Studies for development of hyper osmotic resistant mammalian cells for industrial scale production of recombinant protein

（組換えタンパク質の工業生産のための
高浸透圧耐性細胞開発に関する研究）

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蒲池　泰治

Yasuharu Kamachi

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Chapter 1 Introduction

Biopharmaceutical manufacturing and marketing is increasing worldwide. In 2016, biopharmaceutical sales reached U.S. $163 billion. The commercially available biopharmaceuticals contain recombinant proteins are produced in prokaryotic systems, mainly *Escherichia coli*, or eukaryotic systems based on fungi (*Saccharomyces cerevisiae* and *Pichia pastoris*), mammalian cells, or insect cell lines (1). Microorganisms, especially *E. coli*, have the advantage of low cost in establishing a production strain, quick production cycle, easy in-process control, and high productivity compared to mammalian expression systems. However, there are various limitations for prokaryote systems. Expression of a large complex protein containing multiple subunits, cofactors, disulphide bonds, and posttranslational modifications is a quite challenging (2). Mammalian expression systems for biopharmaceuticals are overcome these limitations. The advantage of mammalian systems for production of recombinant proteins is capacity for post-translational modification and human protein-like molecular structure assembly. Most marketed biopharmaceutical products have been produced in Chinese hamster ovary (CHO) cells, murine myeloma (NS0 and Sp2/0) cells and baby hamster kidney cells (BHK-21) (3). The percentage of approvals of recombinant protein products produced in mammalian systems was approximately 60% from 2010 to 2014. Within

mammalian expression platforms, CHO-based systems remain the most commonly used expression system. The percentage of total biopharmaceutical product approvals by using CHO-based expression system was 35.5 % from 1982 to 2014 (4, 5). CHO cells are widely used as a host to produce various therapeutic proteins including recombinant monoclonal antibodies (mAbs). Jayapal et al. reported that nearly 70 % of all recombinant therapeutic proteins produced by CHO cells (6). There are several reasons why CHO cells are most widely used as the host cells for therapeutic protein production. Firstly, CHO cells can be adapted to growth in the serum-free chemical defined media relatively easily. This is an advantage for production of therapeutic protein to reduce the risk of viral contamination from animal-derived raw materials. Secondly, CHO cells can be adapted to growth in suspension culture, which make it possible for large-scale cell culture in bioreactors. The largest bioreactors in use for CHO cell lines are 20,000 L scale (3). The scalable suspension culture is contributed to reduce the facility size and manufacturing cost. Thirdly, CHO host cells are demonstrated as safe hosts for the past two decades. Berting et al. reported that CHO cells are less permissive to infection by viruses from other cell line used in the production of recombinant proteins, such as BHK cells (7). Finally, CHO cells have the capacity for appropriate

folding and efficient post-translational modification, and they produce recombinant proteins with glycoforms that are compatible with humans (8, 9). For the above reasons, CHO cells are most commonly used as the host cells, which will allow smooth approval of the therapeutic proteins from regulatory agencies. In expression system of CHO cells, dihydrofolate reductase (DHFR) and glutamine synthetase (GS) are common metabolic markers for production cell line selection. In both cases, selection occurs in the absence of the appropriate metabolite (hypoxanthine and thymidine, in the case of DHFR, glutamine in the case of GS), preventing growth of nontransformed cells (3, 6, 10 and 11).

The overall dominance of mAb approvals since the end of the 1990s has continued into the second decade of the twenty-first century. At the end of the 1980s, mAbs represented just a little over 10% of all biologic products approved, whereas between 2010 and April, 2014 they represent almost 27% of all approvals (5). In 2016, the sales of the mAbs reached U.S. $106.9 billion, which was nearly 66% of the total sales of biopharmaceuticals. The top 10 best-selling biopharmaceuticals in 2016 included eight Abs (six mAbs and two Fc-based fusion proteins). “Global Protein Therapeutics Market Outlook 2020,” reported that biopharmaceuticals market may reach U.S. $ 208 billion by the end of 2020

(1).

Due to the increase in demand for the biopharmaceutical manufacturing and marketing, the cell culture process with high productive cell lines generation has been developing. In 1986, cells typically reached a maximal density of about 2×10^6 cells/mL with a batch process production phase of about 7 days and the mAb concentration reached about 50 mg/L. In 2004, cells reached more than 10×10^6 cells/mL about 2 weeks and the mAb concentration reached about 4.7 g/L (13). Productivity of mammalian cell processes has dramatically improved and reached 10 g/L in mAb production (14). To improve productivity, various approaches have been developed for cell culture processes i.e., improved feeding methods, optimization of cell culture media, control of environmental parameters such as pH, temperature and dissolved oxygen. Besides development of cell culture process, the generation of stable, high mAb expressing cell lines is very important for commercial production. To generate the high mAb expressing cell lines, host cell lines are improved by genetic engineering. In engineering CHO host cells, various approaches i.e., overexpression of beneficial genes, repression of disadvantageous gene products by genomic knock-out or siRNA-mediated knockdown have been reported to improve productivity (15). Recently, the number of promising microRNA candidates for

engineering CHO host cells has significantly increased. microRNAs play a critical role for transcriptome regulations in animal cells (16-24).

The purpose of the present study is developing hyper osmotic resistant mammalian cells for industrial scale production of recombinant protein.

Figure 1 shows the general overview of cell culture process for industrial scale production of recombinant protein in fed-batch production culture. Firstly, Master Cell Bank (MCB) or Working Cell Bank (WCB) is thawed and inoculated to the shake flask. Next, the cells are expanded in shake flasks and bioreactors. In expansion cultures, the passage number and working volumes depends on the final production bioreactor scale. Finally, the cells are transferred to the production bioreactor. In fed-batch production culture, feed medium are fed based on consumption rate of nutrients such as glucose. MCB and WCB are defined as follows. MCB is an aliquot of a single pool of cells which generally has been prepared from the selected cell clone under defined conditions, dispensed into multiple containers and stored under defined conditions. The MCB is used to derive all working cell banks. WCB is prepared from aliquots of a homogeneous suspension of cells obtained from culturing the MCB under defined culture conditions (12).

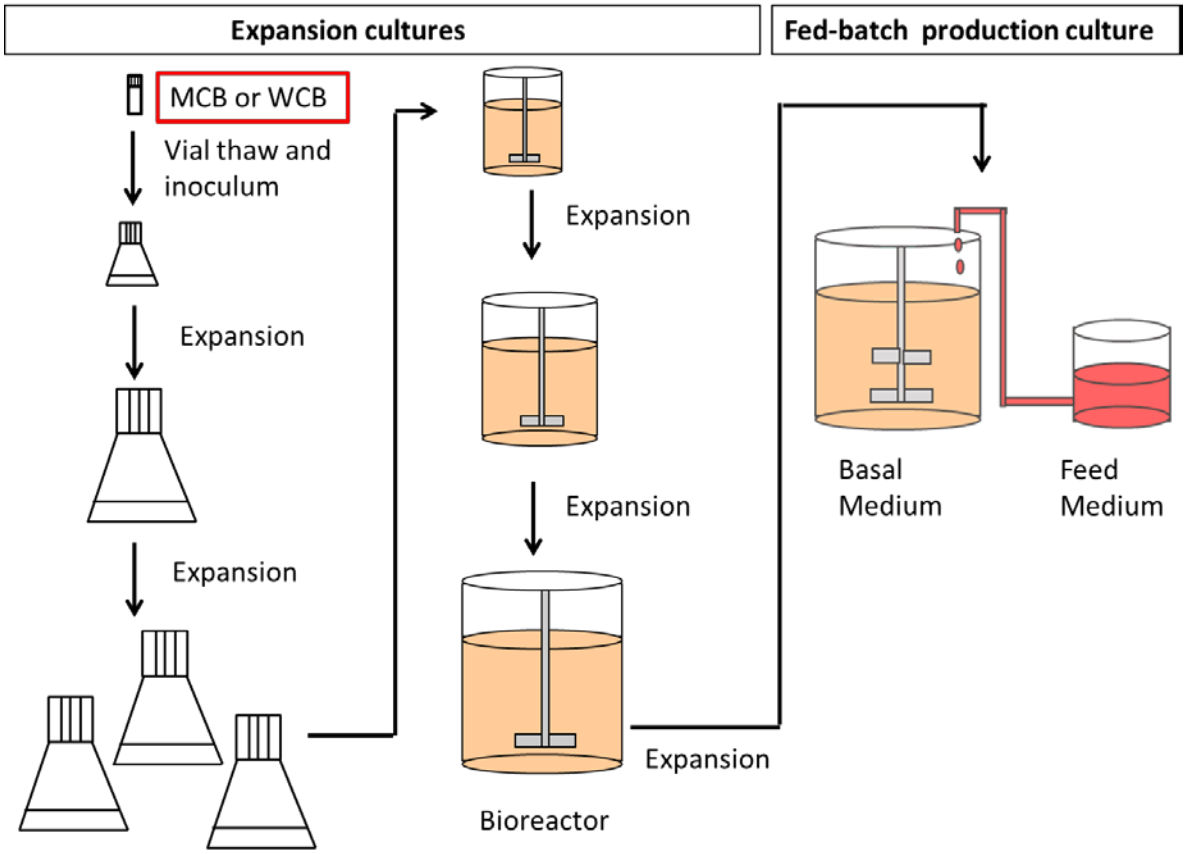

FIG. 1. General overview of cell culture process for industrial scale recombinant protein production in fed-batch culture

Figure 2 shows the overview of generation of MCB and WCB for recombinant protein production. In generation of recombinant protein expressing cell lines, recombinant protein expression vector is transfected into the host cells. After the transfection, high recombinant protein expressing cell lines are selected from transfectant population in the screening and cloning steps. Research Cell Bank (RCB) is prepared from the high recombinant protein expressing cell lines. To select candidate cell line for MCB, cell line stability test and product quality tests are conducted. In this step, final production concentration of each candidate cell line will be confirmed in fed-batch culture. Sub cloning is conducted before or after the creation of RCB. The timing of sub cloning depends on the each company policy and clinical phase. MCB is generated after these evaluations.

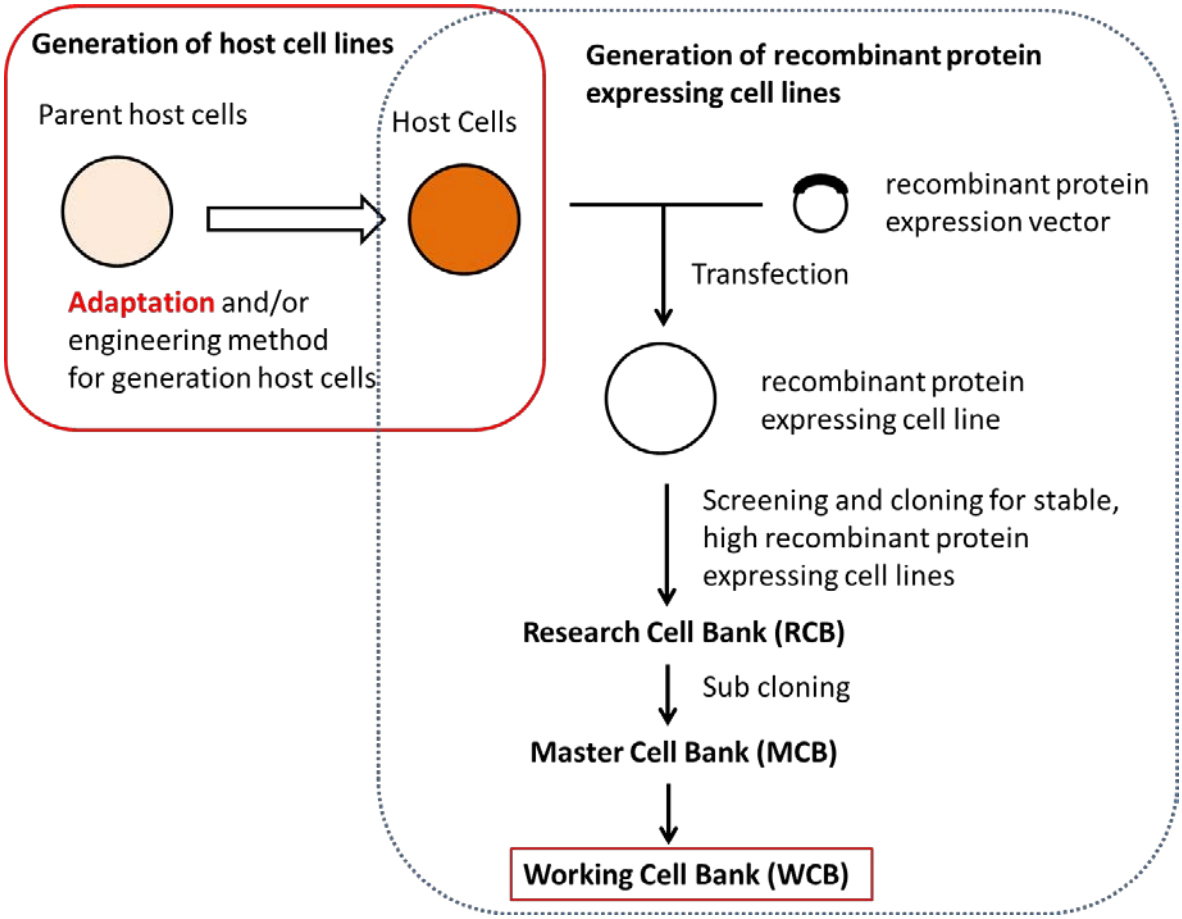

FIG. 2. Overview of generation of MCB and WCB

In generation of host cell lines, host cells are developing by adaptation and/or engineering method such as CHO-K1SV cells (Lonza Biologics, Slough, UK) and Free style CHO-S cells (Life Technologies, CA, USA). Both host cells had been adapted to serum-free suspension culture. In this study, hyper osmotic resistant CHO host cells are developing by using adaptation method. Figure 3 shows the strategy to establish hyper osmotic resistant host cells. The strategy will be available for the development of other host cells, which can handle osmotic stress better than parent host cells. The advantage of the hyper osmotic resistant host cells is as follow.

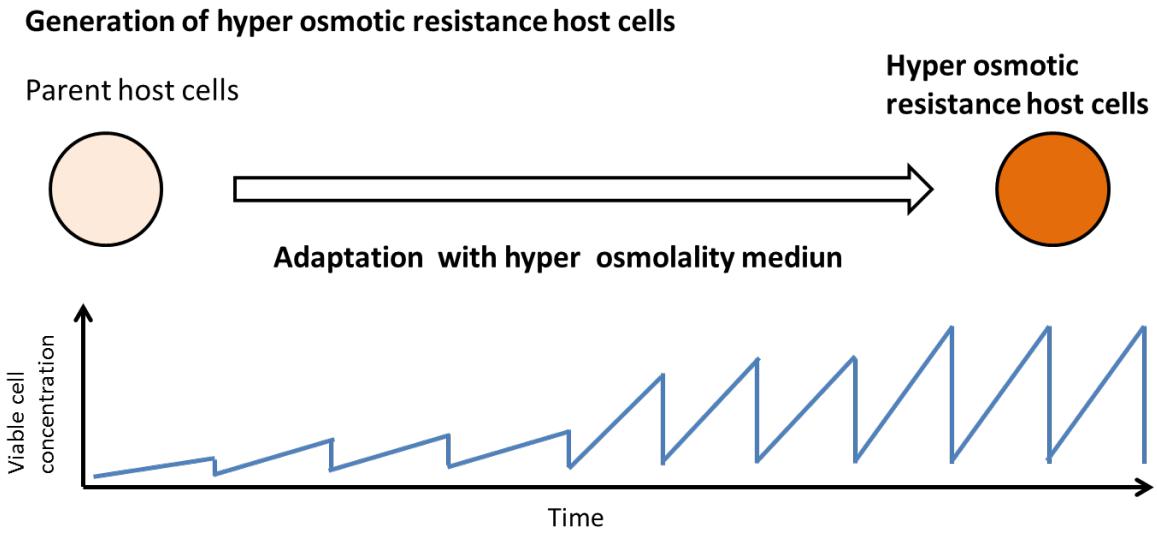

FIG. 3. Strategy to establish hyper osmotic resistant host cells

The final concentration of a recombinant protein in a fed-batch culture is affected by the viable cell density, viable cell culture longevity and specific production rate of the recombinant protein. In fed-batch culture, concentrated nutrient are fed throughout the culture period to maintain nutrient sufficiency. The fed-batch process with bolus feeding is the most widely used method for industrial large-scale mammalian cell culture. However, the bolus feeding of concentrated nutrient and addition of base solution to control pH throughout the culture period leads to increase in medium osmolality. It is well known that hyper osmotic pressure suppresses cell growth (25-27). However, few studies have investigated the development of host cells that do not suppress cell growth in hyperosmotic cultures. In this study, hyper osmotic resistant CHO host cells were developed to overcome growth suppression effects by hyper osmotic pressure. Detailed kinetic and metabolic analyses were performed, and stability of the hyper osmolality resistance of the osmotic resistant CHO host cells was evaluated. The hyper osmotic resistant CHO host cells are expected to increase Integral Viable Cell Concentration throughout the culture period with an increase in medium osmolality. The use of hyper osmotic resistance CHO host cells to create a monoclonal antibody production cell line might be a new approach to increase final antibody concentrations with a fed-batch process.

Chapter 2 Establishment of hyper osmotic resistant CHO host cells

2.1 INTRODUCTION

Chinese Hamster Ovary (CHO) cells are widely used as a host to produce various therapeutic proteins including recombinant monoclonal antibodies. A fed-batch cell culture process with concentrated nutrient feeding throughout the culture period is commonly used for the industrial scale production of therapeutic proteins. There are two types of feeding strategy for fed-batch cultures: i) continuous feeding; and ii) bolus feeding. The fed-batch process with continuous feeding is suitable for maintaining exact nutrient concentrations during cell culture by controlling the feed rate of feed medium based on the consumption of nutrients. Therefore, cells in a fed-batch process with continuous feeding will have less stress from environmental conditions including osmolality during the culture period compared with bolus feeding. Currently, the fed-batch process with bolus feeding is the most widely used method for industrial large-scale mammalian cell culture because of its simple operation, reduced facility requirements and inexpensive manufacturing. Therefore, it is important to develop a simplified platform process with simple operation that meets GMP manufacturing standards, which will reduce the risk of human error and contamination. High productivity of the platform process is also important to meet increasing market demand and reduce the cost of goods.

To improve productivity, various approaches have been developed for cell and cell culture processes, i.e., construction of highly-productive cell lines (15-24, 28, 29), effective strategies for cell line selection (30, 31), improved feeding methods (32-35), optimization of cell culture media (14, 36-40), usage of perfusion seed culture (41), environmental parameters such as pH (42), temperature (43), and dissolved oxygen (39). In general, the final concentration of a recombinant protein in a fed-batch culture is affected by the viable cell density, viable cell culture longevity and specific production rate of the recombinant protein. Osmolality strongly influences these three important parameters. It is well known that hyperosmotic pressure suppresses cell growth. Zhu et al. showed that an increase of osmolality resulted in a linear reduction of the specific growth rate and led to a 60% decrease at 450 mOsm/kg compared with control medium at 316 mOsm/kg (25). However, hyperosmotic stress increases specific productivity in CHO cell lines (26) and hybridoma cell lines (27). Wu et al. reported a decrease in the growth rate and an increase in the specific production rate of hyperosmotic (450 mOsm/kg) cultures compared with iso-osmotic (290 mOsm/kg) cultures (27). Omasa al. reported that the inhibition of hybridoma cell growth was mainly caused by osmotic pressure while lactate production from glucose was inhibited by lactate itself because of

an increase in osmotic pressure (44). Furthermore, increased osmotic pressure increased the productivity of hybridoma cultures (27, 44). However, an increase in the specific production rate does not result in a substantial increase of the final product concentration because of depressed cell growth and decreased cell viability (26, 27). A substantial increase of specific productivity with an increase of osmolality was observed in a batch culture in response to significant metabolic changes. Specific glucose and glutamine consumption rates were increased in a dose-dependent manner in batch cultures. However, the final product concentration did not substantially increase because of a decrease in viable cell density (26). Shen et al. also reported that hyper osmolality slightly repressed the growth rate of fed-batch cells and no significant change in productivity and final product concentration was observed (26). Several studies have reported intracellular changes in response to osmotic stress. In one study, over 600 genes related to cell cycle distribution, growth, transcription and translation regulation in the GS-N0 cell line were affected by hyperosmotic conditions during cell culture (27). However, few studies have investigated the development of host cells that do not suppress cell growth in hyperosmotic cultures.

In a previous study, a fed-batch process with continuous feeding for

monoclonal antibody (IgG) production by CHO cells was developed for pilot plant scale (data not shown). This platform process maintained high cell density and produce high antibody concentrations. However, a more simplified process is required for industrial production to reduce the risk of microbial contamination and operation failure by human error. Therefore, we developed a new bolus feeding method instead of conventional continuous feeding. The bolus feeding method lead to a greater increase in medium osmolality compared with the conventional continuous feeding method because of the large amount of feed volume required to be added in a single administration. Higher osmolality in the culture with the bolus feed method might have a negative effect on cell growth. In this study, hyper osmotic resistant CHO host cells were developed to overcome growth suppression effects by hyper osmotic pressure. To establish these hyper osmotic resistant CHO host cells, an adaptation method with hyper osmotic medium was applied. Batch cultures under osmotic stress conditions were used to confirm the osmotic resistance of the hyper osmotic resistant CHO host cells with CHO-S host cells as controls. Detailed kinetic and metabolic analyses were performed, and stability of the hyper osmolality resistance of the osmotic resistant CHO host cells was evaluated.

2.2 MATERIALS AND METHODS

2.2.1 Cell line and cell culture conditions of the bioreactor

A serum-free adapted recombinant GS-CHO cell line producing human monoclonal antibody was grown in a fed-batch culture. Fed-batch cultures were grown in a 5-L bioreactor (Sartorius, NY, USA) with a 3-L working volume. A 5-L bioreactor was equipped with a pitched blade (diameter = 7.0 cm). The bioreactor temperature was maintained at 37°C, and the agitation speed was 400 rpm. A gas mixture containing air and O_2 was provided. The dissolved oxygen concentration (DO) was controlled using an on-line feedback control at 30 mmHg by varying the oxygen pressure in the gas mixture. Air was supplied through the headspace of the bioreactor for CO_2 removal. pH control was performed by sparging with CO_2 gas into the gas mixture or the addition of 7.5% $NaHCO_3$ solution into the medium.

Protein-free chemical defined in-house media were used for the fed-batch culture. For the fed-batch operations, continuous feeding or bolus feeding methods were used. In continuous feeding, feed medium containing glucose and nutrients was continuously fed based on the glucose concentration to maintain the glucose concentration at a target level of 2 g/L. In the bolus feeding method, the same feed medium was used based on the glucose

concentration once every two or three days to maintain a glucose concentration of > 2 g/L.

2.2.2 Cell line and cell culture conditions of shake flasks

CHO-S host cells (Life Technologies, CA, USA) were used to develop osmotic resistant CHO host cells. In the fed-batch study, we used GS-CHO cells, but we changed the CHO host cell to CHO-S for the adaptation experiments because of the limitations of the host cell license. CD-CHO medium (Life Technologies, CA, USA) containing 8 mM glutamine and NaCl were used to develop osmotic resistant CHO host cells using repeated batch cultivation and developed CHO cell lines were evaluated using batch cultivation. Culture media with different osmolalities were prepared by varying the amount of NaCl added. A sterile 2 M NaCl solution was added into medium to increase the osmolality to the target level. For batch culture evaluation, CHO-S and osmotic resistant CHO cells in the exponential growth phase were inoculated at 2.0×10^5 cells/mL into a 125 mL shake flask with a 40 mL working volume and the time-course of cell growth was analyzed. The cells were cultured at 37°C and 125 rpm in a humidified 5% CO_2 incubator.

2.2.3 Viable cell density, viability, productivity and osmolality

Viable cell density and viability were measured using Vi-Cell Automated Cell Counters (Beckman Coulter, CA, USA). Antibody concentration was measured using Protein A HPLC. Medium osmolality was measured by Vapor Pressure Osmometer (WESCOR, UT, USA) for the fed-batch cultures and by an osmometer (Arkray Osmostat OM-6040, Arkray, MN, USA) for the batch cultures. Both osmometers used the freezing point method for measurement.

2.3 RESULTS AND DISCUSION

2.3.1 Comparison of cell culture performance with continuous feed and bolus feed methods

To establish a simplified platform cell culture method, the feeding method was changed from continuous feed to bolus feed. Figure 4 shows the comparison between continuous and bolus fed-batch cultures. Recombinant GS-CHO cell line A producing human monoclonal antibody was used in both cultures. The continuous feed and bolus feed began at 44 hours after inoculation to maintain a glucose concentration > 2 g/L. Glucose was selected as a surrogate to represent the overall nutrient consumption. In the continuous feed culture, the feed rate was determined by the glucose consumption rate and the pre-determined next sampling time was based on maintaining a glucose concentration between 2 and 3 g/L. In the bolus feed culture, a fixed feed volume was added when the glucose concentration was predicted to be < 2 g/L at the day after sampling. The glucose concentration was maintained between 2 and 10 g/L (Figure 4A). GS-CHO cell line A with the continuous feeding method reached stationary phase at 187 hours after inoculation and a maximum cell concentration of 19.6 × 10^6 cells/mL, whereas with the bolus feeding method cell growth was suppressed after 115 hours and the viable cell concentration was unchanged until 211 hours

after inoculation. The maximum cell concentration was 11.1 × 10^6 cells/mL with the bolus feeding method (Figure 4B). The maximum cell concentration of the GS-CHO cell line A with bolus feeding was 55% of the control culture with continuous feeding. The culture osmolality gradually increased in the continuous feed culture (Figure 4C) to > 440 mOsm/kg at 115 hours and 524 mOsm/kg at 335 hours after inoculation. A significant difference in viable cell density between the continuous and bolus fed-batch cultures was observed at 115 hours after inoculation. However, there was no difference in the viability of the GS-CHO cell line A with continuous feeding or bolus feeding throughout the culture period. The viability was 82.1% for continuous feeding and 76.9% for bolus feeding at 355 hours (Figure 4D). The Integral Viable Cell Concentration (IVC) at 355 hours after inoculation was 2.04 × 10^8 cells·hour/mL for the continuous feeding method and 1.11 × 10^8 cells·hour /mL for the bolus feeding method (Figure 4E). The IVC with bolus feeding was 54% of the control culture with continuous feeding. The cell-specific productivity, q_p, was calculated as the antibody concentration divided by the IVC over the culture duration (Figure 4F). The q_p at 355 hours was 1.49 pg/cell/hour for the continuous feeding method and 2.08 pg/cell/hour for the bolus feeding method. These results showed that increased osmolality in the bolus feed method led to a 40% increase in q_p at 355

hours compared with the control culture with continuous feeding. However, the increase of q_p did not result in a substantial increase in the final monoclonal antibody (mAb) concentration. The final mAb concentrations in the bolus-fed and continuous-fed cultures were 5.6 g/L and 7.3 g/L, respectively (Figure 4G). The final mAb concentration was 77% of the control culture product concentration because of suppressed cell growth after 115 hours and a 46% decrease of IVC over the culture duration compared with the control culture with continuous feeding. The final mAb concentration might be increased using the bolus feed method if cell growth is not be suppressed by increased osmolality (> 450 mOsm) and if the IVC is increased over the culture duration with a higher q_p compared with the continuous feed method.

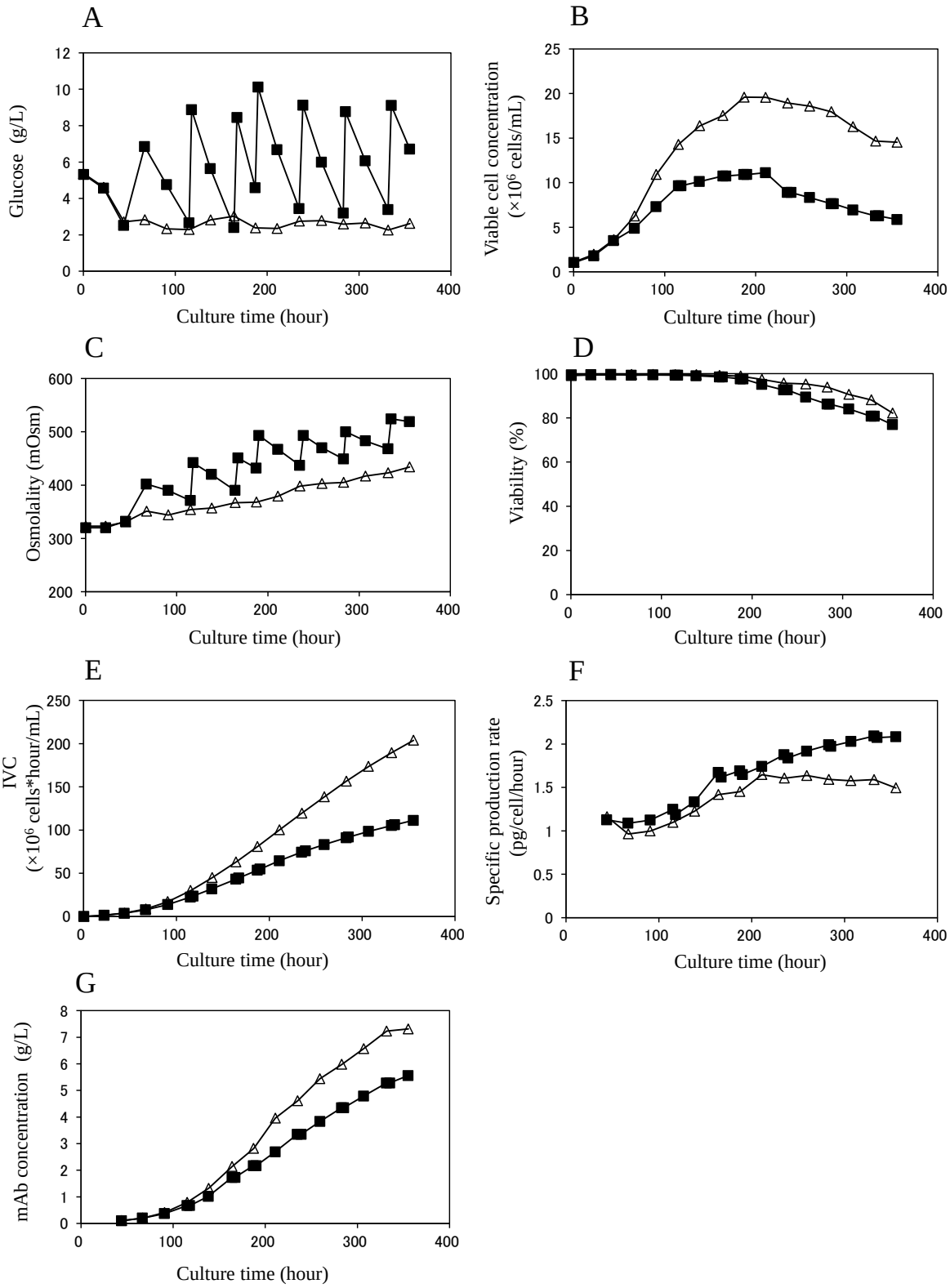

FIG. 4. Comparison of cell culture performance with continuous feed and bolus feed methods using a cell line expressing mAb. (A) Glucose, (B) viable cell

concentration, (C) osmolality, (D) viability, (E) Total Integral Viable Cell Concentration (IVC), (F) specific production rate and (G) mAb concentration. Closed squares represent cell culture with bolus feeding. Open triangles represent cell culture with continues feeding. The data was obtained from a single experiment.

2.3.2 Effect of elevated osmolality on viable cell concentration and viability of CHO host cells

To confirm the relationship between osmolality and cell growth, batch cultures were performed with various initial osmolality conditions (325–517 mOsm/kg) with CHO-S host cells in shake flasks (Figure 5A). Table 1 shows that the specific growth rate from day 3 to day 5 was significantly decreased under hyper osmolality conditions (> 450 mOsm/kg). The viable cell concentration was 6.6 × 10^6 cells/mL with 325 mOsm/kg on day 5, and the viable cell density was 2.05 × 10^6 cells/mL and 0.98 × 10^6 cells/mL, respectively at 456 and 517 mOsm/kg. The viable cell concentration was 31% and 15% of the control culture with 325 mOsm/kg. However, no significant decrease in cell viability was observed with hyper osmolality (> 450 mOsm/kg). The viability of the shake flask cultures was over 80% on day 12 after inoculation (Figure 5B).

Further batch cultures were performed with hyper osmolality conditions (> 450 mOsm/kg), passaged from previous cultures (Figure 5C). Shake flask cultures with 456 mOsm/kg were inoculated from a previous culture with 456 mOsm/kg on day 7 (Figure 5A). A shake flask culture with 517 mOsm/kg was also inoculated from a previous culture with 517 mOsm/kg on day 7 (Figure 5A). Cells with hyper osmolality conditions (> 450 mOsm/kg) had little or no growth

(Figure 5C). Surprisingly, no significant decrease in viability was observed with hyper osmolality (> 450 mOsm/kg) (Figure 5D). The viability of the shake flask cultures was > 80% on day 12 (Figure 5B) and day 7 (Figure 5D) after inoculation. These results confirm the data from a previous report that cell growth was suppressed by increasing osmolality, especially > 400 mOsm/kg, whereas viability was mostly not affected (25). Because viability was unaffected under hyper osmolality conditions suggests that hyper osmotic resistant CHO host cells can be established by adaptation using hyper osmolality medium (450 ± 10 mOsm/kg).

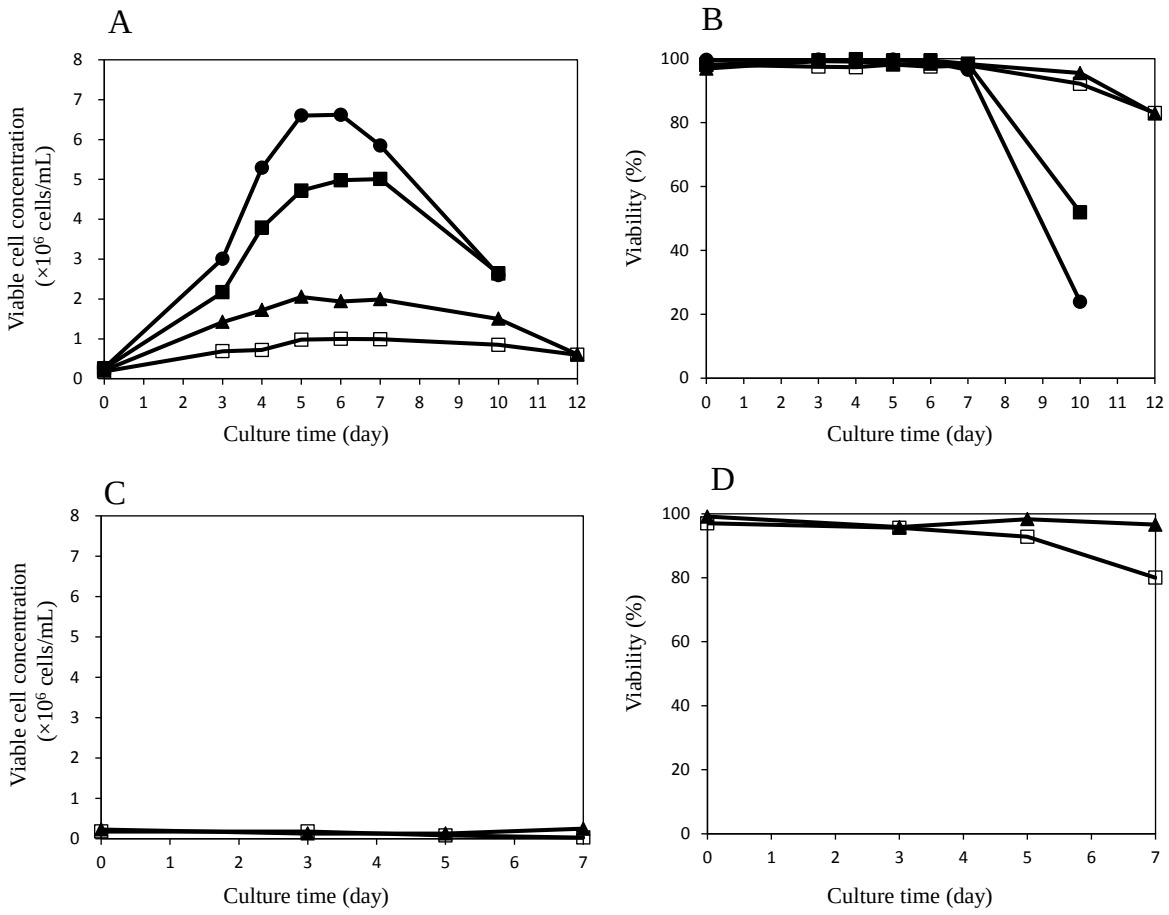

FIG. 5. Effect of elevated osmolality on (A) viable cell concentration and (B) viability of CHO host cells at first passage, (C) viable cell concentration and (D) viability of CHO host cells with the indicated osmotic stress at second passage. Cells from the culture at first passage with 456 mOsm/kg on day 7 were transferred into a new shake flask at second passage. The cells from the culture at first passage with 517 mOsm/kg on day 7 were transferred into a new shake flask at second passage. Closed circles represent 325 mOsm/kg culture. Closed squares represent 391 mOsm/kg culture. Closed triangles represent 456

mOsm/kg culture. Open squares represent 517 mOsm/kg culture. The data was obtained from a single experiment.

TABLE 1. Comparison of viable cell concentration and specific growth rate of CHO-S host cells under different osmolality conditions.

Osmolality (mOsm/kg)	VCC on day 0 (1×10^6 cells/mL)	VCC on day 3 (1×10^6 cells/mL)	VCC on day 5 (1×10^6 cells/mL)	Specific growth rate from day 0 to 3 (h^{-1})	Specific growth rate from day 3 to 5 (h^{-1})
325	0.27	3.01	6.6	0.0335	0.0164
391	0.26	2.17	4.72	0.0295	0.0162
456	0.21	1.42	2.05	0.0265	0.0076
517	0.18	0.69	0.98	0.0187	0.0073

VCC, viable cell concentration.

2.3.3 Establishment of hyper osmotic resistant CHO host cells

To establish hyper osmotic resistant CHO host cells, cells from a previous culture with 456 mOsm/kg on day 7 were transferred into a new shake flask with 446 mOsm/kg medium. Then, the cells were passaged more than 10 times in hyper osmotic basal media (450 ± 10 mOsm/kg). The cultivation period for one passage was 7–10 days in the early adaptation phase (3–5 passages) and 3–4 days in the late adaptation phase. In the late phase, the cells grew faster compared with 2^{nd} passage cells (non-adaptation phase) in hyper osmolality medium (450 ± 10 mOsm/kg).

In the 10^{th} passage, cells were inoculated at 3×10^5 cells/mL in hyper osmolality medium (458 mOsm/kg) and grew to 25×10^5 cells/mL on day 4 after inoculation (data not shown). After 10 passages with hyper osmolality medium (450 ± 10 mOsm/kg), the osmotic resistant CHO-S, named CHO-S-OR host cells were established.

2.3.4 Comparison of viable cell concentration and viability of CHO-S host cells and CHO-S-OR host cells under different osmolality conditions

To evaluate the differences in cell growth and viability between CHO-S-OR host cells and CHO-S host cells as controls, batch cultures were performed using three different levels of osmolality (Figure 6). CHO-S-OR host cells were inoculated after the 15^{th} passage with hyper osmolality medium (450 ± 10 mOsm/kg) for the adaptation period. Figure 6A and 3B show the results of batch cultures with iso-osmolality medium (328 mOsm/kg). Surprisingly, the maximum viable cell concentration of CHO-S-OR host cells was 130% of the CHO-S host cells (control) with 328 mOsm/kg medium. The maximum viable cell concentrations of CHO-S-OR host cells and CHO-S host cells were 9.1 × 10^6 cells/mL and 7.0 × 10^6 cells/mL, respectively (Figure 6A). The CHO-S-OR host cells died more slowly compared with the control CHO-S host cells (Figure 6B). Figure 6C and D show the result of batch cultures with increased osmolality medium from 328 mOsm/kg to 465 ± 3 mOsm induced by osmotic stress on day 3 after inoculation. In a previous study, fed-batch cultures typically showed increased osmolality by feeding on day 3 after inoculation (data not shown). Therefore, osmotic stress was induced on day 3 after inoculation. Figure 6C shows CHO-S host cells reached a maximum viable cell concentration (5.7 ×

10^6 cells/mL) on day 4, which decreased after day 4, whereas the cell growth of CHO-S-OR host cells was not suppressed after day 4 and reached a maximum viable cell concentration (7.5×10^6 cells/mL) on day 6. The maximum viable cell concentration of CHO-S-OR host cells was 132% of control CHO-S host cells. The viabilities of CHO-S host cells and CHO-S-OR host cells were 95.1% and 92.1% on day 6 (Figure 6D). Figure 6E and F show the results of batch cultures with hyper osmolality medium (465 ± 3 mOsm/kg). CHO-S host cells reached a maximum viable cell concentration (3.0×10^6 cells/mL) on day 4 and this decreased after day 5, whereas the cell growth of CHO-S-OR host cells was not suppressed after day 4 and reached a maximum viable cell concentration (4.3×10^6 cells/mL) on day 7. The maximum viable cell density of CHO-S-OR host cells was 143% of CHO-S host cells. The viabilities of CHO-S host cells and CHO-S-OR host cells were 94.8% and 97.1% on day 6 (Figure 6F).

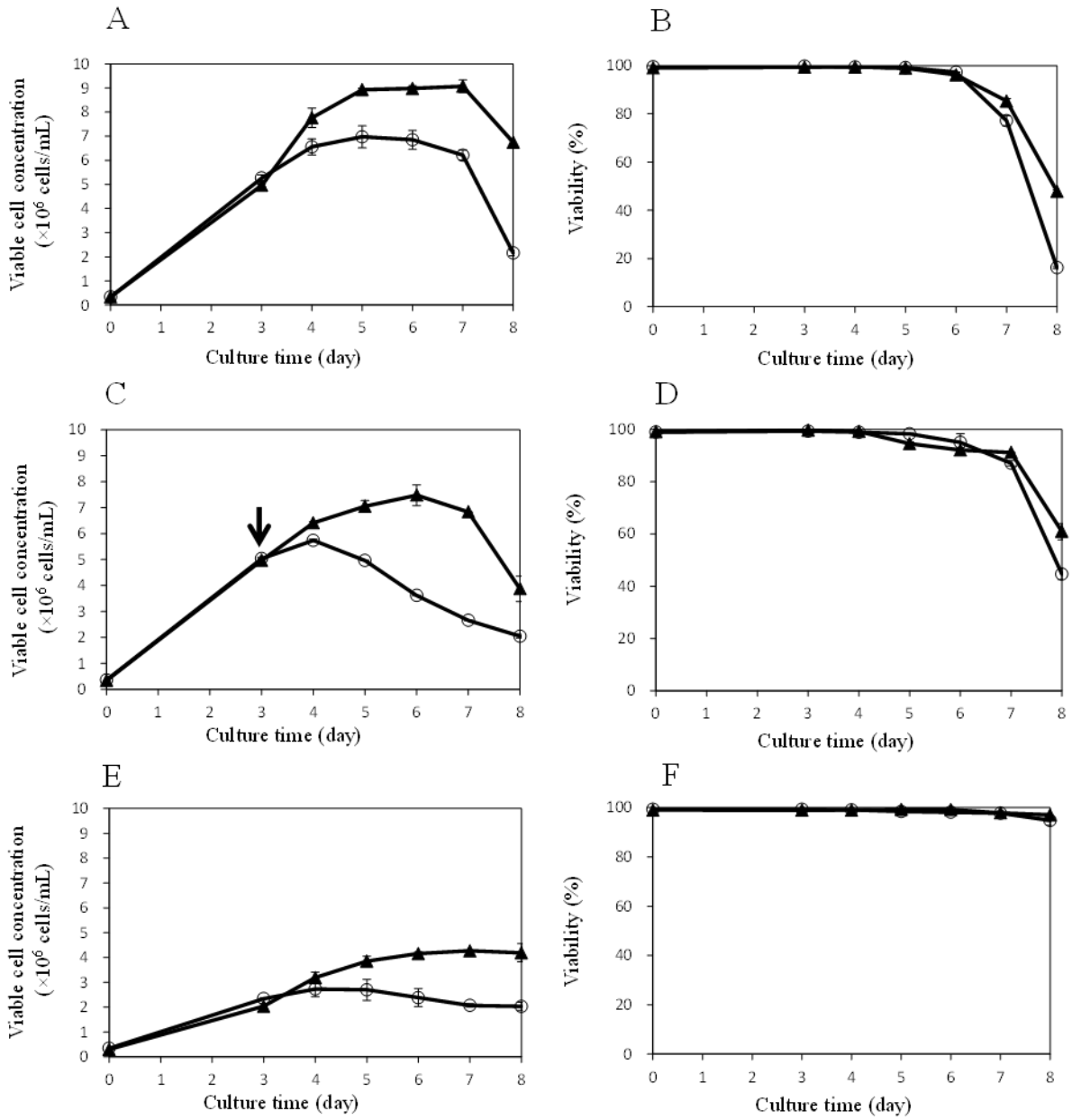

FIG. 6. Comparison of viable cell concentration and viability of CHO-S host cells and CHO-S-OR host cells under different osmolality conditions. (A) Viable cell concentration and (B) viability in batch culture with iso-osmolality medium. (C) Viable cell concentration and (D) viability of batch culture with elevated osmolality. The osmolality of the medium was increased from 328 mOsm/kg to

465 ± 3 mOsm/kg by the addition of NaCl on day 3 after inoculation. The arrow indicates the time point at which NaCl was added to the iso-osmolality medium. (E) Viable cell concentration and (F) viability of the batch culture with hyper osmolality medium (465 ± 3 mOsm/kg). Open circles represent viable cell concentration and viability of CHO-S host cells. Closed triangles represent viable cell concentration and viability of CHO-S-OR host cells. Error bars indicate the standard deviations calculated from data obtained two shake flasks. The data was obtained from a single experiment.

2.3.5 Metabolic profiling of CHO-S host cells and CHO-S-OR host cells with or without osmotic stress

To understand the metabolic difference between CHO-S host cells and CHO-S-OR host cells, metabolic analyses were conducted. Samples for metabolic analysis were taken on day 5 and day 7 with 328 mOsm medium (Figure 6A). The time points were selected based upon the stationary phase of CHO-S host cells and CHO-S-OR host cells. Additionally, samples were taken daily except for day 1 and day 2 with the initiation of osmotic stress on day 3 after inoculation (328 mOsm/kg to 465 ± 3 mOsm) (Figure 6C). Figure 7A and B show the metabolic profiling of sorbitol and erythritol, respectively. It is known that polyols or sugar alcohols such as sorbitol and erythritol function as osmolytes. During the initiation of osmotic stress on day 3 after inoculation, the yield of sorbitol and erythritol from CHO-S-OR host cells were 2.7 times and 1.9 times higher, respectively compared with CHO-S host cells on day 7. Of note, the yield of sorbitol and erythritol from CHO-S-OR host cells were 3.9 times and 2.1 times higher, respectively compared with CHO-S host cells on day 7 with iso-osmolality medium (328 mOsm/kg). These different osmolyte productions suggest that the CHO-S-OR host cells have a greater capacity to generate osmolytes and handle osmotic stress. As shown in Figure 7C, the yield

of oxidized glutathione (GSSG) from CHO-S host cells was 3.9 times higher compared with CHO-S-OR host cells on day 6 after the initiation of osmotic stress on day 3. In iso-osmolality medium (328 mOsm/kg), the yield of oxidized glutathione (GSSG) from CHO-S host cells was 2.9 times higher compared with CHO-S-OR host cells on day 5. GSSG is generated when glutathione (GSH) acts as an antioxidant to reduce reactive species, thereby exerting a tight control on the redox status (45). GSSG is subsequently extruded from the cell by pumps, and when GSH is not replenished quickly, its depletion is often associated with the onset of apoptosis (46, 47). The increased extracellular GSSG level of CHO-S host cells might indicate higher amounts of free radicals and/or greater reactive oxygen/nitrogen species scavenging. This reflects the increased oxidative stress in CHO-S host cells and suggests that CHO-S-OR host cells handle stress better than CHO-S host cells. The difference in the yield of GSSG between CHO-S host cells and CHO-S-OR host cells as shown in Figure 7C might explain the results shown in Figure 6A and C. The increased level of GSSG from CHO-S host cells was higher than in CHO-S-OR host cells (Figure 7C). The increased level of extracellular GSSG might decrease the intracellular GSH levels in CHO-S host cells. Previous observations showed that cellular GSH depletion reduced the rate of cell proliferation in human lungs (48) and

colon carcinoma cells (49), as well as in rodent fibroblasts (50, 51). Schnelldorfer et al also showed that GSH depletion inhibited cell growth and enhanced apoptosis in pancreatic cancer cells (52). Decreased intracellular GSH in CHO-S host cells might cause the inhibition of cell growth. However, increased levels of GSSG in CHO-S-OR host cells were lower than in CHO-S host cells (Figure 7C). This suggests that intracellular GSH in CHO-S-OR host cells might be higher than that in CHO-S host cells, which might explain why the maximum viable cell concentration of CHO-S-OR host cells was higher than CHO-S host cells (Figure 6A and C). Furthermore, the viabilities of CHO-S-OR host cells and CHO-S host cells were 47.8% and 16.2% on day 8, (Figure 6B) and 60.9% and 44.7% on day 8 (Figure 6D). Chong et al. showed that the addition of GSSG to CHO cell line expressing mAb cells resulted in a 3.9-fold increase in caspase activity (36). Apoptosis is a form of programmed cell death executed by a family of cysteine proteases known as caspases (53, 54). The increased level of GSSG in CHO-S host cells was higher than in CHO-S-OR host cells with iso-osmolality medium (328 mOsm/kg) and the initiation of osmotic stress on day 3 (Figure 7C). The increased level of extracellular GSSG of CHO-S host cells might have induced caspase activity and decreased cell viability on day 8.

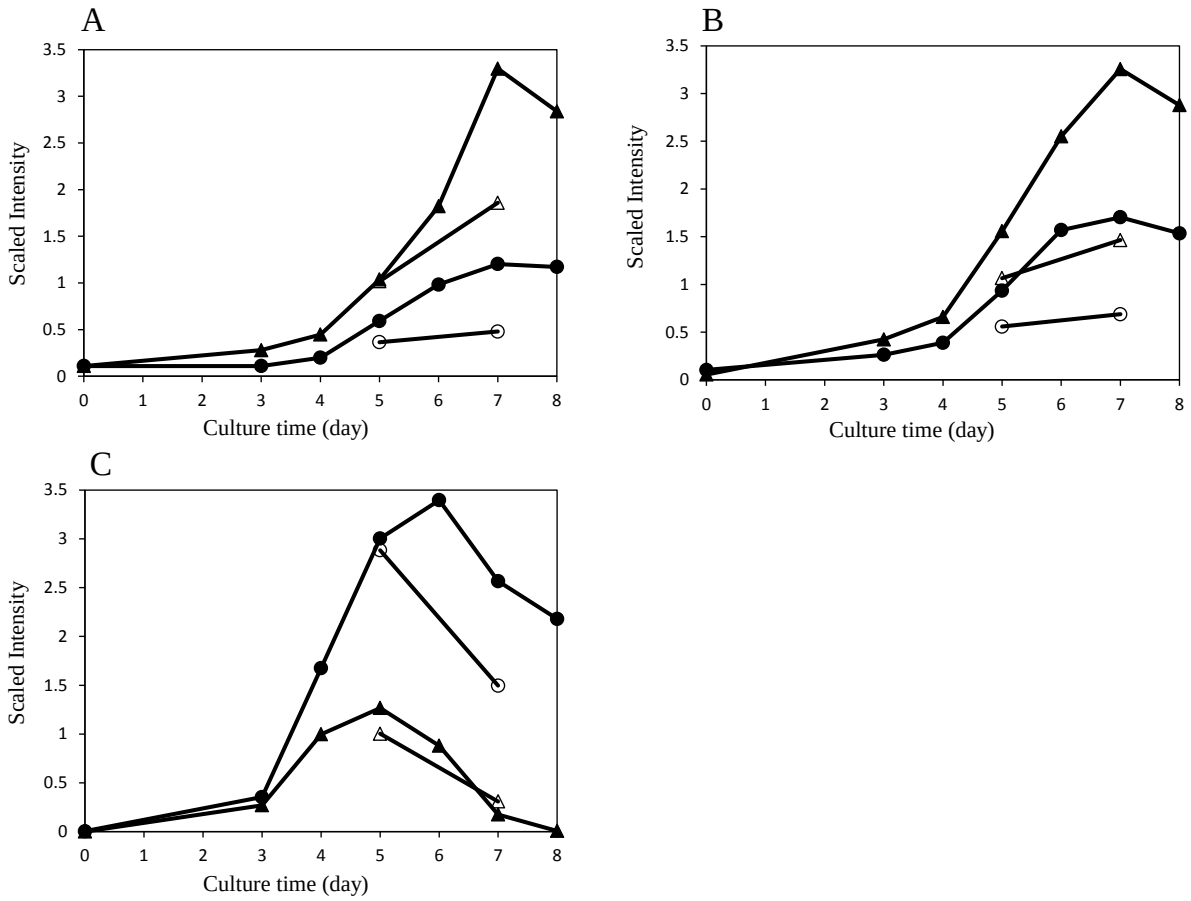

FIG. 7. Metabolic profiling for (A) sorbitol, (B) erythritol and (C) oxidized glutathione (GSSG) from CHO-S host cells and CHO-S-OR host cells with or without osmotic stress. Closed triangles represent the metabolic profile of CHO-S-OR host cells with osmotic stress. The osmolality of the medium was increased from 328 mOsm/kg to 465 ± 3 mOsm/kg on day 3 after inoculation. Closed circles represent the metabolic profile of CHO-S host cells with osmotic stress. The osmolality of the medium was increased from 328 mOsm/kg to 465 ± 3 mOsm/kg on day 3 after inoculation. Open triangles represent the metabolic

profile of CHO-S-OR host cells with iso-osmolality medium (328 mOsm/kg). Open circles represent the metabolic profile of CHO-S host cells with iso-osmolality medium (328 mOsm/kg). The data was obtained from a single experiment.

2.3.6 Stability of hyper osmolality resistance of CHO-S-OR host cells with iso-osmolality basal medium

After the 20th passage, a cell bank of CHO-S-OR host cells was established. To confirm the stability of hyper osmolality resistance, the CHO-S-OR host cell bank was thawed and passaged five or seven times in iso-osmolality basal medium before the batch cultures were performed. These numbers of passages were determined based on the minimal passages required for pilot production culture and commercial production culture, respectively. After five passages with iso-osmolality basal medium (320 ± 5 mOsm/kg), one batch culture was initiated with osmotic stress on day 1 after inoculation (323 mOsm/kg to 446 mOsm/kg) (Figure 8A) and another batch culture was simultaneously initiated with osmotic stress on day 3 (323 mOsm/kg to 446 mOsm/kg) (Figure 8C). The timings of osmotic stress induction were used to evaluate the effect of osmotic stress on the cell growth and viability of CHO-S host cells and CHO-S-OR host cells in the early or middle growth phase. After the initiation of osmotic stress on day 1 (early growth phase), CHO-S host cells reached a stationary phase on day 3 and a maximum viable cell concentration of 2.4 × 10^6 cells/mL, whereas the cell growth of CHO-S-OR host cells was not suppressed after day 3 and reached a maximum viable cell concentration (5.1 × 10^6 cells/mL) on day 6. The

maximum viable cell concentration of the CHO-S-OR host cells was 213% of CHO-S host cells. The viabilities of CHO-S host cells and CHO-S-OR host cells were 93.3 % and 94.0% on day 8 (Figure 8B). After the initiation of osmotic stress on day 3 (middle growth phase), CHO-S host cells reached a maximum viable cell concentration (5.2×10^6 cells/mL) on day 4, which declined on day 5, whereas the cell growth of CHO-S-OR host cells was not suppressed after day 4 and reached a maximum viable cell concentration (6.3×10^6 cells/mL) on day 5, which then declined on day 8 (Figure 8C). The maximum viable cell concentration of CHO-S-OR host cells was 121% of CHO-S host cells. The viabilities of CHO-S host cells and CHO-S-OR host cells were 84.5% and 82.5% on day 8 (Figure 8D). These data had similar profiles to the data in Figure 6C and D. After the initiation of osmotic stress on day 3, CHO-S host cells reached a maximum cell concentration on day 4, which decreased on day 5, whereas the viable cell concentration of CHO-S-OR host cells increased after day 4 (Figure 6C and Figure 8C). The viability of CHO-S host cells and CHO-S-OR host cells was > 90% on day 6, which indicated the osmotic stress on day 3 did not directly affect cell viability (Figure 6D and Figure 8D). After seven passages with iso-osmolality basal medium (320 ± 5 mOsm/kg), batch cultures were started with the initiation of osmotic stress on day 1 after

inoculation (328 mOsm/kg to 457 mOsm/kg) (Figure 8E). The maximum viable cell concentration of CHO-S host cells was 3.1×10^5 cells/mL on day 4, compared with 5.5×10^5cells/mL for CHO-S-OR host cells on day 6 after the induction of osmotic stress. The maximum viable cell concentration of CHO-S-OR host cells was 177% of CHO-S host cells. The viabilities of CHO-S host cells and CHO-S-OR host cells were 98.5% and 96.3% on day 7, respectively (Figure 8F). This study demonstrated that osmotic resistance was maintained in CHO-S-OR host cells even after seven passages in iso-osmolality basal medium.

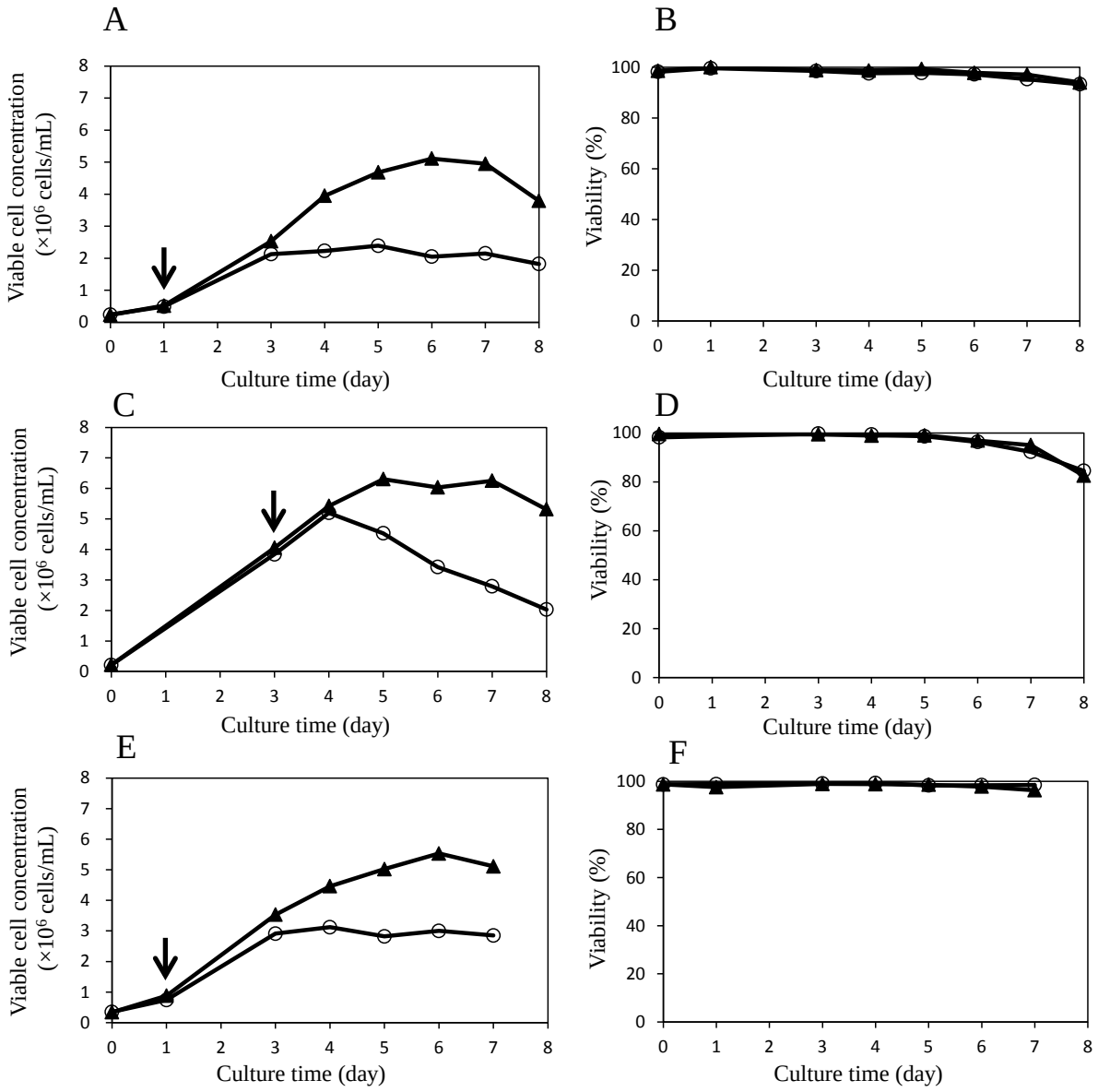

FIG. 8. Comparison of viable cell concentration and viability of CHO-S host cells and CHO-S-OR host cells under different osmolality conditions after five passages or seven passages with iso-osmolality basal medium. (A) Viable cell concentration and (B) viability of the batch culture with elevated osmolality after five passages in iso-osmolality basal medium. The osmolality of the

medium was increased from 323 mOsm/kg to 446 mOsm/kg by the addition of NaCl on day 1 after inoculation. (C) Viable cell concentration and (D) viability of the batch culture with increased osmolality after five passages in iso-osmolality basal medium. The osmolality of the medium was increased from 323 mOsm/kg to 446 mOsm/kg by the addition of NaCl on day 3 after inoculation. (E) Viable cell concentration and (F) viability of the batch culture with increased osmolality after seven passages in iso-osmolality basal medium. The osmolality of the medium was increased from 323 mOsm/kg to 457 mOsm/kg by the addition of NaCl on day 1 after inoculation. The arrow indicates the time point at which the NaCl was added to the iso-osmolality medium. Open circles represent viable cell concentration and viability of CHO-S host cells. Closed triangles represent viable cell concentration and viability of CHO-S-OR host cells. The data was obtained from a single experiment.

2.3.7 Summary

It has been widely reported that cell growth is suppressed under hyper osmotic medium (26, 27 and 55). Data from Figure 4 shows that markedly increased osmolality with the bolus feed method led to a 40% increase in q_p on day 15 compared with the control culture with continuous feeding. However, the increase of q_p did not result in a substantial increase in the final mAb concentration. Data from Figure 5 shows that cell growth was suppressed by increasing osmolality, especially > 450 mOsm/kg. In this study, hyper osmotic resistant CHO host cells were developed to overcome growth suppression effects by hyper osmotic pressure. CHO-S-OR host cells were established by adaptation with hyper osmolality medium. The cell growth of CHO-S host cells was dramatically affected by the induction of osmotic stress, whereas the cell growth of CHO-S-OR host cells appeared to be unaffected. The results of metabolic analysis between CHO-S host cells and CHO-S-OR host cells suggests that CHO-S-OR host cells have a greater capacity to generate osmolytes and handle osmotic stress. CHO-S-OR host cells might also express different levels of mRNAs, proteins and microRNAs related to osmotic resistance compared with CHO-S host cells. Shen et al. reported that the transporter systems were generally activated in response to elevated osmolality

and ionic strength in CHO cell cultures. They found that nine transporter genes were significantly regulated and the majority of these genes were upregulated. These genes included lipid transporter (*Cd36*), amino acid transporter (*Slc38a2*), protein transporter (*Stx3*) and ion transporters (*Slco4a1*). In addition, sodium/potassium ATPase, Atp 1b3, was upregulated (26). In CHO-S-OR host cells, the different regulation of these transporters may contribute to handle osmotic stress compared with CHO-S host cells. The analysis of extracellular GSG and GSSG levels in CHO-S host cells and CHO-S-OR host cells was used to determine the difference in cell growth (Figure 6A and C). The use of hyper CHO-S-OR host cells to create a mAb production cell line should generate higher final antibody concentrations compared with a mAb production cell line established from CHO-S host cells (non-adaptation with hyper osmolality medium) because the cell growth of a mAb production hyper osmotic resistance cell line will not be suppressed in response to increased osmolality after bolus feeding; thus the IVC will increase over the culture duration. Moreover, the specific production rate might increase under increased osmolality medium as shown in Figure 4F.

In conclusion, CHO-S-OR host cells were established by adaptation using hyper osmolality medium (450 ± 10 mOsm/kg). The cell growth of CHO-S-OR

host cells was affected less by osmotic stress compared with CHO-S host cells. Metabolic analysis showed that CHO-S-OR host cells displayed a preferential capacity to generate osmolytes such as sorbitol and erythritol. The difference in levels of extracellular GSSG between CHO-S host cells and CHO-S-OR host cells suggests that CHO-S-OR host cells handle stress better. Additionally, the osmotic resistance of CHO-S-OR host cells was maintained even after seven passages in iso-osmolality basal medium. The development of host cells that do not suppress cell growth in a hyperosmotic culture might be a potential solution to increase IVC and cell-specific productivity using the bolus feeding method with an increase in medium osmolality. Further experiments are required to validate these findings.

Chapter 3 General conclusion

To meet increasing biopharmaceutical demand in worldwide, development of high productivity of the cell culture platform process is very important. Currently, fed-batch process with bolus feeding is the most common cell culture method for industrial large-scale because of its simple operation. The simple manipulation of large-scale GMP manufacturing will contribute to reduce facility requirements, the risk of human error and contamination. In fed-batch culture process, concentrated nutrient are fed throughout the culture period to maintain high viable cell density and viability. However, the concentrated nutrient feed with bolus feed will cause rapidly increase of osmolality in medium. It is reported a decrease in the growth rate and an increase in the specific production rate of hyperosmotic (450 mOsm/kg) cultures compared with iso-osmotic (290 mOsm/kg) cultures (27).

In the present study, a significant difference in viable cell density between the continuous and bolus fed-batch cultures was observed. The IVC with bolus feeding was 54% of the control culture with continuous feeding. Conversely, the increased osmolality in the bolus feed method led to a 40% increase in the specific production rate (q_p) compared with the control culture**.** However, the final mAb concentration was 77% of the control culture product concentration because of decreased IVC. These results suggests that the final mAb

concentration might be increased using the bolus feed method if the cell growth is not be suppressed by increased osmolality and if the IVC is increased over the culture duration with a high q_p under hyper osmolality. Although the hyper osmotic resistant animal host cells might be available to improve productivity, few studies have investigated the development of host cells that do not suppress cell growth by hyper osmotic pressure.

Figure 9 schematically depicts the generation of recombinant protein expressing cell lines. In this study, hyper osmotic resistant CHO-S host cells (CHO-S-OR) were established by adaptation with hyper osmolality medium (450 ± 10 mOsm/kg). The maximum viable cell concentration of CHO-S-OR host cells was 132 % of the CHO-S host cells (parent host cells) by osmotic stress on day 3 after inoculation as shown in Figure 6C. In future step, a recombinant protein expression vector will be transfected into the CHO-S-OR host cells with hyper osmolality medium. The established recombinant protein expressing CHO-S-OR cells might have a greater capacity to generate osmolytes and handle osmotic stress.

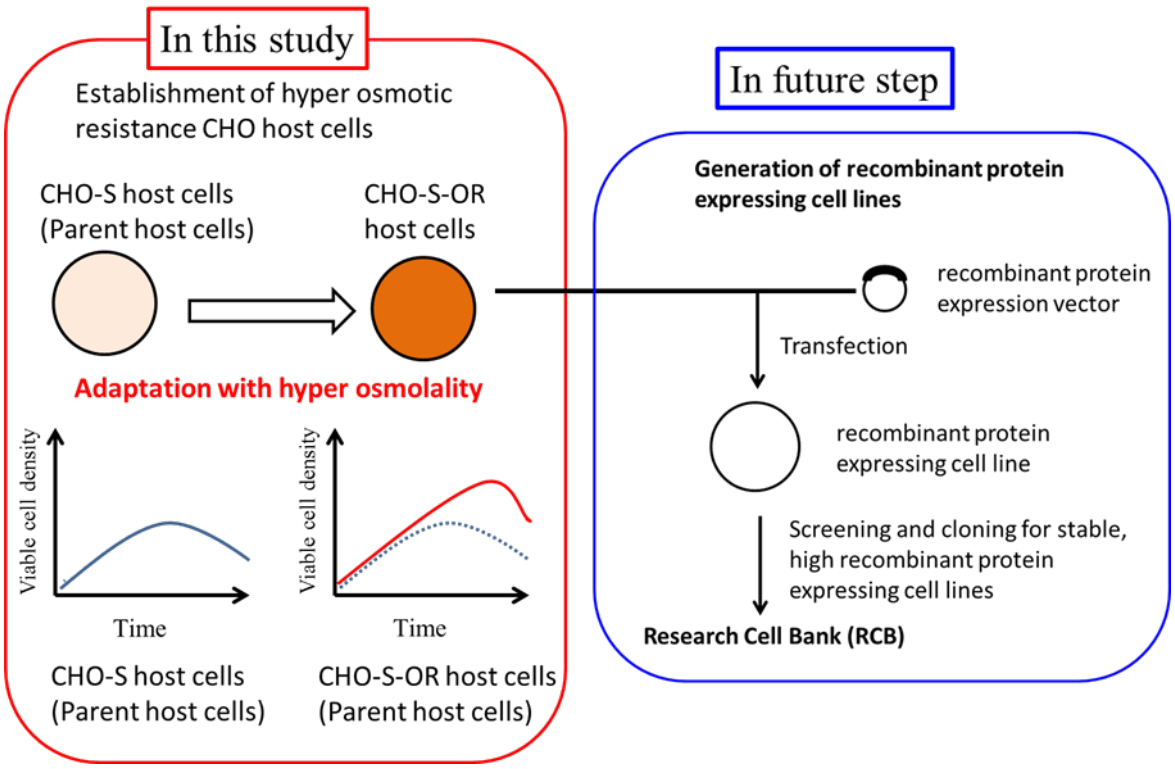

FIG. 9. Schematic depiction of the generation of recombinant protein expressing in this study and future step.

The osmotic resistance of CHO-S-OR host cells was maintained even after seven passages in iso-osmolality basal medium (320 ± 5 mOsm/kg). Seven passages will be required for expansion steps from the vial thaw to commercial production scale. This result implies that mAb production cell line by using CHO-S-OR host cells can be passaged with iso-osmolality basal medium in expansion step. After the expansion step, the mAb production cell line might not be suppressed under hyperosmotic stress with bolus feeding in production step.

Omasa et al. reported that product concentration can be approximated as a function of two parameters: the specie production rate and integral value of viable cell concentration (44). Golabgir et al. also reported the IVCC and peak VCC were identified as important factors for predicting final product concentrations (56). Figure 10 shows a new approach to increase the product concentration by using recombinant protein expressing hyper osmotic resistant CHO cell line. The recombinant protein production hyper osmotic resistant CHO cells will not be suppressed in response to increased osmolality in fed-batch culture. The IVC will increase throughout the culture period. Moreover, the specific production rate might increase under increased osmolality medium. Therefore, the use of hyper osmotic CHO host cells to create a recombinant protein production cell line should generate higher final product concentrations

compared with a recombinant production cell line established from CHO host cell (non-adaptation with hyper osmolality medium).

The new approach by using the hyper osmotic resistant host cells is expected to be used for industrial scale production of recombinant protein. In general, it will take time to generate the hyper osmotic resistant host cells by using the adaptation method. However, once the desirable host cells are established by adaptation method, the host cells will be able to use to create different recombinant protein expressing cell lines. The technologies developed in this study could be a powerful tool to improve product concentration in fed-batch culture. It will contribute to meet increasing market demand and reduce the cost of goods. Furthermore, the technologies might be available to generate recombinant protein production osmotic resistant mammalian cell lines.

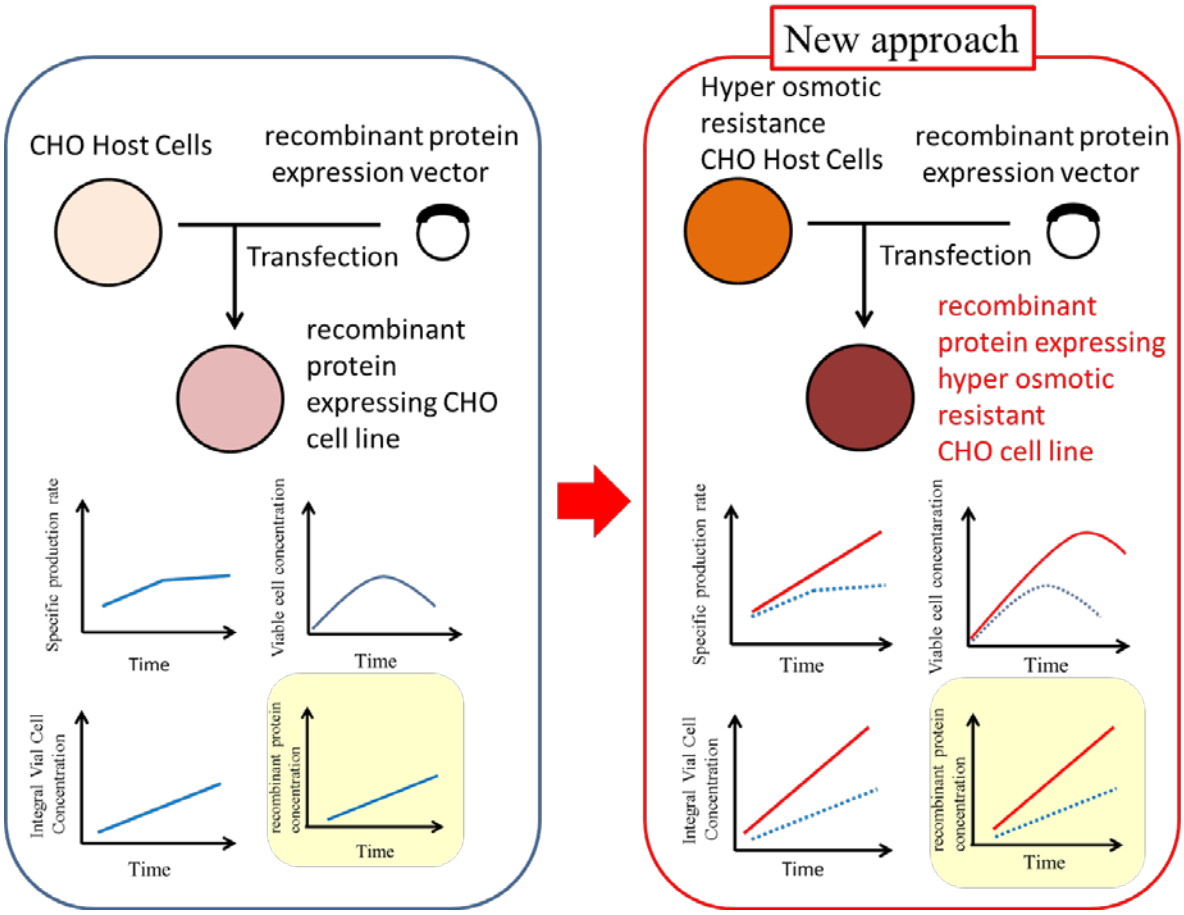

FIG. 10. Strategy of increase the product concentration by using recombinant protein expressing hyper osmotic resistant CHO cell line.

Genetic engineering is a more direct approach for host-cell improvement. The timeline for generation of desirable CHO host cells with genetic engineering will be shorter than conventional adaptation method if the target genes are identified. To establish hyper osmotic resistant CHO host cells with genetic engineering method, overexpression and repression of some genes related with osmotic stress will be needed. The analysis of DNA array, microRNA array and proteome analysis with both CHO-S host cells and CHO-S-OR host cells might be available to determine these candidate genes to establish hyper osmotic resistant CHO host cells by using genetic engineering method contain microRNA. The hyper osmotic resistant animal host cells might contribute to enhance cell performance, i.e. increase cell growth, IVC, recombinant protein production under hyper osmotic pressure in bolus fed batch culture.

Currently, a more simplified platform cell culture process is required for industrial production to reduce the risk of microbial contamination and operation failure by human error. If the number of bolus feed timing is reduced throughout fed-batch culture period, which will contribute to establish a simplified platform cell culture process. To reduce the number of feed timing throughout fed-batch culture period, more amount of feed volume will be needed at once time to avoid nutrient depletion compared with conventional feed volume. More amount

of feed volume at once time will cause dramatically increase osmolality in the medium. The hyper osmolality will suppress the cell growth and IVC will decrease throughout culture period. The use of hyper osmotic resistance animal host cells to create recombinant proteins including mAb production cell line might be a new approach to establish a more simplified platform cell culture process for large-scale GMP manufacturing. Further experiments are required to validate these hypotheses.

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