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ORIGINAL RESEARCH PAPER

Culture temperature modulates monoclonal antibody charge variation distribution in Chinese hamster ovary cell cultures

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Abstract
Objective To investigate the effect of lowering culture temperature on monoclonal antibody charge variation distribution in Chinese hamster ovary cell cultures.
Results In both batch and fed-batch cultures, lowering the culture temperature decreased the antibody acidic variant levels. The acidic variant levels (defined as variants eluting earlier than the main peak of an antibody during HPLC) at 32 °C were about 10 % lower than those at 37 °C at the end of both batch and fed-batch cultures. Additionally, lowering the culture temperature increased the lysine variant level, which further increased basic variant level. The lysine variant levels at 32 °C were about 8 % (batch culture) and 3 % (fed-batch culture) higher than those at 37 °C at the end of cultures. Real-time PCR results suggests that the decrease in carboxypeptidase B transcription level might be partially responsible for the increased lysine variant level at sub-physiological temperatures.
Conclusion Culture temperature exhibits noticeable impact on antibody charge variation distribution, especially the acidic variants and lysine variants.

Keywords Basic carboxypeptidase · Charge variation · Chinese hamster ovary cells · Culture temperature · Lysine variation · Monoclonal antibodies · Weak cation exchange chromatography

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Introduction

Therapeutic monoclonal antibodies (mAbs) derived from mammalian cells are currently one of the best-selling and fastest-growing product classes within the biopharmaceutical market (Aggarwal 2014). To achieve robust cell culture process for mAbs with high quantity and quality, the effects of various process parameters and media components on cell growth, mAb production and product quality have been

extensively studied (Aghamohseni et al. 2014; Borys et al. 2010).

Temperature is a critical process parameter during culture of mammalian cells. It has a direct influence on cell growth, nutrient consumption and productivity (Fan et al. 2010; Marchant et al. 2008). Moreover, temperature is to be associated with product quality, including glycosylation patterns (Ahn et al. 2008), aggregation (Gomez et al. 2012) and cysteine variants (Gomez et al. 2010). However, the effect of culture temperature on mAb charge variation and the mechanism of how hypothermic condition affects charge variation are rarely known.

Charge heterogeneity is commonly observed during mAb manufacturing processes and has gained increasing attention due to its potential influence on product stability and biological activity (Khawli et al. 2010). Isomerization of Asp102 of one heavy chain, which generated a basic variant, led to approx. 70–88 % decrease in bioactivity (Harris et al. 2001). Since charge heterogeneity is mainly generated by post-translational modifications (PTMs) and chemical degradations during culture process, it is expected to be influenced by certain process parameters.

In this study, we investigated the effect of lowering culture temperature on mAb charge variation distribution, especially the lysine variant, in both batch and fed-batch cultures of a Chinese hamster ovary (CHO) cell line.

Materials and methods

Cell line and cell culture

A CHO cell line producing a chimeric anti-CD20 monoclonal antibody was used in this study (Sun et al. 2013). The basal medium was a proprietary chemically defined medium derived from a mixture of DMEM/F12 (1:1, v/v) supplemented with ethanolamine, sodium selenate, cholesterol, Pluronic F68, vitamins, trace metals, and sodium bicarbonate. Cultures were grown in 250 ml shake-flasks with 80 ml medium. Exponentially growing seed cells were harvested by centrifugation ($300\times g$ for 5 min) and inoculated in fresh medium at 10^6 cells/ml. For batch cultures, the temperature was initially controlled at 37 °C and shifted to 32 or 35 °C on day 3. For fed-batch cultures, a proprietary chemically-defined feed medium was added daily at 2 % (v/v) from day 2 and the temperature shift was performed on day 8. Sampling was performed daily to determine viable cell density and the supernatant was kept at −80 °C for further analysis. The sample pH was measured immediately after sampling using a pH meter.

Routine analytical methods

Viable cell density was determined with a hemacytometer, and viable cells were distinguished from dead cells using the Trypan Blue dye exclusion method. The mAb concentration in supernatant was determined by Protein A HPLC assay. The specific mAb production rate (q_{mAb}) was calculated from a plot of the cumulative mAb concentration against the integral of viable cell concentration with time (*IVCC*) (Yoon et al. 2003a).

Weak cation exchange chromatography (WCX) analysis

WCX was conducted to determine the mAb charge variation distribution. Before WCX analysis, mAb in the supernatant was purified by Protein A affinity chromatography. WCX was performed at room temperature on an Agilent 1260 HPLC system using a ProPac WCX-10 column, 4 × 250 mm. Mobile phase A was 10 mM Na_2HPO_4, pH 7.5, and mobile phase B was 100 mM NaCl in 10 mM Na_2HPO_4, pH 7.5. A linear gradient elution from 30 to 75 % B over 20 min was used at 1 ml/min to separate mAb charge variant with detection at 280 nm. The level of each charge variant was expressed as the percentage of total peak area.

Real-time PCR analysis

The mRNA levels of basic carboxypeptidases were quantified by real-time PCR analysis. Total mRNA was extracted with Trizol. The primers used for real-time PCR were as follows: GAPDH, 5′-AGGTTGT CTCCTGCGACTTCA-3′ and 5′-GAGGTCCACCA CTCTGTTGCT-3′; carboxypeptidase B, 5′-CAGCG AGAATCCACAACTC A-3′ and 5′-ATCAATGTT-GACCACAGGC A-3′; carboxypeptidase H, 5′-TG TGCTCTCCGCCAATCT-3′ and 5′-AACTACTGTC ATCGTCGTTC TT-3′. Real-time PCR reactions were performed and monitored using the SYBR Green PCR

Master mix and the ABI Prism 7700 Sequence Detection System.

Results and discussion

Effect of culture temperature on cell growth and mAb production

Figure 1 shows the profiles of cell growth at different temperatures. In batch cultures, both cell density and viability were maintained higher at sub-physiological temperatures than those at 37 °C (Fig. 1a, b). However, in fed-batch cultures, no significant difference was found in cell growth and cell viability among different culture temperatures (Fig. 1c, d). Lowering culture temperature was reported to reduce nutrient consumption and extend culture longevity (Yoon et al. 2003b). Thus, in batch cultures, a more rapid cell death was expected in cultivations at 37 °C since cells were more likely to suffer from the deficiency of nutrient (which was alleviated in fed-batch cultures), when compared with those at sub-physiological temperatures. In fed-batch cultures, cells tended to aggregate and adhere to the sidewall of shake-flasks since high cell density was achieved, which led to a rapid decrease of viable cell density (viable cells in suspension were estimated only) while cell viability remained high (70 %) after day 8.

mAb production at different temperatures is shown in Table 1. In batch cultures, lowering culture temperature significantly increased mAb concentration, since the beneficial effect of lowering culture temperature on *IVCC* outweighed its detrimental effect on q_{mAb} (Table 1). Contrarily, in fed-batch cultures, mAb concentration was decreased with lowering culture temperature due to the rapid decreased in q_{mAb}. Lowering the temperature can improve specific productivity (Marchant et al. 2008); however, this effect varies between target proteins and among both cell lines and expression systems (Mason et al. 2014). In this study, q_{mAb} was decreased with lowering culture temperature in both batch and fed-batch cultures (Table 1).

Effect of culture temperature on mAb charge variation

Acidic variants are defined as the antibody variants that elute earlier than the main peak during WCX (HPLC) analysis. In both batch and fed-batch cultures, the acidic variant levels were increased throughout the

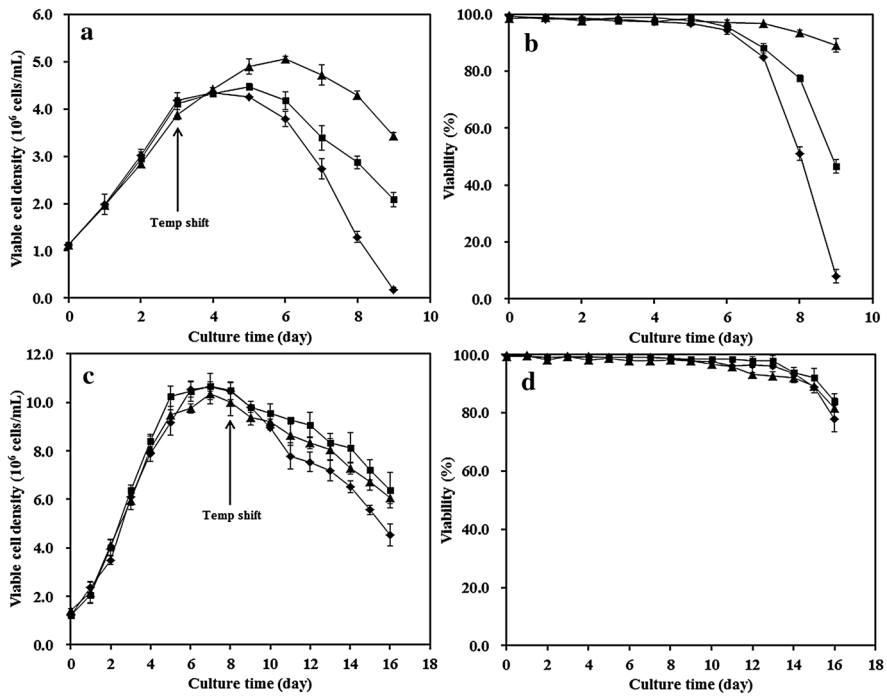

Fig. 1 Profiles of cell growth and cell viability at different culture temperatures. **a** Cell growth in batch culture; **b** cell viability in batch culture; **c** cell growth in fed-batch culture; **d** cell viability in fed-batch culture. (*filled triangle*) 32 °C; (*filled square*) 35 °C; (*filled diamond*) 37 °C. The *error bars* indicate the standard deviations from three independent experiments

Table 1 Effect of culture temperature on mAb production

Temperature (°C)	Batch culture			Fed-batch culture		
	32	35	37	32	35	37
mAb concentration (g/L)	0.59 ± 0.02*	0.51 ± 0.03	0.45 ± 0.02	2.44 ± 0.04*	3.01 ± 0.02*	3.53 ± 0.03
IVCC (10^9cell day/L)	34.3 ± 0.8*	29.7 ± 1.1*	25.9 ± 0.6	116.9 ± 3.2	127.8 ± 3.8	121.3 ± 4.5
q_{mAb} [mg/(10^9cells day)]	18.26 ± 0.32*	19.43 ± 0.41	20.91 ± 0.23	12.34 ± 0.62*	20.25 ± 0.53*	29.81 ± 0.86

Values are means ± standard deviations of three independent experiments

IVCC integral of viable cell concentration with time, q_{mAb} specific mAb production which was calculated from the cultures after temperature shift

* $p < 0.05$ relative to 37 °C

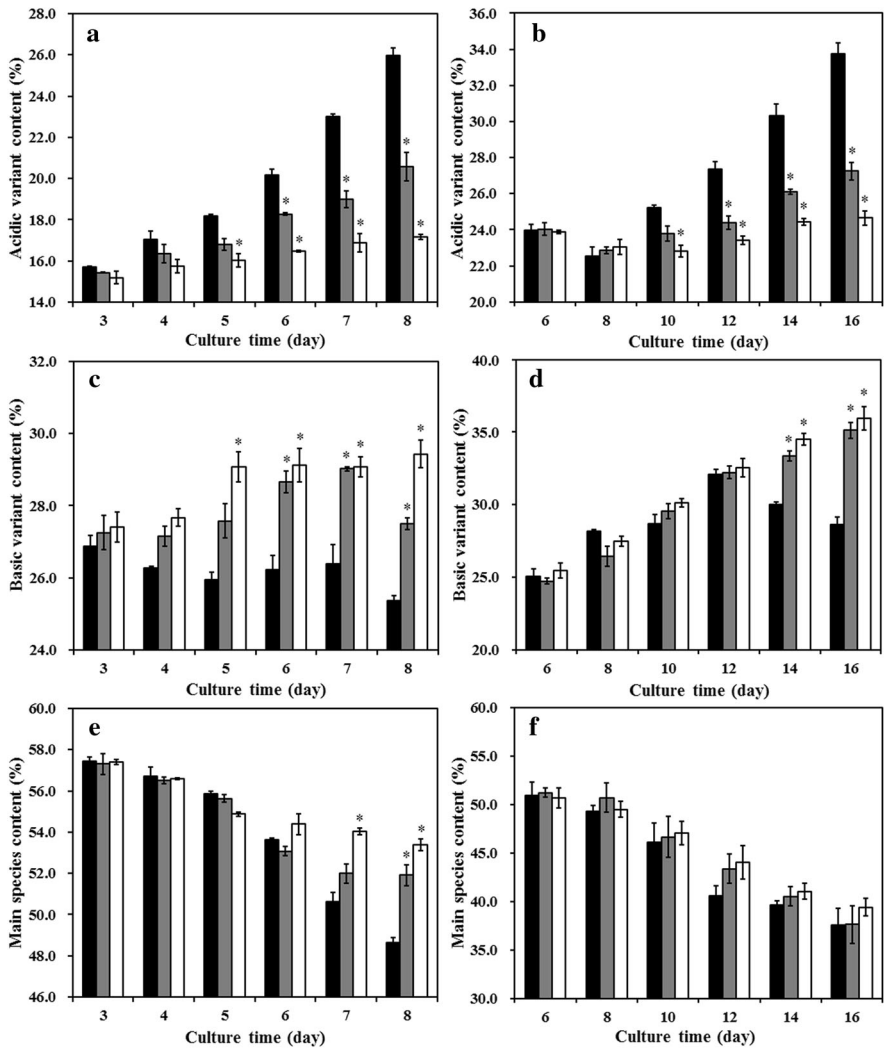

Fig. 2 Profiles of mAb charge variation distribution at different culture temperatures. **a** Acidic variant level in batch culture; **b** acidic variant level in fed-batch culture; **c** basic variant level in batch culture; **d** basic variant level in fed-batch culture; **e** main species level in batch culture; **f** main species level in fed-batch culture. (*white bar*) 32 °C; (*shaded bar*) 35 °C; (*black bar*) 37 °C. The *error bars* indicate the standard deviations from three independent experiments and *p* values were estimated by two-tailed Student's *t* test. *$p < 0.05$ relative to 37 °C

cultures regardless of culture temperature. Nevertheless, a higher temperature led to a higher level of acidic variant (Fig. 2a, b). At the end of both batch and fed-batch cultures, the acidic variant levels at 37 °C were about 10 % higher than those at 32 °C. Acidic charge variants were found to be generated by lots of modifications, such as glycation, glycosylation (sialic acid), deamidation and reduced disulfide bonds (Du

et al. 2012). Generally, more than one modification mentioned above will occur and lead to several acidic variants in one mAb, and the deamidation of asparagine is regarded as a major cause of acidic variants (Harris et al. 2001). Deamidation is a spontaneous modification and reported to strongly depend on pH and temperature during mAb purification and formulation (Pace et al. 2013). Therefore, high temperature is expected to accelerate the deamidation rate and further lead to a high acidic variant level during culture process. However, whether the deamidation of asparagine occurred in the present processes should be further examined and the detailed mechanism of how culture temperature affects such a modification is also required to be explored.

Basic variants are defined as the antibody variants that elute later than the main peak during WCX analysis. In batch cultures, the basic variant levels at three temperatures fluctuated between 25 and 29 % throughout the cultures, while those in fed-batch cultures were continually increased (from 25 to 35 %). Lowering culture temperature led to a higher level of basic variant in batch cultures (Fig. 2c) and the same phenomenon was found in the later course of fed-batch cultures (Fig. 2d). Basic charge variants were found to be generated by a number of modifications, such as *C*-terminal modifications (lysine cleavage and proline amidation), methionine oxidation and aspartate isomerization (Du et al. 2012). In the present study, the main cause for the higher basic variant levels obtained at sub-physiological temperatures will be further elucidated in the following section.

The main species is the antibody that elutes as the major peak on chromatograms. Generally, the level of main species is regulated and determined by the levels of acidic and basic variant of the mAb. In both batch and fed-batch cultures, the main species levels were decreased throughout the cultures regardless of culture temperature (Fig. 2e, f), which was consistent with the trend of the increased acidic and basic variant level mentioned above. Furthermore, as discovered above, lowering culture temperature decreased acidic variant levels but increased basic variant levels in both batch and fed-batch cultures. However, the influence of lowering culture temperature on the basic variants was weaker than on the acidic ones in the later course of batch culture, while in fed-batch cultures the influences on both sides seemed equal. Therefore, a lower culture temperature led to a higher main species level in the later course of batch cultures (i.e. days 7 and 8) while no significant difference in the main species was found among the fed-batch cultures with different temperatures (Fig. 2e, f).

pH is another key process parameter which is associated with mAb quality. pH values during the cultures were monitored at each sampling time and no significant difference was found among different temperatures (data not shown), suggesting that culture pH should not be responsible for the different mAb charge variants distribution profiles.

Effect of culture temperature on mAb lysine variation

Incomplete *C*-terminal lysine residue cleavage leads to the presence of lysine variation, which is a major basic variant commonly detected during mAb manufacturing. To find the cause for the higher basic variant levels obtained at sub-physiological temperatures in the present study, lysine variant levels under different temperatures were further investigated. As shown in Fig. 3, sub-physiological temperatures led to higher levels of lysine variant compared with those at 37 °C in both batch and fed-batch cultures. This result is consistent with the higher basic variant levels obtained at sub-physiological temperatures, suggesting that the higher basic variant levels observed under hypothermic conditions were mainly attributed to the higher lysine variant levels when compared with those at 37 °C.

Since heterogeneity of *C*-terminal lysine residues is due to the varying degree of proteolysis by basic carboxypeptidases, the transcription levels of basic carboxypeptidases in batch cultures were investigated by real-time PCR analysis. In our previous study, carboxypeptidase B and carboxypeptidase H have been confirmed as two major basic carboxypeptidases responsible for *C*-terminal lysine cleavage in CHO cells (Zhang et al. 2015). GAPDH, which was used as an internal control, was reported not to be affected by culture temperature (Mason et al. 2014). As shown in Fig. 4, lowering culture temperature significantly decreased the transcription level of carboxypeptidase B (Fig. 4a). Specifically, the transcription level of carboxypeptidase B was stable throughout the culture at 37 °C but decreased by approximately 35 % once the temperature was shifted to 32 °C ($p < 0.05$). On the other hand, the transcription level of carboxypeptidase

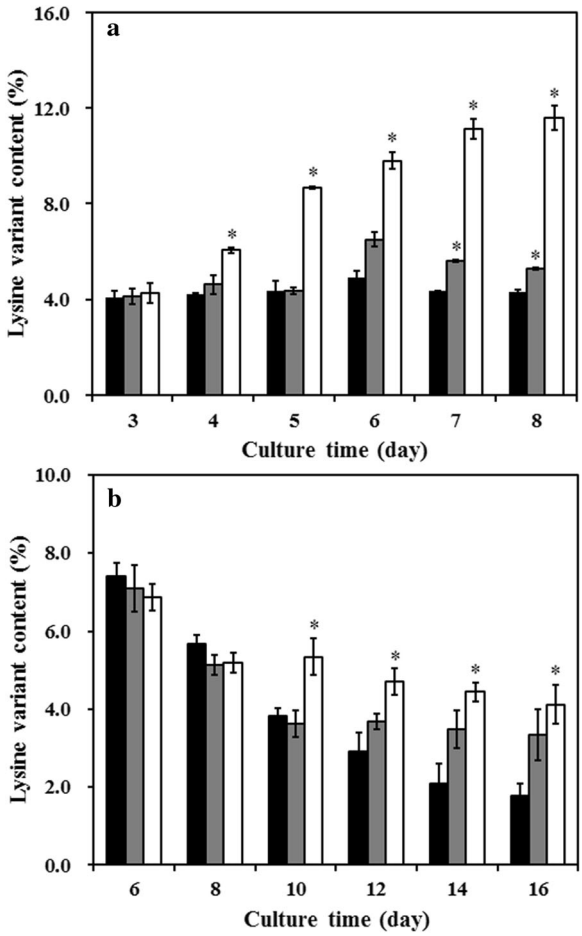

Fig. 3 Profiles of mAb lysine variant level at different culture temperatures. **a** Lysine variant level in batch culture; **b** lysine variant level in fed-batch culture. (*white bar*) 32 °C; (*shaded bar*) 35 °C; (*black bar*) 37 °C. Lysine variant level was quantified by comparing the basic variant levels obtained from WCX with or without carboxypeptidase B treatment. The *error bars* indicate the standard deviations from three independent experiments and *p* values were estimated by two-tailed Student's *t* test. $*p < 0.05$ relative to 37 °C

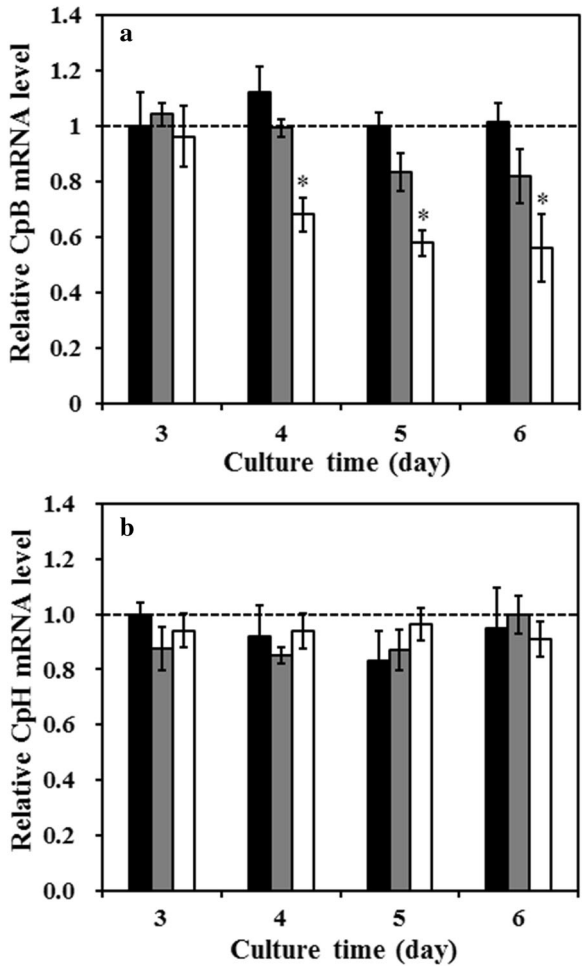

Fig. 4 Transcription levels of basic carboxypeptidases at different culture temperatures in batch cultures. **a** Carboxypeptidase B transcription level; **b** carboxypeptidase H transcription level. (*white bar*) 32 °C; (*shaded bar*) 35 °C; (*black bar*) 37 °C. The relative levels of basic carboxypeptidases mRNAs were analyzed using the $2^{-\Delta\Delta C_t}$ method with GAPDH as an internal control and normalized to 37 °C on day 3. The *error bars* indicate the standard deviations from three independent experiments and *p* values were estimated by two-tailed Student's *t* test. $*p < 0.05$ relative to 37 °C

H was not affected by temperature shift (Fig. 4b). Accordingly, the decrease in the transcription level of carboxypeptidase B might be partially responsible for the increased lysine variant level at sub-physiological temperatures. In addition, the decrease in the activity of basic carboxypeptidases caused by lowering culture temperature might be another cause for the increased lysine variant level (Bradley et al. 1996).

Apart from the culture temperature itself, the time point of temperature shift also showed noticeable effect on cell growth, mAb production and charge variation distribution in cell cultures, especially in fed-batch cultures (data not shown). A later time point of temperature shift led to a lower mAb concentration while the acidic variant level was lower and the main species level was higher. In the present study, culture temperature in fed-batch cultures was shifted on day 8 since it was the optimum time to balance the mAb concentration and charge variation distribution.

Conclusions

Charge variation is one of the most important heterogeneities during mAb manufacturing. Lowering culture temperature, which Is traditionally used to regulate cell growth and productivity, was investigated in respect of its potential roles in mAb charge variation distribution. In both batch and fed-batch cultures, cultivations at sub-physiological temperatures decreased mAb acidic variant levels but increased the basic ones. Simultaneously, lowering culture temperature increased lysine variant level, which might be the main cause for the increased basic variant level and partially attributed to the decrease in carboxypeptidase B transcription level. Taken together, culture temperature exhibits significant impact on mAb charge variation distribution, especially the acidic variants and lysine variants.

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