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Efficient folding/assembly in Chinese hamster ovary cells is critical for high quality (low aggregate content) of secreted trastuzumab as well as for high production: Stepwise multivariate regression analyses

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When developing cell culture processes for therapeutic antibodies, the low content of aggregated proteins is the most critical because administering aggregated antibody molecules might result in adverse effects such as immunogenicity. To characterize cells with high productivity and quality, we determined factors that are closely related to antibody titer, which is a productivity indicator, and the area percentage of high molecular weight species in cultivated media, which is equivalent to aggregate content and is used as a quality indicator. We examined the factors influencing antibody titer and aggregate content using various data from 28 cell lines throughout their culture periods from growth to death phases. Our study using correlation analysis revealed that statistically significant correlations between factors and indicators changes with sampling points, hence we thought that various factors would influence each indicator simultaneously. To understand the relationship between these factors and titer/aggregates contents, we performed stepwise multiple linear regression analyses and deduced a multiple linear model for each indicator. The titer was found to positively associate with specific growth rate and specific production rate and negatively with intracellular heavy chain content. The aggregate content was found to positively associate with protein disulfide isomerase mRNA level and negatively with light chain secreted into culture media, specific production rate, intracellular light chain content, and specific growth rate. Our observations suggest that correct and efficient assembling and/or folding of an antibody molecule in an endoplasmic reticulum are important for high titer and low aggregates contents.

[**Key words:** Therapeutic antibody; Antibody productivity; Antibody quality; Cell line; Antibody aggregate; Chinese hamster ovary cell; Endoplasmic reticulum stress; Protein disulfide isomerase; Heavy chain binding protein; Stepwise multiple linear regression]

Therapeutic monoclonal antibodies (mAbs) have been widely used owing to their high antigenic specificity, long serum half-life, and low incidence of undesirable side effects. Moreover, the market for mAbs is growing annually worldwide (1).

Because the administration of high doses of therapeutic mAbs is required, production processes with high productivity (titer) of mAbs are needed to reduce the expense to patients. Titers of recombinant antibodies produced by cell lines have been improved by selecting hosts, improving expression vectors, optimizing gene-coding sequences, selecting cell lines suitable for large-scale production, and optimizing culture media (2–5). Although several mammalian cell lines are used for manufacturing glycoproteins, therapeutic mAbs are commonly manufactured using Chinese hamster ovary (CHO) cell lines for which clinical safety has been established and high productivity has been reported. Final titers of 1–5 g/L mAbs are currently achieved with CHO cell lines (5–7).

In addition to their productivity, the quality of therapeutic mAbs is also critical because mAb molecules produced from CHO cells are heterogeneous owing to aggregation (8,9) and various post-translational modifications (10,11) such as oxidation (12), fragmentation (13,14), deamidation (15,16), epimerization (17,18), glycation (19,20), and glycosylation (21–23). These aggregations and modifications of antibodies might lead to reduction of biological activity and/or increase of undesirable effects under certain circumstances (8,24–27).

Among various qualities of mAbs that produce heterogeneity, the content of aggregated species is the most important because protein aggregates might induce immunogenic responses and cause adverse events on administration (28,29). The relation between aggregate contents in intravenous immune globulin and various side effects has been reported (30). U.S. Food and Drug Administration (FDA) recommends that it is critical for manufacturers of therapeutic protein products to minimize protein aggregation to the extent possible (31). Therefore, antibody aggregate levels must to be controlled precisely (8,24–27).

Because the productivity and quality of mAbs depend on cell line properties, selecting cell lines suitable for large-scale production is a very important stage in process development for mAb production. In fact, this selection process is time consuming as well

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as labor intensive (32), and various factors must be evaluated during this screening. Factors affecting antibody production in the cultivation processes of cell lines are found to be cell growth rate, viable cell density (VCD), and specific production rate (Q_p); in particular, cell lines suitable for large-scale production have been reported to exhibit high VCD and Q_p (5).

The final goal in developing a cell culture process for therapeutic mAbs is high quality and high productivity. To achieve this goal, the effects of various factors (e.g., Q_p, VCD, levels of heavy/light chains, levels of proteins involved in protein folding/assembly) on the quality and productivity should be analyzed simultaneously and systematically. Although the effects of these factors may change with culture period, the correlations between an indicator and various factors were analyzed based on the data obtained at a single culture point in previous reports (32,33). In fact, we found that even the sign of many correlations changes with culture period as described below. In addition, simultaneous analysis on the correlation of various factors with aggregate contents has not been made as far as we know, and it was not known which factor dominantly affects the productivity and quality. So we carried out stepwise multivariate regression analyses on the titer and quality at the culture end point using many factors at the time points that influences most strikingly on these indices.

In this study, we determined factors closely related to titer, which is a productivity indicator, and the area percentage of high molecular weight species [HMWS(%)], which is equivalent to aggregate content and is used as a quality indicator, to characterize cells that have high productivity and low aggregates contents. Twenty-eight stable CHO cell lines that produce trastuzumab [trade names Herclon, Herceptin (34)] were generated and their properties were analyzed, such as titer, HMWS(%) in culture media, Q_p, specific growth rate (μ), low molecular weight species (LMWS) in culture media, heavy chain (HC)/light chain (LC) mRNA levels, protein disulfide isomerase (PDI)/heavy chain binding protein (BiP) mRNA levels, and intracellular HC/LC protein content levels. To understand the relationship between various factors and titer/HMWS(%), we performed stepwise multiple linear regression analyses. The cell lines that exhibited a high titer were characterized by high μ, high Q_p, and low HC protein content. The cell lines that exhibited low HMWS(%) were characterized by a low PDI mRNA level, high LMWS(%), high Q_p, high μ, and high LC protein content.

The contributions of high Q_p and μ to high productivity have been demonstrated by many reports (2,5,32). However, although the effect of low intracellular HC content on titer has been suggested, a clear associate between them was demonstrated in this study for the first time. On the other hand, the researches that reported the factors affecting the aggregate content of mAbs are limited: Lee et al. (32) suggested the effect of the LC/HC mRNA ratio on aggregation, Gomez, et al. (33) indicated the effect of temperature on aggregation, and Bhoskar et al. (35) reported that free light chain in culture media reflects antibody productivity and quality. In this study, various factors that related to the efficient assembling and/or folding of an antibody heterotetramer in an endoplasmic reticulum (ER) were demonstrated to be crucial for the high mAb quality, by stepwise multivariate regression analyses. In addition, the contribution of high titer to low HMWS(%) was demonstrated.

MATERIALS AND METHODS

Cell culture Twenty-eight single clonal cell lines were created from the CHO cells that produce trastuzumab. Clonal isolation was carried out using limiting dilution. These cells were inoculated at 0.3×10^6 cells/mL with a working volume of 30 mL in 125 mL Erlenmeyer flasks. Basal medium and feed-culture medium (serum-free) were prepared in-house respectively. The cultures were shaken at 100 rpm at 37°C and under a 5% CO_2 atmosphere. On day 3, daily feeding (the volume equivalent to 3% of the media that remained in the flask at the time) of the feed medium was started. Sampling for analysis was performed on days 5, 7, 10, 12, and 14.

Antibody concentration determination The titer of the antibody in a multiple-culture media was determined using the Protein A column (4.6 × 50 mm, Applied Biosystems, Foster City, CA, USA) at ambient temperature. Mobile phase A consisted of 20 mM sodium phosphate and 300 mM sodium chloride (pH 7.0), and mobile phase B consisted of 20 mM sodium phosphate and 300 mM sodium chloride (pH 2.8). The Protein A column was first equilibrated with 100% mobile phase A for 0.3 min, then eluted with 100% mobile phase B. Detection of antibodies was performed at 214 nm with an ultraviolet (UV) detector, and their titers were determined from the calibration curve that was calculated using standard samples.

Calculation of growth and production rates μ was calculated using the following formula:

$$\mu = \frac{\ln\left(\frac{X_{t2}}{X_{t1}}\right)}{t_2 - t_1} \tag{1}$$

where X_{ti} is the viable cell density on day t_i.

Q_p was calculated using the following formula:

$$Q_p = \rho = \frac{(P_{t2} - P_{t1})}{\int_{t_1}^{t_2} X dt} \tag{2}$$

where P_{ti} is the concentration of the production on Day ti and X_{ti} is the viable cell density on Day ti. The trapezoidal rule was used for estimating an approximate value of the integral, and the area under the growth curve from Time t_2 to Time t_1 was determined using the following equation.

$$\int_{t_1}^{t_2} X dt \approx S = \sum \frac{(t_2 - t_1)(X_{t2} - X_{t1})}{2} \tag{3}$$

Product concentration was plotted against the integral viable cell density (IVCD) from day 0 to 7, and Q_p was determined as the slope calculated by a least-square method. To consider the effects of cell death in the late stage of the culture, Q_p in each culture period was also calculated.

mRNA analysis of heavy/light chains and chaperones A culture fluid containing 1×10^6 cells was centrifuged at 1500 rpm for 1 min, the supernatant was removed, the cell pellets were re-suspended in phosphate buffered saline (PBS) and centrifuged again, the supernatant was discarded, and the precipitated cells were stored at −20°C. RNA extraction was performed with the EZY RNA Cell mini-kit (Qiagen, Venlo, Netherlands) according to the manufacturer's protocol, and its amount was determined using NanoDrop (Thermo Fisher Scientific, Salt Lake City, UT, USA). Primers matching the designed TaqMan probes were developed using a Primer-Express software (Applied Biosystems). Trastuzumab LC primers: forward, 5′-TCACTTGTCGGGCGAGTCA-3′ and reverse, 5′-TGCCTGGTTTCTGCTGATACC-3′. Trastuzumab HC primers: forward, 5′-GGACAA GAAAGTTGAGCCCAAA-3′ and reverse, 5′-GGTCCCCCCAGGAGGAGTTCA-3′. BiP primers: forward, 5′-ACTACAGCCTGTTGCTGGACTTC-3′ and reverse, 5′-GCCACCA TAGGGAACTTCATCT-3′. PDI primers: forward, 5′-TGATGGCAACCTGAAGAGATACC-3′ and reverse, 5′-TTTCTGCTACCACAACCTTGACA-3′. TM probe sequences were as follows. LC: 5′-ACGTGAACACCGCCGTGGCC-3′; HC: 5′-TGACAAAACTCA CATGCCCACCG-3′; BiP: 5′-AGACTGCAGACGGACCGACCGC-3′; PDI: 5′-CAAGTCT GAACCTATCCCAGAGACCAACGA-3′. The TM probes were labeled with 6-carboxy fluorescein at the 5′ end and with 6-carboxytetramethyl rhodamine at the 3′ end. For real-time-PCR (RT-PCR) analysis, TaqMan one-step RT-PCR Master Mix Reagent (Applied Biosystems) and TM Ribosomal RNA Control Reagent (Applied Biosystems) were used according to the manufacturer's protocol. Each mRNA expression levels were normalized to the housekeeping gene 18S rRNA. RT-PCR samples were processed using the HT7900 system (Applied Biosystems), and RT-PCR conditions were a reverse transcription at 48°C for 30 min and an activation at 95°C for 10 min followed by 40 cycles at 95°C for 0.25 min and at 60°C for 1 min. The relative fold change of expression of the transcripts of target genes was quantified as $2^{-(\Delta Ct)}$, where ΔCt was Ct (target genes) − Ct (housekeeping gene, 18S rRNA).

Size-exclusion chromatography To analyze the content of aggregates and low molecular weight protein components and antibody molecules in culture media, size-exclusion chromatography (SEC) analyses were performed. The culture media were filtered with a 0.22 μm filter and were stored at −80°C until analysis. Preliminary experiments have confirmed that the SEC patterns of the culture media do not change significantly by this storage procedure (data not shown). In culture media, SEC was used to estimate the percentages of HMWS (substances with molecular weights greater than that of the antibody), LMWS (substances with molecular weights lower than that of the antibody), and the main species exhibiting the same molecular weight as the antibody.

SEC was performed with a TSK-gel SuperSW3000 column (4.0 mm i.d. × 30 cm; Tosoh, Tokyo, Japan) preceded by a guard column. The mobile phase consisted of 50 mM sodium phosphate, 500 mM sodium chloride, and 5% v/v ethanol (pH 7.0), and the flow rate was 0.175 mL/min. Detection was performed with the UV detector set at 215 nm.