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Titer of trastuzumab produced by a Chinese hamster ovary cell line is associated with tricarboxylic acid cycle activity rather than lactate metabolism

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Achieving high productivity and quality is the final goal of therapeutic antibody development, but the productivity and quality of antibodies are known to be substantially dependent on the nature of the cell lines expressing the antibodies. We characterized two contrasting cell lines that produce trastuzumab, namely cell line A with a high titer and a low aggregate content and cell line B with a low titer and a high aggregate content to identify the causes of the differences. We observed the following differences: cell growth (A > B), proportion of defucosylated oligosaccharides on antibodies (A < B), and proportion of covalent antibody aggregates (A > B). Our results suggest that the high monoclonal antibody (mAb) titers in cell line A is associated with the high proliferation and is not caused by the lactate metabolism shift (switching from lactate production to net lactate consumption). Rather, these differences can be accounted for by the following: levels of tricarboxylic acid cycle intermediates (A > B), ammonium ion levels (A ≤ B), and oxidative stress (A > B).

[**Key words:** Antibody aggregate; Antibody productivity; Antibody quality; Cell line; Fucosylation; Lactate metabolism shift; Chinese hamster ovary cell; Oxidative stress; Therapeutic antibody; Tricarboxylic acid cycle intermediates]

Therapeutic monoclonal antibodies (mAbs) are widely used for their high antigenic specificity, long serum half-life, and low incidence of undesirable side effects, accounting for the annual growth of the global market for therapeutic mAbs (1). Because large doses of therapeutic mAbs are usually required to achieve clinical success, mAb production processes should have high productivity to reduce expense to patients. In addition, given that in the Guidance for Industry: Immunogenicity Assessment for Therapeutic Protein Products (2), FDA recommends that manufacturers of therapeutic protein products minimize protein aggregation to the extent possible, high-quality therapeutic mAb is required.

Recently, analyses of metabolites (metabolome analyses) have attracted attention as methods for evaluating the status of cells, and the utility of metabolome analysis has been reported: ophthalmic acid is an oxidative stress biomarker (3); lactate consumption in a fed-batch culture of mammalian cells is an outcome of reduced glycolysis flux (4), and glutamine is utilized more efficiently than glucose for both anaplerotic replenishment and lactate production during the exponential phase (5). Lactate is an important metabolite of glucose but suppresses the growth of cells and the production of recombinant proteins (6). However, a positive correlation has been observed between lactate metabolism shift (switching from lactate production to net lactate consumption in a late culture stage) and the productivity of recombinant protein in CHO cells (4), and the lactate metabolism shift in the cell culture process depends not only on the nature of the cell line, but is greatly affected by medium composition (7). Cell growth (as well as mAb productivity) is reported to be associated with the lactate metabolism shift (7).

Because the productivity and quality of antibodies are known to be substantially dependent on the nature of cell lines expressing the antibodies, we characterized two contrasting cell lines that produce trastuzumab (trade names Herclon and Herceptin) by metabolome analyses: cell line A (high productivity and low aggregate content) and cell line B (low productivity and high aggregate content). We previously observed that the difference between two cell lines in the productivity (titer) was attributed mainly to differences in the number of total cells (8). We accordingly investigated whether the high proliferative potency and high mAb titer of cell line A are associated with the lactate metabolism shift. With respect to the qualities of mAbs secreted from the two cell lines, we observed that there was a cell-type difference in the proportions of defucosylated oligosaccharides (A < B), which are related to the antibody-dependent cell cytotoxicity (ADCC) activity of mAbs (9,10). In addition, in a previous study, we observed different proportions of covalent aggregates (A > B) although the total aggregate content was higher for cell line B than for cell line A (8). We investigated whether these differences were associated with the cell's metabolic state.

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MATERIAL AND METHODS

Cell culture Two single clonal cell lines (designated as A and B) were selected from 28 cell lines that had been created by transfecting CHO cells with vectors expressing heavy and light chains. Two cell lines were inoculated at 0.3×10^6 cells/mL with a working volume of 800 mL in 2-L glass bioreactors (Able Co., Tokyo, Japan). Serum free basal media containing 8.6 g/L glucose and 4 mM glutamine (pH 7.5) and serum free feed-culture media containing 60 g/L glucose and 34 mM glutamine were prepared in-house. The cultures were stirred at 85 rpm at 37°C under a 5% CO_2 atmosphere and 50% dissolved oxygen for 14 days. The pH of media was not controlled during the culture period. On day 3 and thereafter, daily feeding (a volume equivalent to 3% of the media that remained in the reactor) of the feed medium was initiated. Sampling for various analytical methods was performed daily, and samples were stored at −20°C until further use.

Analytical methods for monitoring cell cultures were performed on the sampling day as follows: The number of viable cells was determined using a Vi-Cell XR (Beckman Coulter, Fullerton, CA, USA); measurements of glutamine, lactate, and ammonium ion were performed using a Bioprofile 400 (Nova Biomedical, Waltham, MA, USA). Culture was performed for 14 days, and culture supernatants were stored at −20°C until purification. The cells sampled for metabolomic were stored at −80°C after washing them twice with phosphate-buffered saline using a centrifugal separator. Culture was performed using three reactors for each cell line.

The titers of the antibody in a media were determined using a protein A column (4.6 × 50 mm, Applied Biosystems, Foster City, CA, USA) at an ambient temperature (25°C). Mobile phase A was composed of 20 mM sodium phosphate and 300 mM sodium chloride (pH 7.0), and Mobile phase B was composed of 20 mM sodium phosphate and 300 mM sodium chloride (pH 2.8). The protein A column was washed with 100% mobile phase A after the sample was injected at a flow rate of 3 mL/min and was eluted with 100% mobile phase B. Antibodies were detected by their absorbance at 214 nm using an ultraviolet (UV) detector, and their titers were determined from a calibration curve generated using standard samples.

Calculation of specific mAb production rates Specific mAb production rate (Qp) was calculated using the following formula:

$$Q_p = \rho = \frac{(P_{t2} - P_{t1})}{\int_{t_1}^{t_2} X dt} \quad (1)$$

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.

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

Purification of antibodies using affinity chromatography To compare the characteristics of antibodies between two cell lines, the antibody in culture media was purified using a protein A affinity chromatography. The culture medium was loaded onto a protein A (MabSelect Sure) column (1 × 5 cm, GE Healthcare Life Sciences, Piscataway, NJ, USA) equilibrated with 10 mM sodium phosphate buffer (pH 6.0, washing solution) to absorb the antibody to the protein A column. After the column was washed with 5 column volumes of washing solution, the adsorbed antibody was eluted with 10 mM sodium citrate buffer (pH 3.4), and the pH of the eluent from the column was adjusted to 5.5 with 1.5 M Tris. This solution was exchanged and concentrated using a formulation buffer containing 10 mM glutamate, pH 5.5, 262 mM sorbitol, with an Amicon Ultra 10 K centrifugal concentrator (Millipore, Billerica, MA, USA). The antibody concentration in each cell line was determined from the absorbance based on the extinction coefficient 1.48 $(mg/mL)^{-1}\ cm^{-1}$ at 280 nm, which was estimated using the formula by March et al. (11).

Liquid chromatography-mass spectrometry Liquid chromatography-mass spectrometry (LC-MS) was used to identify post-translational modification. Reverse-phase separation was performed using an Acquity UPLC (Waters, Milford, MA, USA) with a MassPREP Micro Desalting column (4.0 mm i.d. × 2.5 cm; Waters), and the column temperature was maintained at 80°C. After the protein sample (5 μg) was injected into the column, the flow rate was maintained at 0.5 mL/min for 0.5 min, followed by 0.2 mL/min. To prepare the samples for analysis, the purified antibody sample was diluted with the formulation buffer. The column was equilibrated with 95% mobile phase A (0.1% formic acid, purified water) and 5% mobile phase B (0.1% formic acid, acetonitrile) for 0.5 min after sample injection. The column was eluted with a linear gradient of 5%–90% mobile phase B for 10 min.

MS analyses were performed using an Xevo TQ MS (Waters), and analyses conditions were as follows: ESI source; positive ion mode; capillary voltage, 2.5 kV; drying gas flow rate, 1000 L/h; vaporizing temperature, 350°C; cone gas flow rate, 50 L/h; ion source temperature, 120°C. The conditions of deconvolution of electrospray ionization mass spectra in BiopharmaLynx (Waters) were determined according to a previous report (12). The percentage of each peak in the purified antibody samples was calculated using BiopharmaLynx (Waters).

Analysis of *N*-linked oligosaccharides The *N*-linked oligosaccharides adorning the antibody molecules produced in this study were analyzed using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). Samples were prepared by adding 2-mercaptoethanol (1.7 μL) to an antibody solution containing 25 μg purified antibody diluted to 85 μL with purified water, and then incubating the mixture for 5 min at 37°C. Protein *N*-glycosidase F aqueous solution (1 unit/μL, 4.5 μL; Roche, Mannheim, Germany) was added to the mixture, and the mixture was incubated for another 12–15 h at 37°C. After the addition of 150 μL cold (−20°C) methanol, the sample was centrifuged at 15,000 ×*g* for 15 min, and the supernatant was transferred to a new tube. The supernatant was evaporated to dryness using a centrifugal evaporator. Derivatization of the oligosaccharides with 2-aminobenzoic acid (2-AA) was performed according to a slightly modified method of a previous report (13), as follows. In brief, purified water (20 μL) was added to the dried sample. Reaction reagent solution was prepared by dissolving 2-AA (30 mg) and sodium cyanoborohydride (20 mg) in methanol containing 4% sodium acetate trihydrate and 2% boric acid immediately before use. Reaction reagent solution (100 μL) was added to the sample solutions, and the mixture was incubated at 80°C for 50 min. The mixture was centrifuged after cooling, and purified water (30 μL) was added. A 1-mL aliquot of aqueous acetonitrile [95:5 (v/v)] was added, and the solution was mixed. It was applied to a 1 cc Oasis HLB cartridge (Waters) that had been washed with 1 mL aqueous acetonitrile [95:5 (v/v)]. After the cartridge was washed twice with 1 mL of aqueous acetonitrile [95:5 (v/v)], the 2-AA-derivatized oligosaccharides were eluted with 0.4 mL of diluted aqueous acetonitrile [20:80 (v/v)]. The eluted solution was evaporated to dryness using a centrifugal evaporator and was redissolved in purified water (50 μL). A matrix solution containing 10 mg/mL 2,5-dihydroxybenzoic acid in 50% aqueous methanol was prepared. The matrix solution (4 μL) was mixed with sample solutions (1 μL), and 1 μL of the mixture was applied to a standard steel target (Bruker Daltonik, Bremen, Germany). After drying, the samples were analyzed using an Autflex II (Bruker Daltonik) MALDI-TOF MS instrument. The analytical conditions were as follows: ion source voltage, 19.0 KV; lens voltage, 8.5 KV; and pulsed ion extraction, 100 ns.

Analysis of metabolites To prepare samples for HPLC using reverse-phase liquid chromatography (RP-LC) and hydrophilic interaction liquid chromatography (HILIC-LC), metabolites were extracted from the cells as follows: After the addition of a 50% (v/v) aqueous solution of methanol (−20°C, 992 μL), 2.5 mM methylsuccinic acid (4 μL) and 2.5 mM L-methionine sulfone (4 μL) were added to 1.0×10^7 cells stored at −80°C, and the mixture was vortexed, frozen at −80°C, and thawed to rupture the cells. Chloroform (250 μL) was added to the solution, the solution was then vortexed vigorously, centrifuged at 2280 ×*g* for 15 min, and the aqueous phase was isolated and transferred to a clean tube. The solution was centrifuged at 6000 ×*g* for 15 min, and the supernatant was transferred to an Amicon Ultra 10 K (Millipore) that had been washed with purified water, and centrifuged at 14,000 ×*g* for 15 min. The concentrated solution was freeze-dried and stored at −80°C.

Analysis of metabolites by LC-MS using RP-LC To estimate the concentration of major metabolites, such as TCA cycle intermediates, ribo- and deoxyribonucleotides, and antioxidant-related substances, LC-MS with RP-LC was performed. RP-LC was performed using an Acquity UPLC (Waters) with a Sunniest RP-AQUA column (2.0 mm i.d. × 15.0 cm, 3 μm particle size; ChromaNik, Osaka, Japan), and the column temperature and flow rate were maintained at 37°C and 0.2 mL/min throughout the run. To prepare the samples for analysis, the freeze-dried sample was reconstituted with 100 μL of purified water. The column was equilibrated with 100% mobile phase A (10 mM ammonium formate:purified water), and the column was eluted with a linear gradient of mobile phase B (mobile phase A:acetonitrile; 70:30, v/v) from 0% to 42% over 25 min after sample injection. MS analyses were performed using Xevo TQ MS (Waters), and analyses conditions were as follows: ESI source; negative ion mode; capillary voltage, 2.7 kV; drying gas flow rate, 800 L/h; vaporizing temperature, 350°C; cone gas flow rate, 50 L/h; ion source temperature, 120°C. The RP-LC data are presented in Table 1 with cone voltage. The standard solution was prepared from each metabolite (Table 1), and methyl succinic acid and L-methionine sulfone were added to the same final concentration as the sample. The intracellular concentration of these metabolites was calculated from the calibration curves.

LC-MS analysis of metabolites using HILIC-LC To estimate the concentration of major metabolites, such as amino acids and monosaccharides, LC-MS with HILIC-LC was performed. HILIC-LC was performed using an Acquity UPLC (Waters) using a ZIC-HILIC column (2.0 mm i.d. × 15.0 cm, Merck, Darmstadt, Germany), and the column temperature and flow rate were maintained at 37°C and 0.1 mL/min, respectively, throughout the run. To prepare the samples for analysis, the freeze-dried sample was reconstituted with 100 μL of acetonitrile:water (50:50, v/v), and the solution was diluted twice with acetonitrile:water (50:50, v/v) containing methyl succinic acid (200 μM) and L-methionine sulfone (200 μM). The column was equilibrated with 83% mobile phase A (acetonitrile) and 17% mobile phase B (5 mM ammonium acetate aqueous solution; pH 6.8), and the column was eluted with a linear gradient of mobile phase B from 17% to 45% for 25 min after sample injection. MS analyses were performed using a Xevo TQ MS (Waters), and analytical conditions were as follows: ESI source; negative ion mode; capillary voltage, 2.7 kV; drying gas flow rate, 800 L/h; vaporizing temperature, 350°C; cone gas flow rate, 50 L/h; ion source temperature, 120°C. Metabolites quantified using HILIC-LC are presented in