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Generation of a chickenized catalytic anti-nucleic acid antibody by complementarity-determining region grafting

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ABSTRACT

In contrast to a number of studies on the humanization of non-human antibodies, the reshaping of a non-human antibody into a chicken antibody has never been attempted. Therefore, nothing is known about the animal species-dependent compatibility of the framework regions (FRs) that sustain the appropriate conformation of the complementarity-determining regions (CDRs). In this study, we attempted the reshaping of the variable domains of the mouse catalytic anti-nucleic acid antibody 3D8 (m3D8) into the FRs of a chicken antibody ("chickenization") by CDR grafting, which is a common method for the humanization of antibodies. CDRs of the acceptor chicken antibody that showed a high homology to the FRs of m3D8 were replaced with those of m3D8, resulting in the chickenized antibody (ck3D8). ck3D8 retained the biochemical properties (DNA binding, DNA hydrolysis, and cellular internalizing activities) and three-dimensional structure of m3D8 and showed reduced immunogenicity in chickens. Our study demonstrates that CDR grafting can be applied to the chickenization of a mouse antibody, probably due to the interspecies compatibility of the FRs.

1. Introduction

The reshaping, or "humanization," of the variable regions of an antibody is required for reducing the immunogenicity of monoclonal antibodies derived from non-human sources that occurs when these antibodies are repeatedly administered to humans during antibody-based therapy for human diseases (Kim et al., 2005). In most antibodies conventionally humanized from non-human sources (most commonly mouse), six complementarity-determining regions (CDRs) of the heavy chain variable region (V_H) and light chain variable region (V_L) are grafted into the framework regions (FRs) of a human antibody scaffold by antibody engineering (Ahmadzadeh et al., 2014). Along with the rapid development of humanization methods for variable regions (Safdari et al., 2013), a large number of non-human antibodies with specificity for antigens of therapeutic interest have been humanized from antibodies of mouse, rat, rabbit, and camelid origin (Finlay et al., 2009; Gorman and Clark, 1990; Vincke et al., 2009; Yu et al., 2010). So far, all of these approaches have been applied to the humanization of antibodies for therapeutic uses, and the reshaping of a non-human antibody into other non-human antibodies has been limited to camelization (Davies and Riechmann, 1995) and caninization [offered by the company Creative Biolabs (Shirley, NY)]. There have not been any reports of the chickenization of human/non-human antibodies.

We previously humanized the mouse antibody 3D8 (m3D8) by CDR grafting (Kim et al., 2009). This antibody, a catalytic

Abbreviations: CDR, complementarity-determining region; scFv, single chain variable fragment; FR, framework region; V_H, variable region of heavy chain; V_L, variable regions of light chain; FRET, fluorescence resonance energy transfer; ELISA, enzyme-linked immunosorbent assay; RMSD, root mean square deviations; RMSF, root mean square fluctuations.

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anti-nucleic acid antibody that contains a single chain variable fragment (scFv) and can induce DNA/RNA hydrolysis and cellular internalization (Jang et al., 2009; Kim et al., 2006), was humanized to determine whether its DNA-binding and hydrolyzing activities are derived from an intrinsic property of the catalytic antibody CDRs or whether the FRs are involved. In terms of the antibody engineering, studies on the reshaping of an antibody from one species into another may increase our understanding of the species-dependent compatibility of FRs, which sustain the appropriate conformation of the CDRs, and will reveal whether the strategies used for antibody humanization can be easily applied to the reshaping of antibodies derived from non-humans.

In the present study, we attempted the reshaping of the m3D8 antibody into a chicken antibody ("chickenization") by CDR grafting based on FR homology. The 3-dimensional structure and structural stability by computational analysis, the DNA-binding/hydrolyzing activities, the cellular internalization, and the antibody responses induced by administration to chickens of the parental m3D8 and the chickenized 3D8 (ck3D8) were compared. The ck3D8 was shown to retain the structural/biochemical properties of m3D8 and to result in reduced immunogenicity in chickens, as expected. This study suggests that it may be possible to reshape antibody variable domains between other non-human species using various humanization strategies.

2. Material and methods

2.1. Preparation of scFv proteins

Approximately 800 bp of the ck3D8 *scFv* gene, in which the V_H and V_L sequences are connected by a $(Gly4/Ser1)_3$ linker, was synthesized by Bioneer Co. (Korea). The ck3D8 gene with a stop codon at the 3′ end was subcloned into the pIg20 expression vector (Kim et al., 2006) using the *Xma*I and *Nco*I restriction sites. ck3D8 and m3D8 scFv proteins with both a $(His)_5$ tag and a Protein A tag at the C-terminus, or scFv proteins lacking the Protein A tag, were expressed in *Escherichia coli* BL21DE3 (pLysE) and prepared through solubilization of the inclusion body and refolding. Briefly, proteins were expressed in BL21DE3 (pLysE) by inducing a 1 L culture in LB broth plus 100 μg/ml ampicillin and 25 μg/ml chloramphenicol with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 6 h at 37 °C once it had reached an A_{600} (the absorbance at 600 nm) of 1.0. After preparation of inclusion bodies from the cells (Zhao et al., 2010), the denatured scFv protein was purified from the solubilized inclusion bodies by chromatography on a HisPur Cobalt column (Thermo Scientific) according to the manufacturer's instructions. Subsequently, the denatured/purified protein was renatured by slowly diluting the purified protein 80-fold in 8 M urea/150 mM imidazole at 4 °C over a period of 30 h. Finally, the urea was completely removed from the protein by dialysis in cold PBS (pH 7.4). Unless otherwise specified, scFv proteins with both a His tag and a Protein A tag at the C-terminus were used.

2.2. Enzyme-linked immunosorbent assay (ELISA)

To assay DNA-binding activity, scFv was incubated on wells of a 96-well ELISA plate that had been coated with pUC57 plasmid DNA (2 μg/ml). Bound scFv proteins were detected using rabbit IgG (Sigma) followed by alkaline phosphatase (AP)-conjugated anti-rabbit IgG (Pierce), as described previously (Kim et al., 2006). To determine the immunogenicity of scFv in chickens, scFv lacking the C-terminal Protein A tag was used as the antigen to exclude immune responses against the tag. In an indirect ELISA, wells coated with m3D8 or ck3D8 (2 μg/ml) were incubated with either serially diluted pre-immune sera or immune sera from the immunized chickens. Bound chicken IgY was detected using AP-conjugated anti-chicken IgY (Abcam. 1/500 dilution). In a competitive ELISA, wells coated with m3D8 (2 μg/ml) were incubated with a mixture of sera from mice immunized with m3D8 scFv diluted at 1:1,000 and various concentrations of competitors (m3D8 or ck3D8 protein), followed by incubation with AP-conjugated anti-chicken IgY.

2.3. Fluorescence resonance energy transfer (FRET)-based DNA cleavage assay

A DNA substrate comprised of 21 nucleotides labeled with 6-carboxyfluorescein (FAM) at the 5′-terminus and a black hole quencher (BHQ) at the 3′-terminus (5′FAM-CGATGAGTGCCATGG ATATAC-BHQ 3′) was generated by M-Biotech Inc. scFv (1 μM) or DNase I (1 U) were either pre-incubated or not with heparin (a competitor molecule; 10 μg/ml) for 10 min at room temperature and then loaded onto the wells of a 96-well black plate containing DNA substrate (250 nM). Immediately after the addition of the DNA substrate, the fluorescence intensity was read in real time over a period of 6 h at 37 °C in a fluorescence detector (molecular devices). Each reaction was carried in a final volume of 100 μl with TBSM buffer [100 mM Tris–HCl (pH 7.4), 150 mM NaCl, 2 mM $MgCl_2$].

2.4. Flow cytometry

HeLa cells seeded in 6-well plates at a density of 5×10^5 cells/well were incubated with 5 μM scFv in serum-free medium for 6 h at 37 °C. The cells were washed three times with ice-cold PBS and fixed with 4% paraformaldehyde in PBS for 10 min at 4 °C. After washing with PBS, cell membranes were permeabilized with *P* buffer (1% BSA, 0.1% saponin, and 0.1% sodium azide in PBS) and incubated with rabbit IgG (10 μg/ml) followed by Alexa Fluor 647-conjugated anti-rabbit IgG (Life Technologies) at a dilution of 1:500. *P* buffer was used for the dilution of all antibodies. Each incubation step was performed for 1 h at 4 °C, followed by washing with ice-cold PBS. Finally, the cells were suspended in 4% paraformaldehyde and analyzed using a FACSCanto II flow cytometer (Becton Dickinson).

2.5. Confocal microscopy

HeLa cells were seeded on glass coverslips in 24-well plates at a density of 4×10^4 cells/well and incubated with 5 μM scFv in serum-free medium for 6 h at 37 °C. Cells were washed, fixed, permeabilized, and labeled with antibodies as described for flow cytometry experiments. The cell nuclei were stained with Hoechst 33342 (Vector Laboratories) for 30 min at room temperature. Then, cells on the coverslips were mounted with Vectashield mounting medium (Vector Laboratories). Images were obtained using a laser scanning confocal fluorescence microscope (model LSM710, Carl Zeiss).

2.6. Computational analysis of ck3D8

The 3-dimensional structure of ck3D8 cFv was generated using the "Build homology models" algorithm, available in Discovery Studio (DS) 3.5 software (Accelrys). The crystal structure of m3D8 scFv [Protein Databank (PDB) ID: 2GKI, 2.88 Å] was used as the template. The molecular dynamics (MD) simulations were performed using the GROMACS program (version 4.5.3) (Berendsen et al., 1995; Pronk et al., 2013) with an assisted model building with energy refinement (AMBER) 03 force field (Duan et al., 2003). All systems were neutralized by the addition of Cl^- counterions, and the particle mesh Ewald (PME) method was used to calculate long-range electrostatic interactions (Darden et al., 1993). Each system has approximately 39,200 atoms, including approximately 3600 atoms