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Glycosylation analysis of an aggregated antibody produced by Chinese hamster ovary cells in bioreactor culture

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***N*-Glycosylation of therapeutic antibodies contributes not only to their biological function, but also to their stability and tendency to aggregate. Here, we investigated the impact of the glycosylation status of an aggregated antibody that accumulated during the bioreactor culture of Chinese hamster ovary cells. High-performance liquid chromatography analysis showed that there was no apparent difference in the glycosylation patterns of monomeric, dimeric, and large aggregated forms of the antibody. In contrast, lectin binding assays, which enable the total amounts of specific sugar residues to be detected, showed that both galactose and fucose residues in dimers and large aggregates were reduced to 70–80% of the amount in monomers. These results strongly suggest that the lack of *N*-linked oligosaccharides, a result of deglycosylation or aglycosylation, occurred in a proportion of the dimeric and large aggregated components. The present study demonstrates that glycosylation heterogeneities are a potential cause of antibody aggregation in cell culture of Chinese hamster ovary cells, and that the lack of *N*-glycosylation promotes the formation of dimers and finally results in large aggregates.**

[**Key words:** Chinese hamster ovary cell; Cell culture; Antibody production; Antibody aggregation; Glycosylation heterogeneity; Bispecific diabody]

Immunoglobulin G (IgG) antibody molecules consist of Fab and Fc regions, and the Fc region contains two *N*-glycosylation sites in the C_H2 domains. *N*-Glycosylation enables the interaction of Fc receptors and C1q proteins, which is involved in effector functions such as antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity. The *N*-linked oligosaccharides show marked heterogeneities in their terminal galactose and sialic acid and core fucose residues, which influence the expression of the effector functions (1,2). In addition to functional aspects, *N*-glycosylation of antibodies can influence their structural stability and aggregation. The trimming of *N*-linked oligosaccharides, deglycosylation, and aglycosylation reduce antibody structural stability, and make IgG molecules less resistant to aggregation (3–6). Aggregation of therapeutic antibodies can have a serious impact on their safety and quality (7,8). Aggregated antibodies lose their biological activity and exhibit reduced potency. In addition, it has been shown that the aggregation and misfolding of therapeutic proteins can induce a new and cryptic epitope presentation, which results in an unexpected immune response after administration (9,10).

Chinese hamster ovary (CHO) cells are important for the industrial production of therapeutic glycoproteins because of its wide usage in GMP-certified recombinant protein production, the development of industrial serum-free media, and its high-level production of recombinant proteins (11–16). In the manufacturing of therapeutic antibodies, antibody aggregation can occur at different stages, including cell culture fermentation, purification, formulation, and long-term storage. Although much is known regarding antibody aggregation during purification and formulation processes, it is less well understood how antibodies aggregate during cell culture fermentation. Media pH, temperature, oxidation status, and cultivation time may also possibly induce antibody aggregation during the cell culture process (7,8). It is well established that the glycosylation heterogeneity of recombinant glycoproteins is largely caused by environmental cell culture conditions (17–20).

Here, we suggest the hypothesis that the heterogeneity of *N*-linked oligosaccharides is a possible cause of antibody aggregation during the cell culture process. Various studies have investigated the contribution of *N*-glycosylation to the stability and aggregation of antibodies, as described above. Conversely, the difference in the *N*-glycosylation status between intact and aggregated antibodies has not been reported. In this study, we analyzed the glycosylation status of an aggregated antibody accumulated during the cell culture process to assess the relationship between glycosylation heterogeneity and aggregation. We used the humanized anti-EGFR × anti-CD3 bispecific diabody with an Fc portion, Ex3-scDb-Fc (Fig. 1A) (21,22), as the model antibody. Ex3-scDb-Fc retargets lymphokine-activated killer cells with a T-cell phenotype against epidermal growth factor receptor-positive cells and shows remarkable antitumor activity *in vitro* (21,22). We previously cultivated a CHO Top-H cell line expressing the bispecific diabody and analyzed the glycosylation pattern of Ex3-scDb-Fc (23). In

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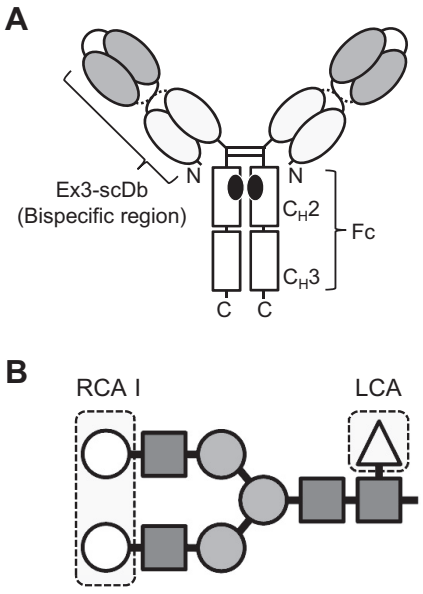

FIG. 1. (A) Schematic diagram of Ex3-scDb-Fc (21,22). The black spheres in the C_H2 domains represent the *N*-glycosylation sites focused on in this article. (B) The G2F glycoform of *N*-linked oligosaccharide. Closed square: *N*-acetylglucosamine; closed circle: mannose; open triangle: fucose; open circle: galactose. *Ricinus communis* agglutinin I (RCA I) and *Lens culinaris* agglutinin (LCA) used in this article preferentially recognize terminal galactose and core fucose residues, respectively.

addition, we used α-2,6 sialyltransferase derived from CHO cells (CHO ST6Gal I) for the α-2,6 sialylation of the bispecific antibody (24). Ex3-scDb-Fc is a promising candidate for a next-generation therapeutic antibody because of its dual functionalities. Despite its promising potency, the biophysical properties of the bispecific diabody remain to be elucidated. Factors affecting its aggregation in the manufacturing process need to be clarified and controlled. In this study, we investigated the glycosylation status of monomeric, dimeric, and large aggregated forms of the bispecific diabody in terms of their glycosylation patterns and their total amounts of sugar residues.

MATERIALS AND METHODS

Cell culture, antibody production, and purification The CHO Top-H cell line producing the Ex3-scDb-Fc bispecific diabody (23,24) was cultivated in suspension culture using serum-free ExCD medium [a mixture of ExCell 302 (SAFC Bioscience, St. Louis, MO, USA) and IS CHO-CD (Irvine Scientific, Santa Ana, CA, USA) supplemented with 1000 nM MTX and 1 mM G418]. The cells were cultivated in a 1-L glass bioreactor (Biott, Tokyo, Japan) containing 750 mL of the medium for approximately 2 weeks. The temperature was maintained at 37°C during cultivation. The agitation speed was 70 rpm and the headspace of the vessel was aerated with air supplied at a flow rate of 100 mL/min. The pH was controlled at 7.1. The dissolved oxygen (DO) concentration was measured with a DO sensor (InPro 6880; Mettler Toledo, Zurich, Switzerland) and was always kept above 40% air saturation. In operation, 25 mL of feeding solution containing 60 g/L glucose, 60 mM L-glutamine, and Yeastolate Ultrafiltrate (Life Technologies, Carlsbad, CA, USA) was added to the bioreactor every 3 days. The Ex3-scDb-Fc antibody was purified from the culture medium by HiTrap protein A affinity chromatography on an AKTA Prime Plus system (GE Healthcare, Buckingham, UK) with a single-step pH gradient from the equilibration buffer [25 mM Tris, 100 mM NaCl, 1 mM EDTA (pH 7.5)] to the elution buffer (1 M Arg-HCl, pH 4.2). Eluted fractions containing the antibody were dialyzed against the equilibration buffer [25 mM Tris, 100 mM NaCl, 1 mM EDTA (pH 7.5)], and further purified with a Sephacryl S-300 prepacked column (GE Healthcare). The purities of the fractionated monomeric, dimeric, and large aggregated forms of the antibody were checked by SDS-PAGE, and the antibody concentrations were determined using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific Inc., Rockford, IL, USA). A deglycosylated antibody was prepared by glycopeptidase F (GPF) digestion (Takara Bio, Otsu, Japan) of 0.5 mg of a monomeric bispecific antibody with 10 mU GPF in 100 mM Tris−HCl buffer (pH 8.6), and the antibody was purified with Protein A Mag Sepharose Xtra (GE Healthcare). Deglycosylation was checked by SDS-PAGE.

Circular dichroism and fluorescence spectrum measurements Far-UV CD spectra were measured using a Jasco J-820 spectropolarimeter (Jasco, Tokyo, Japan) with a quartz cell of 1-mm path length. Antibody concentrations were prepared at 0.4 mg/mL in buffer comprising 25 mM Tris, 100 mM NaCl, and 1 mM EDTA (pH 7.5). The temperatures of the samples were kept at 20°C using a Peltier temperature controller (PYC-347WI; Jasco). Thermal denaturation experiments were performed by monitoring CD signals at 218 nm as the temperature increased. ANS binding fluorescence spectra were measured with a Hitachi F-7000 fluorescence spectrophotometer (Hitachi, Tokyo, Japan). 1-Anilinonaphthalene-8-sulfonate (ANS) was purchased from Sigma. The excitation wavelength was 365 nm, and each measurement sample contained 0.1 mg/mL antibody and 10 μM ANS in 25 mM Tris, 100 mM NaCl, and 1 mM EDTA (pH 7.5). The temperature of the sample was kept at 20°C by using circulating temperature-controlled water.

High-performance liquid chromatography analysis of *N*-linked oligosaccharides *N*-linked oligosaccharides were released from 0.5 mg of monomeric, dimeric, and large aggregated forms of the antibody by glycopeptidase F digestion (Takara Bio) and purified with a cellulose cartridge Glycan preparation kit (Takara Bio). The reducing ends of the oligosaccharides were derivatized with 2-aminopyridine-acetic acid. Pyridylaminated (PA) oligosaccharides were repurified with a Blot Glyco cleanup column (Sumitomo Bakelite, Tokyo, Japan). Prepared PA oligosaccharide was analyzed with an ODS C18 COSMOSIL 5C18-AR-II column (Nacalai Tesque) attached to a Shimadzu LC-20AD high-performance liquid chromatography (HPLC) system (Shimadzu Corporation, Kyoto, Japan). Variants were estimated by comparing their retention times with those of pyridylaminated standards (Masuda Chemical Industries, Takamatsu, Japan) and further identified by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectroscopy measurements using an Autoflex mass spectrometer (Bruker Daltonics, Billerica, MA, USA).

Lectin binding assay The total amounts of galactosylated and fucosylated oligosaccharides on the antibodies were estimated using an enzyme-linked lectin binding assay (24,25). The wells of a 96-well plate (Corning Inc., NY, USA) were coated with the monomeric, dimeric, and aggregated forms of the bispecific antibody (100 μL/well, 0.5−2.5 μg/mL). To prepare the denatured samples, each sample of bispecific antibody was heated at 60°C with 0.1% SDS and 5 mM DTT for 5 min. After immobilization and BSA blocking, 100 μL of 18 μg/mL biotinylated *Ricinus communis* agglutinin I (RCA I, RCA_{120}) and biotinylated *Lens culinaris* agglutinin (LCA) solutions (Funakoshi, Tokyo, Japan) were added to each well. After 12 h incubation at 4°C, streptavidin-conjugated horseradish peroxidase (HRP) was used for detection. Absorbance at 405 nm was measured with an Infinite M200 microplate reader (Tecan, Grödig, Austria).

RESULTS AND DISCUSSION

Characterization of the aggregated bispecific diabody that accumulated during the CHO cell culture process The CHO Top-H cell line producing Ex3-scDb-Fc was cultivated in a 1-L glass bioreactor. The cells were inoculated at 3×10^5 cells/mL from the preculture in the mid-exponential growth phase. The maximum cell density reached 50×10^5 cells/mL. The culture medium was harvested after 15 days when the cell viability reached 60%. The final concentration of Ex3-scDb-Fc was approximately 40 mg/L (Supplementary Fig. S1). Ex3-scDb-Fc was purified by protein A affinity chromatography. In the affinity purification, we used the 1M Arg-HCl solution (pH 4.2) as an eluent, because arginine solution can avoid the antibody aggregation in the purification process (26).

The oligomeric state of the bispecific antibody was examined by size exclusion chromatography (Fig. 2A). The elution profile showed multiple peaks, and the peaks at elution volumes of 71, 58, and 42 mL corresponded to the monomeric, dimeric, and high-order aggregated forms, respectively. The homogeneity of the polypeptides was checked by SDS-PAGE in a reduced condition (Fig. 2B). Single bands at approximately 90 kDa, the size of a single-chained bispecific antibody, indicated that these oligomeric states were derived from the full length antibody. It should be noted that the dimeric and the high-order aggregated (large aggregate) forms were not formed in the purification process but in the cell culture process, because monomeric Ex3-scDb-Fc re-purified on a protein A column showed no tendency to form aggregates and retained the monomeric state (data not shown). The ratio of three oligomeric states relative to total Ex3-scDb-Fc was calculated based on the peak area of the size exclusion chromatography analysis (Fig. 2A).