mAb production and mAb quality is studied. The basal medium and feed media were developed using a single CHO cell line. The applicability of these fed-batch culture media to other CHO cell lines were examined in chapter 4.

Chapter 3 Effect of choline enrichment on product titer and product quality

3.1 Introduction

In chapter 2, choline was found to be one of the potential limiting nutrients in the fed-batch culture using the basal medium and feed media developed. In this chapter, to determine whether choline was a limiting nutrient in the fed-batch culture, 2-fold and 4-fold choline-enriched feed media were prepared and evaluated in fed-batch cultures using cell line A. An effect of choline enrichment on product titer and product quality is reported.

3.2 Materials and methods

Cell lines and culture medium

Recombinant CHO cell line A expressing mAb A were used in this study. The cells were passaged in CD CHO medium (Life Technologies, Carlsbad, CA, USA) containing methionine sulfoximine for selective pressure. For fed-batch production cultures, the basal medium and feed media developed in chapter 2 were used in this study.

Culture conditions

Fed-batch production cultures were conducted using 3-L glass bioreactors equipped with an ABLE Pack controller (ABLE Corp., Tokyo, Japan). Agitation was maintained

at 127 rpm in the 2-L bioreactor. The temperature was maintained at 37 °C using a heating blanket. The pH was controlled by CO_2 sparging and by adding 7.5% sodium bicarbonate. Dissolved oxygen was controlled at 20% air saturation using an air overlay and oxygen sparging. Starting from day 2 or later, feed medium containing glucose was added continuously into the bioreactors to maintain the glucose concentration at 2 g/L. The feed rates of all the feed media were determined in proportion to the feed rate of glucose.

Routine bioreactors offline measurements

Offline dissolved oxygen, dissolved CO_2, and pH were measured daily with a RAPIDLab 348 blood gas analyzer (Siemens Healthcare Diagnostics, Frimley, UK). Cell viability was measured by trypan blue exclusion using a Vi-CELL XR cell counter (Beckman Coulter, Brea, CA, USA). Cell density was determined with a Z2 Coulter counter (Beckman Coulter). Viable cell density was calculated from the cell density and the cell viability. A portion of the broth from each daily sampling was centrifuged to obtain the supernatant for metabolite and product titer measurements. Glucose and lactate concentrations were determined with a BF-5 bioanalyzer (Oji Keisoku Kiki, Hyogo, Japan). Osmolality was measured with an Osmostat OM-6040 (Arklay, Kyoto,

Japan).

The mAb titer was measured using a high-performance liquid chromatography (HPLC) system (Waters, Milford, MA, USA) with a UV detector and a POROS Protein A affinity column (Applied Biosystems, Foster City, CA, USA).

Antibody product quality analysis

To analyze the antibody quality, the antibody fraction in the culture supernatant was purified using a Protein A HP Spin-Trap (GE Healthcare, Buckinghamshire, UK). The percentage of antibody aggregates was determined by size-exclusion chromatography using an HPLC system (Waters) with a UV detector and a TSK Gel G3000 SW XL column (Tosoh, Tokyo, Japan).

Antibody deglycosylation was performed using glycopeptidase F (Takara Bio, Shiga, Japan). The obtained oligosaccharides were derivatized with 2-aminopyridine. After purification, pyridinylamino derivatives of the oligosaccharides were analyzed by reverse-phase HPLC with a fluorescence detector and Shim-pack CLC-ODS18 column (Shimadzu, Kyoto, Japan).

3.3 Results

In chapter 2, choline was found to be one of the potential limiting nutrients in the fed-batch culture. To determine whether choline was a limiting nutrient in the fed-batch culture, 2-fold and 4-fold choline-enriched feed media were prepared and evaluated in fed-batch cultures using cell line A. The 2-fold and 4-fold choline enriched feed medium respectively contained 2-fold and 4-fold choline concentration of the original feed medium. The cells were seeded at 1.4×10^6 cells/mL in a 2-L bioreactor, and they reached a peak viable cell density of 1.7×10^7 cells/mL at 166 h of cultivation (Fig. 6A). A 6-fold increase in the initial viable cell density resulted in the cell density peaking 3 days earlier compared with the previous cultures shown in Fig. 5A. Although there was no difference in the peak viable cell density among the three different choline conditions, the 2-fold and 4-fold choline-enriched cultures showed a milder decrease in the slope of the cell viability compared with the 1-fold choline culture (Fig. 6B). The cell viabilities at 358 h of cultivation were 43.7%, 55.0%, and 56.4%, respectively, for the 1-fold, 2-fold, and 4-fold choline-enriched cultures. A 16% increase in the mAb titer was observed for the 2-fold and 4-fold choline-enriched cultures compared with the 1-fold choline culture (Fig. 6C). Both the 2-fold and 4-fold choline-enriched cultures yielded a 6.4 g/L mAb titer at 358 h of cultivation. Thus, the choline-enriched fed-batch culture process increased the mAb production titer 12-fold compared with the batch culture in

CD CHO medium, in which the final mAb titer was 510 mg/L. The time courses of the lactate concentration in the culture were very similar among the three choline concentrations. However, the lactate concentrations of the two choline-enriched cultures were slightly lower than that of the 1-fold choline culture after 300 h of cultivation.

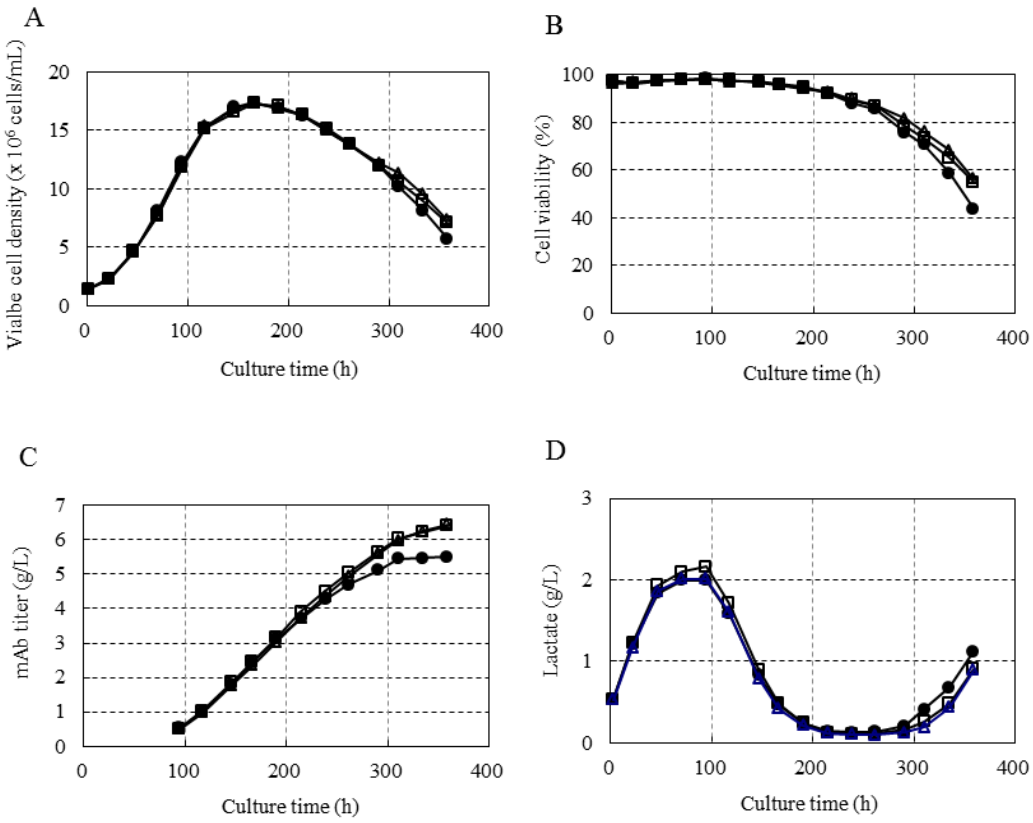

Fig. 6 Fed-batch cultures of cell line A under three different choline concentrations. Cells were seeded at 1.4 × 10^6 cells/mL in a 2-L bioreactor and cultured at 37 °C. A, cell growth; B, cell viability; C, the mAb titer; and D, the lactate concentration in the spent medium. The 1×, 2×, and 4× choline-enriched fed batch cultures are represented by (●), (□), and (△), respectively.

Because the choline-enriched cultures improved cell viability and the mAb titer, the effect of the choline-enriched culture on the quality of mAb A was evaluated. Among the quality attributes, the author paid attention to both the mAb aggregate content and the glycosylation profile because unusually high levels of mAb aggregates and mannose-5 were observed during the development of multiple cell lines expressing mAb A (data not shown). As shown in Table 2, the aggregate content of mAb A decreased with increasing choline contents in the feed medium. The aggregate content decreased from 13.3% to 9.1% by enriching choline 4-fold in the feed medium. For the glycosylation profile, the mannose-5 content of mAb A also decreased from 6.62% to 3.63% following a 4-fold enrichment of choline in the feed medium (Table 3).

Table 2. Effect of enriched choline on the mAb titer and aggregate content

Relative level of choline in the feed medium	1×	2×	4×
mAb titer (g/L)	5.5	6.4	6.4
Aggregates (%)	13.3	10.5	9.1

Table 3. Effect of choline on the glycosylation profile of mAb A.

Relative level of choline in the feed medium	1×	2×	4×
Mannose 5	6.62	3.74	3.63
G0 [a]	16.67	17.28	17.66
G1 [a]	3.90	3.22	2.98
G2 [a]	0.69	0.31	0.32
G0F [b]	54.13	59.08	60.02
G1F [b]	11.30	12.01	11.35
G2F [b]	1.02	1.11	0.97

[a]G0, G1 and G2 are non-fucosylated oligosaccharides terminated by 0, 1 and 2 galactoses respectively.
[b]G0F, G1F and G2F are fucosylated oligosaccharides terminated by 0, 1and 2 galactoses respectively.

The results described above show that the choline enrichment improved the mAb titer and product quality. A metabolome analysis of the 4-fold choline-enriched fed-batch culture showed that the choline concentrations in the cells and the spent medium were well maintained throughout the cultivation (data not shown). These results suggest that choline was a limiting nutrient in the first platform fed-batch process. Other potential limiting nutrients that were found by the metabolome analysis were evaluated in the same manner as for choline, but no other limiting nutrients were found (data not shown). Thus, the feed formulation with the 4-fold choline enrichment was finally selected and incorporated into the second platform basal medium and feed media.

3.4 Discussion

During the feed media optimization, it was found that the choline limitation during the fed-batch culture caused a lower cell viability, a lower mAb titer, a higher mAb aggregate content, and a higher mannose-5 content. Choline is required for the biosynthesis of phosphatidylcholine, which is a major constituent of cell membranes. Choline limitation may produce defective Golgi membranes, thereby leading to more mAb aggregates and a higher mannose-5 content. Choline is also an essential component of cell culture media, and it has been included as choline chloride in Eagle's minimal essential medium (MEM) (Eagle 1959), Dulbecco's modified Eagle medium (DMEM) (Dulbecco and Freeman 1959), and Ham's F12 nutrient mixture (Ham 1965). However, the ratios of choline chloride to glucose (g-choline chloride/g-D-glucose) in MEM, DMEM, and F-12 were 0.001, 0.00089, and 0.0079, respectively (up to a 9-fold difference). For our feed medium, the ratio of choline chloride to glucose was 0.00285 in the 1-fold choline culture condition (Figure 7), and this ratio was choline-limiting during the fed-batch culture of cell line A. Both the 2-fold and 4-fold choline culture resulted in a 16% increase in the mAb titer and also resulted in the similar mAb quality improvement compared with the 1-fold choline culture. Therefor it was concluded that choline chloride to glucose ratios of 0.0057 and 0.0114 were optimal in terms of the mAb production titer and the mAb quality for the fed-batch culture of cell line A. To

date, no studies have addressed the optimal choline ratio in the medium for CHO cell fed-batch cultures. This is the first report that describes the optimal choline ratio in the medium and its effect on the mAb production titer and mAb quality in a CHO cell fed-batch culture. It was found that both a high mAb aggregate content and a high mannose-5 content are inherent characteristics of mAb A; a high mAb aggregate content and a high mannose-5 content were not observed so far for other mAbs that were produced using the platform basal medium and feed media (data not shown) in Takeda Pharmaceutical Company. Thus, the selection of cell line A that produces mAb A revealed that choline limitation can impact the mAb aggregate content and the mannose-5 content.

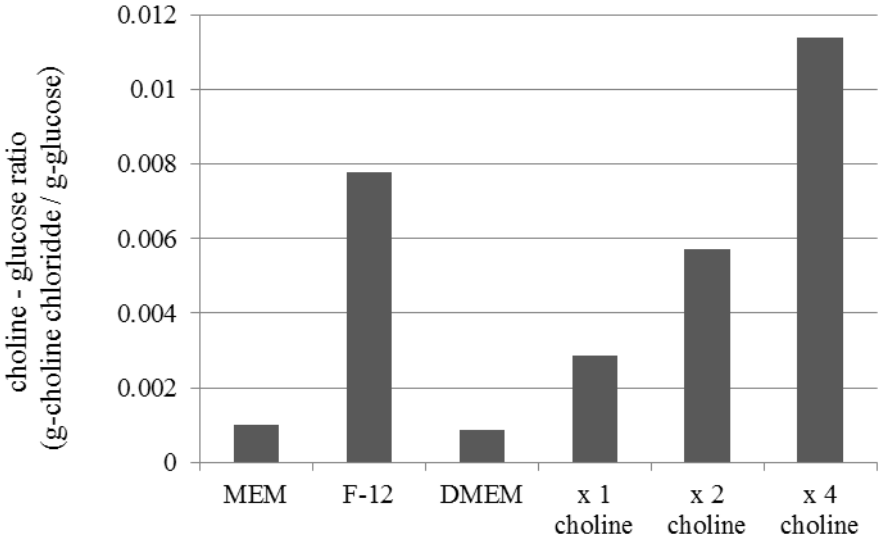

Fig. 7 The choline chloride to glucose ratio. The choline chloride to glucose ratio of the in-house feed media with 1×, 2×, and 4× choline concentrations were compared to Eagle's minimal essential medium (MEM), F-12 medium, and Dulbecco's modified Eagle medium (DMEM)

Chapter 4 Applicability of platform basal medium and feed media

4.1 Introduction

A set of platform basal medium and feed media were developed using a single CHO cell line in chapter 2 and 3. In this chapter applicability of the second platform basal medium and feed media was tested on other three different cell lines.

4.2 Materials and methods

Cell lines and culture medium

Three recombinant CHO cell lines, B, C, and A16, were used in this study. Cell lines B, and C, expressing mAbs B, and C, respectively, were established using CHOK1SV cells as the host cell and the GS Gene Expression System provided by Lonza Biologics (Slough, Berkshire, UK). Cell line A16, expressing mAb A, was obtained from cell line A via subcloning. mAb A is humanized IgG, while mAbs B and C are human IgG.

The cell lines were passaged in CD CHO medium (Life Technologies, Carlsbad, CA, USA) containing methionine sulfoximine for selective pressure. For fed-batch production cultures, the basal medium and the 4-fold choline enriched feed media developed at Chapter 3 were used in this study.

Culture conditions

Fed-batch production cultures were conducted using 3-L glass bioreactors equipped with an ABLE Pack controller (ABLE Corp., Tokyo, Japan). Agitation was maintained at 127 rpm in the 2-L bioreactor. The temperature was maintained at 37 °C using a heating blanket. The pH was controlled by CO_2 sparging and by adding 1.0 M sodium carbonate. Dissolved oxygen was controlled at 20% air saturation using an air overlay and oxygen sparging. Starting from day 2 or later, feed medium containing glucose was added continuously into the bioreactors to maintain the glucose concentration at 2 g/L. The feed rates of all the feed media were determined in proportion to the feed rate of glucose.

Routine bioreactors offline measurements

Offline dissolved oxygen, dissolved CO_2, and pH were measured daily with a RAPIDLab 348 blood gas analyzer (Siemens Healthcare Diagnostics, Frimley, UK). Cell viability was measured by trypan blue exclusion using a Vi-CELL XR cell counter (Beckman Coulter, Brea, CA, USA). Cell density was determined with a Z2 Coulter counter (Beckman Coulter). Viable cell density was calculated from the cell density and the cell viability. A portion of the broth from each daily sampling was centrifuged to obtain the supernatant for metabolite and product titer measurements. Glucose and

lactate concentrations were determined with a BF-5 bioanalyzer (Oji Keisoku Kiki, Hyogo, Japan). Osmolality was measured with an Osmostat OM-6040 (Arklay, Kyoto, Japan).

The mAb titer was measured using a high-performance liquid chromatography (HPLC) system (Waters, Milford, MA, USA) with a UV detector and a POROS Protein A affinity column (Applied Biosystems, Foster City, CA, USA).

4.3 Results

As described in chapter 2 and 3, the platform basal medium and feed media were developed using cell line A. To determine whether the second platform basal medium and feed media can work for other cell lines, fed-batch cultures of three different cell lines, A16, B, and C, were performed using a 2-L bioreactor. Cell line A16 is a cloned cell line from cell line A. Cell lines B and C express mAbs B and C, respectively. The seeding viable cell densities of cell lines A16, B, and C were 1.2×10^6, 5×10^5, and 3×10^5 cells/mL, respectively. The peak viable cell density of each cell line was greater than 1.3×10^7 cells/mL (Fig. 8A). Cell lines A16, B, and C yielded mAb titers of 8.4, 3.3, and 6.2 g/L, respectively, at harvest (Fig. 8C). Although cell line A16 showed the lowest peak viable cell density and the lowest viability after 200 h of cultivation among the three cell lines, it showed the highest mAb titer (Fig. 8B). Lactate was produced by the three cell lines until 100 h of cultivation, but it was consumed after 100 h of cultivation and then its concentration decreased to less than 1 g/L (Fig. 8E). The osmolality was maintained between 300 and 450 mOsm/kg throughout the cultivation of each cell line (Fig. 8F). These results show that the platform basal medium and feed media developed using cell line A worked well for the other CHO cell lines.

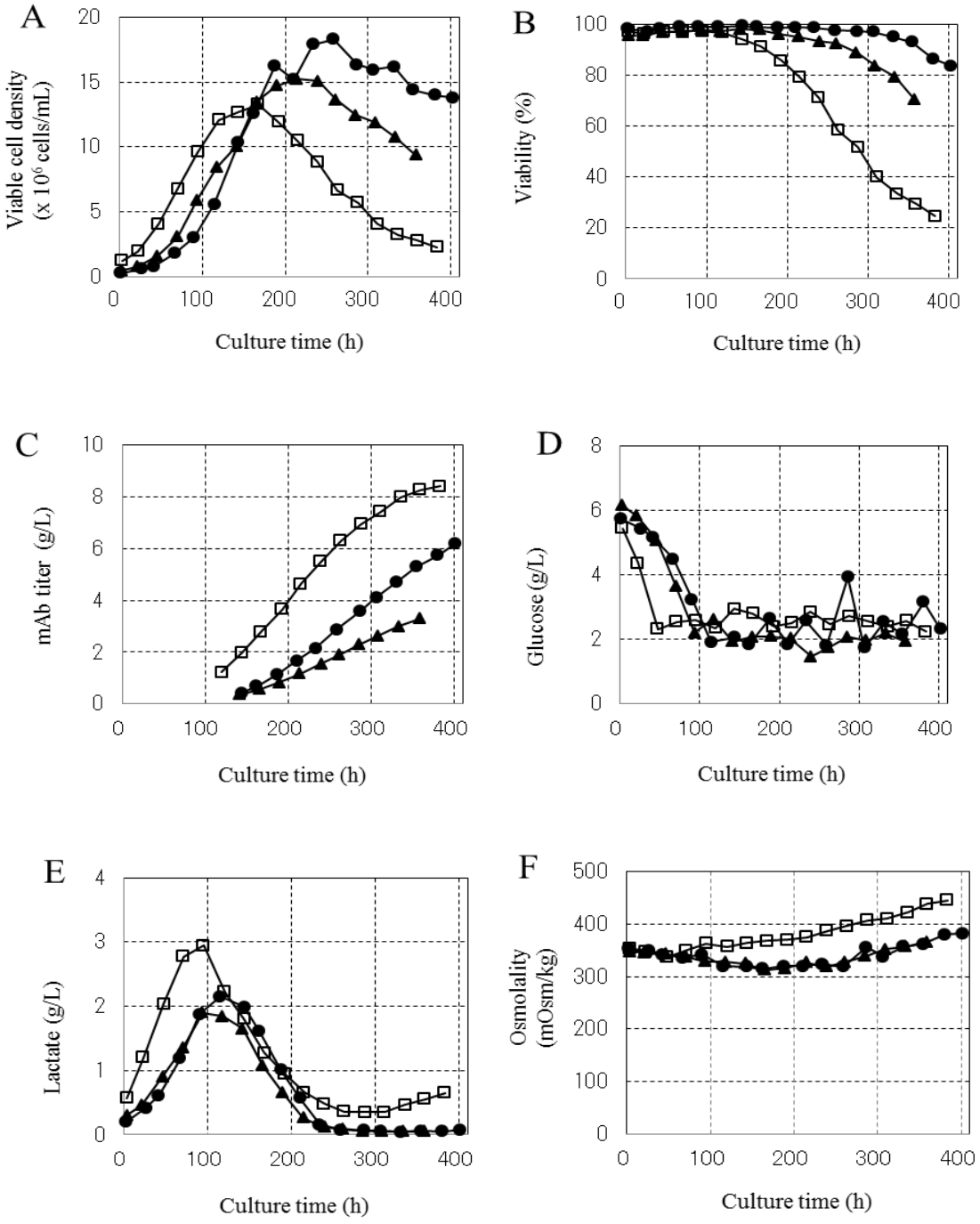

Fig. 8 Fed-batch culture of three different cell lines in the platform basal medium and feeds. Cell lines A16, B, and C were seeded at 1.2×10^6, 5×10^5, and 3×10^5 cells/mL, respectively, and cultured at 37 °C. A, cell growth; B, cell viability; C, the mAb titer; D, the glucose concentration in the spent medium; E, the lactate concentration in the spent medium; and F, the osmolality of the spent medium. Cell lines A16, B, and C are represented by (□), (▲), and (●), respectively.

4.4 Discussion

To shorten the time required to move an antibody drug candidate from the laboratory bench to a clinical trial, the establishment of a high-yield platform basal medium and feed media for cell culture is highly desired because development of cell culture medium takes at least several months for each mAb production cell line. To develop an in-house platform basal medium and feed media, the author used the following three strategies. The first strategy was to develop a chemically defined formulation because it enables the rational development of a stoichiometrically balanced feed to avoid both limiting and excess nutrients during a fed-batch culture. In the second strategy, the author selected a single CHO cell line that showed a high peak viable cell density in a batch culture in a chemically defined medium to develop a platform basal medium and feed formulation, because the author assumed that a cell line that grows well in batch culture has an efficiently balanced nutrient requirement ratio and this balanced nutrient requirement ratio is potentially common to other high mAb-producing CHO cell lines that grow well. Third, the author selected glucose as an indicator of cell metabolism to determine the feed rate of all other nutrients because glucose can be measured easily and accurately off-line and on-line. The successful use of glucose as an indicator of cell metabolism to determine the feed rates of other nutrients was reported previously (Kuwae et al. 2005; Lu et al. 2013). The validity of

these three strategies was proven by this study, as the mAb titers obtained by the three different cell lines in the platform media reached 8.4, 3.3, and 6.2 g/L, respectively, without any optimization for each cell line. The peak viable cell densities of the three cell lines ranged from 1.3×10^7 to 1.8×10^7 cells/mL. The metabolic shift from lactate production to lactate consumption was observed during the fed-batch culture of the three cell lines. This type of lactate metabolic shift was typically observed previously in a nutritionally well-balanced fed-batch culture (Kuwae et al. 2005; Luo et al. 2012; Ma et al. 2009, Zhou et al. 1997). Our platform basal medium and feed media will shorten the media-development time for mAb-producing cell lines.

Chapter 5 Conclusion

Monoclonal antibodies (mAbs) and Fc fusion proteins have become major drug modalities for the treatment of a wide range of diseases, especially in the areas of autoimmune/inflammatory disorders and oncology. In 2016, six of top 10 global prescription drugs were mAbs and Fc fusion proteins. To meet the demands of clinical development and the commercial market a large amount of mAb must be produced, because mAb therapies usually require large doses over a long period of time. For example, to enable phase 1 clinical trial, hundreds gram to 1 kg of purified mAb is generally required for manufacturing process development and supply of investigational medicinal product. In addition, because of the increasing number of therapeutic mAb candidates and to shorten the period of time needed to reach Phase 1 clinical trials, faster development of a high-yield cell culture process has been an area of focus among biopharmaceutical companies. The platform approach is a practical solution to enable faster development of cell culture processes. More specifically, a high yield platform fed-batch culture process that can be utilized for multiple mAb producing cell lines will eliminate a six to twelve month cell culture process development period, multiple production cell culture batches and then will finally enable faster Phase 1 clinical trial.

In this study, the author used the following three strategies to develop an in-house

platform basal medium and feed media. The first strategy was to develop a chemically defined media because it enables the rational development of a stoichiometrically balanced feed media to avoid both limiting and excess nutrients during a fed-batch culture. In the second strategy, the author selected a single CHO cell line that showed a high peak viable cell density in a batch culture in a chemically defined medium to develop a platform basal medium and feed formulation, because the author assumed that a cell line that grows well in batch culture has an efficiently balanced nutrient requirement ratio and this balanced nutrient requirement ratio is potentially common to other high mAb-producing CHO cell lines that grow well. Third, the author selected glucose as an indicator of cell metabolism to determine the feed rate of all other nutrients because glucose can be measured easily and accurately off-line and on-line.

In chapter 2, the author developed a chemically defined platform basal medium and feed media using a single CHO cell line, cell line A producing mAb A. Cell line A, which showed a peak viable cell density of 5.9×10^6 cells/mL and a final mAb titer of 0.5 g/L in batch culture, was selected for the platform media development. Stoichiometrically balanced feed media were developed using glucose as an indicator of cell metabolism to determine the feed rates of all other nutrients. A fed-batch culture of cell line A using the platform fed-batch medium yielded a 5 g/L mAb titer, which was

10-fold higher than that of the batch culture. To further improve the basal medium and feed media, a metabolome analysis was performed on the cell lysate and the spent media of the fed-batch culture. Among the metabolites analyzed, choline was found to be one of the potential limiting nutrients in the fed-batch culture.

In chapter 3, to determine whether choline was a limiting nutrient in the fed-batch culture, 2-fold and 4-fold choline-enriched feed media were prepared and evaluated in fed-batch cultures using cell line A. Then the author found that choline limitation in the fed-batch culture with the original 1-fold choline feed media caused a lower cell viability, a lower mAb titer, a higher mAb aggregate content, and a higher mannose-5 content. Both the 2-fold and 4-fold choline enriched culture resulted in a 16% increase in the mAb titer, 6.4 g/L, and also resulted in the similar mAb quality improvement compared with the 1-fold choline culture. Therefor it was concluded that choline chloride to glucose ratios (g-choline chloride/g-D-glucose) of 0.0057 and 0.0114 were optimal in terms of the mAb production titer and the mAb quality for the fed-batch culture of cell line A. The combination of the developed basal medium and the 4-fold choline enriched feed media were selected as platform media for CHO cell fed-batch culture process.

In chapter 4, the applicability of the platform basal medium and feed media was

examined. Three other different cell lines (A16, B, and C) that produce mAb A, mAb B, and mAb C were cultured using the platform fed-batch media, and they yielded mAb titers of 8.4, 3.3, and 6.2 g/L, respectively, without any optimization for each cell line. The peak viable cell densities of the three cell lines ranged from 1.3×10^7 to 1.8×10^7 cells/mL. These results show that the nutritionally balanced fed-batch basal medium and feed media worked well for other cell lines.

The platform basal medium and feed media developed in this study will eliminate an at least six month media-development time for mAb-producing cell lines and then enable faster phase 1 clinical trials.

Abbreviations

ATA	aurintricarboxylic acid
CHO	Chinese hamster ovary
DMEM	Dulbecco's modified Eagle medium
HPLC	high-performance liquid chromatography
IVC	integral of viable cell concentration over time
mAb	monoclonal antibody
MEM	Eagle's minimal essential medium
TSE	transmittable spongiform encephalopathy

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