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Effects of amino acid substitutions on the biological activity of anti-CD20 monoclonal antibody produced by transgenic silkworms (*Bombyx mori*)

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ABSTRACT

Recombinant monoclonal antibodies (mAbs) have been used in various therapeutic applications including cancer therapy. Fc-mediated effector functions play a pivotal role in the tumor-killing activities of some tumor-targeting mAbs, and Fc-engineering technologies with glyco-engineering or amino acid substitutions at the antibody Fc region have been used to enhance cytotoxic activities including antibody-dependent cellular cytotoxicity (ADCC). We previously reported that the mAbs produced using transgenic silkworms showed stronger ADCC activity and lower complement-dependent cytotoxicity (CDC) activity than mAbs derived from Chinese hamster ovary (CHO) cells due to their unique N-glycan structure (lack of core-fucose and non-reducing terminal galactose). In this study, we generated anti-CD20 mAbs with amino acid substitutions using transgenic silkworms and analyzed their biological activities to assess the effect of the combination of glyco-engineering and amino acid substitutions on the Fc-mediated function of mAbs. Three types of amino acid substitutions at the Fc region (G236A/S239D/I332E, L234A/L235A, and K326W/E333S) modified the Fc-mediated biological activities of silkworm-derived mAbs as in the case of CHO-derived mAbs, resulting in the generation of Fc-engineered mAbs with characteristic Fc-mediated functions. The combination of amino acid substitutions at the Fc region and glyco-engineering using transgenic silkworm made it possible to generate Fc-engineered mAbs with suitable Fc-mediated biological functions depending on the pharmacological mechanism of their actions. Transgenic silkworms were shown to be a promising system for the production of Fc-engineered mAbs.

1. Introduction

Recombinant monoclonal antibodies (mAbs) have been used in various therapeutic applications, including the treatment of cancer, inflammatory diseases, and infections [1,2]. Most therapeutic mAbs are produced by Chinese hamster ovary (CHO) cells [3], which is the best-characterized production platform for glycoprotein biopharmaceuticals. On the other hand, various alternative production systems using transgenic animals, transgenic plants, and transgenic insects have been developed with the aim of reducing the cost of production [4]. Silkworm (*Bombyx mori*) is one of the alternative production substrates for large-scale protein production. This highly domesticated insect has useful characteristics for production of recombinant proteins; it can produce high-molecular-weight proteins, has greater protein biosynthesis speed, and is easy to breed and scale up with low cost [5,6]. The development of a method for stable germline transformation using piggyBac transposon-derived vectors enables the mass production of recombinant proteins in transgenic silkworms [7], and transgenic silkworms are anticipated to be a convenient and economical production system for recombinant proteins including mAbs.

N-glycosylation at the Fc region is one of the posttranslational modifications related to the effector functions of mAbs [8,9], and

Abbreviations: ADCC, antibody-dependent cellular cytotoxicity; ADCP, antibody-dependent cellular phagocytosis; CBB, Coomassie Brilliant Blue; CDC, complement-dependent cytotoxicity; CHO, Chinese hamster ovary; FcγR, Fc gamma receptor; mAb, monoclonal antibody; SPR, surface plasmon resonance.

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the structure of N-glycan differs according to the production system [10]. We previously reported the unique characteristics of anti-CD20 mAb produced by transgenic silkworm [11]: namely, it had unique N-glycan structures (lack of core-fucose and non-reducing terminal galactose) and showed higher antibody-dependent cellular cytotoxicity (ADCC) activity and lower complement-dependent cytotoxicity (CDC) activity compared with the anti-CD20 mAbs produced by CHO cells. These results suggested that transgenic silkworm could be a powerful production system to produce mAbs with strong ADCC activity. On the other hand, there is demand for therapeutic mAbs possessing different Fc-mediated biological functions depending on their pharmacological mechanism of action. For example, Fc-mediated cytotoxicity against antigen-expressing cells is not preferable for mAbs designed to neutralize cytokine receptors. In contrast, enhancement of Fc-mediated cytotoxicity is desired to increase the tumor-killing activities of mAbs that target tumor antigens, although Fc-mediated biological activity that is too strong is sometimes related to adverse reactions [12]. Thus, the optimization of Fc-mediated biological activities by choosing the appropriate IgG subclass and/or Fc-engineering technology is indispensable for the development of therapeutic mAbs with the desired efficacy and safety profiles. Fc engineering with amino acid substitutions, as well as glyco-engineering, has been used to modify the Fc-mediated biological activities of mAbs [13–15].

In this study, we used transgenic silkworms to generate three types of Fc-engineered anti-CD20 mAbs with amino acid substitutions modulating Fc-mediated functions. We analyzed the biological activities of these mAbs and revealed that the Fc-engineering with amino acid substitutions could modify the FcγR receptors (FcγRs)-activation properties and the CDC activities of anti-CD20 mAbs derived from silkworms. These results indicate that the combination of Fc engineering and the silkworm production system is useful for regulating the Fc-mediated functions of mAbs.

2. Materials and methods

2.1. Cell lines and reagents

Raji cells (JCRB9012) and Ramos cells (JCRB9119) were obtained from the JCRB Cell Bank (Osaka, Japan). Jurkat/FcγR/NFAT-Luc cells, which express human FcγRIIa or FcγRIIIa and an NFAT-driven luciferase reporter, were developed previously [16]. The cells were maintained at 37 °C in 5% CO_2 with RPMI1640 medium (Life Technologies, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin-streptomycin (Thermo Fisher Scientific, Rockford, IL). Recombinant human FcγRI, FcγRIIa, and FcγRIIIa were purchased from Sino Biological (Beijing, China).

2.2. Antibody production

The amino acid sequence of human-mouse chimeric anti-CD20 mAb used in this study was identical to that of rituximab (Mabthera®). Wild-type and Fc-engineered variants with G236A/S239D/I332E, L234A/L235A, or K326W/E333S substitutions were produced using CHO-S cells or transgenic silkworms as reported previously [10,15,16]. The anti-CD20 mAbs were purified using a HiTrap Protein G HP column (GE Healthcare, Buckinghamshire, UK) and desalted using a PD-10 column (GE Healthcare) equilibrated with phosphate-buffered saline (PBS). The concentration of mAbs was measured using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific).

2.3. Peptide mapping by liquid chromatography/mass spectrometry

Peptide mapping of anti-CD20 mAbs was performed using liquid chromatography/mass spectrometry (LC/MS). Briefly, anti-CD20 mAbs were reduced by dithiothreitol and carboxymethylated by sodium monoiodoacetate, followed by digestion with trypsin of mass spectrometry grade (Promega, Madison, WI). Tryptic digests were analyzed by LC/MS. HPLC was performed on an UltiMate 3000 RSLCnano LC System (Thermo Fisher Scientific/Dionex, Sunnyvale, CA) equipped with an L-Column 2 ODS (0.075 mm × 150 mm, 3 μm; Chemicals Evaluation and Research Institute, Tokyo) at a flow rate of 300 nl/min. The eluents consisted of water containing 2% (v/v) acetonitrile and 0.1% (v/v) formic acid (pump A) and 90% acetonitrile containing 0.1% formic acid (pump B). The samples were eluted with a linear gradient of 10%–50% of pump B over 300 min. Mass spectrometric analyses were performed using an Orbitrap Elite Mass Spectrometer (Thermo Fisher Scientific). The mass spectrometer was operated in positive ion mode, and full mass spectra were acquired using a full mass range of *m/z* 350–2000 and full mass resolution of 60,000. Glycopeptides were identified by comparison of the experimentally observed mass and theoretical mass of glycopeptides from the Fc region. The percentage distribution of the glycopeptides was calculated using the peak area average values.

2.4. Cell-based competitive CD20-binding assay

Raji cells were suspended with PBS, seeded onto an MSD high-bind plate (Meso Scale Discovery, Rockville, MD) and incubated for 1 h at 37 °C in 5% CO_2. The supernatant was removed, and the cells were incubated with 3% blocker A (Meso Scale Discovery) in PBS for 1 h at room temperature. After washing with PBS, the cells were incubated with a pre-mixed sample (1% blocker A in PBS supplemented with 0.5 μg/mL biotin-labeled rituximab, 0.5 μg/mL SULFO-TAG streptavidin, and serially diluted anti-CD20 mAbs incubated for 1 h at room temperature with shaking) for 1 h at room temperature. The cells were washed, and the binding level of biotin-labeled rituximab was measured using a MESO QuickPlex SQ 120 multiplex imager (Meso Scale Discovery). The percentages of binding were calculated in relation to the binding level of the negative control sample without competitive mAbs.

2.5. CDC assay

Raji cells were cultured with Opti-MEM I Reduced Serum media containing 16% human AB serum (Sigma-Aldrich, St. Louis, MO) and serially diluted anti-CD20 mAbs. After incubation for 2 h at 37 °C in 5% CO_2, cell lysis was measured using a CytoTox-Glo Cytotoxicity Assay kit (Promega) and an Ensight Multimode Plate Reader (PerkinElmer, Waltham, MA) according to the manufacturers' protocols.

2.6. C1q-binding assay

The Raji cells were opsonized with 20 μg/mL of anti-CD20 mAbs for 15 min at room temperature. The opsonized cells were cultured with 5% human AB serum for 30 min at 37 °C (containing 10 μg/mL of anti-CD20 mAbs). The cells were stained with FITC-conjugated anti-human C1q antibody (ab4223; Abcam, Cambridge, UK) for 30 min at room temperature. The C1q-binding level (mean fluorescence intensity) was analyzed by a BD FACSCanto II flowcytometer (BD Biosciences, San Jose, CA). The fluorescence intensities were corrected by the background signal from the sample without mAbs.

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