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The transgenic chicken derived anti-CD20 monoclonal antibodies exhibits greater anti-cancer therapeutic potential with enhanced Fc effector functions

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ABSTRACT

Modern genetic techniques, enable the use of animal bioreactor systems for the production and functional enhancement of anti-cancer antibodies. Chicken is the most efficient animal bioreactor for the production of anti-cancer antibodies because of its relatively short generation time, plentiful reproductive capacity, and daily deposition in the egg white. Although several studies have focused on the production of anti-cancer antibodies in egg white, in-depth studies of the biological activity and physiological characteristics of transgenic chicken-derived anti-cancer antibodies have not been fully carried out. Here, we report the production of an anti-cancer monoclonal antibody against the CD20 protein from egg whites of transgenic hens, and validated the bio-functional activity of the protein in B-lymphoma and B-lymphoblast cells. Quantitative analysis showed that deposition of the chickenised CD20 monoclonal antibody (cCD20 mAb) from transgenic chickens increased in successive generations and with increasing transgene copy number. Ultra-performance liquid chromatography (UPLC) tandem mass spectrometry (LC/MS/MS) analysis showed that the cCD20 mAb exhibited 14 N-glycan patterns with high-mannose, afucosylation and terminal galactosylation. The cCD20 mAb did not exhibit significantly improved Fab-binding affinity, but showed markedly enhanced Fc-related functions, including complement-dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC) compared to commercial rituximab, a chimeric mAb against CD20. Our results suggest that the transgenic chicken bioreactor is an efficient system for producing anti-cancer therapeutic antibodies with enhanced Fc effector functions.

1. Introduction

Recombinant monoclonal antibodies (mAbs) are used therapeutically for cancer and inflammatory diseases [1,2]. For example, the therapeutic mAb, rituximab, which targets CD20 on B cells, is used to treat non-Hodgkin's lymphoma, chronic lymphocytic leukaemia (CLL), and rheumatoid arthritis [3,4]. The mechanisms by which rituximab induces a therapeutic effect include induction of apoptosis, complement-dependent cytotoxicity (CDC), and antibody-dependent cellular cytotoxicity (ADCC) [5,6]. However, the therapeutic efficacy of rituximab is variable and often modest [7]. Therefore, the efficacy of anti-CD20 mAbs needs to be improved.

To produce high-efficacy mAbs in bioreactor systems efficiently, methods involving living organisms, including transgenic plants [8] and insects [9], have been developed. Transgenic animal bioreactor systems have been proposed for producing recombinant proteins [10]. In comparison with other bioreactor systems using living

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organisms, transgenic animals enable the efficient production of complex, biologically active proteins [11]. The use of chicken as an animal model has increased in recent decades due to its relatively short generation time, plentiful reproductive capacity, and suitability for use as a human disease model, including in studies of cancer and inherited disease [12–14]. Regarding the production of recombinant protein, a single egg white of chicken contains ~6.5 g of protein and is composed of 10 major proteins [15]. In addition, more than half of egg-white proteins are encoded by a single gene, ovalbumin (OV), which means that chicken bioreactor systems are more competitive for mass production and subsequent purification of exogenous proteins compared to systems based on other animals [15,16]. More importantly, genetic stability among generations is an advantage of the chicken system for the production of therapeutic antibodies [15,17,18].

Previous studies on the development of transgenic birds for the production of recombinant proteins relied on virus-mediated methods, which involve injection of viral particles into the subgerminal cavity of embryos [15,18,19]. However, selecting progeny with the desired genotype is difficult due to the low germline transmission efficiency (approximately 1–4%), although viral infection of embryonic stem cells (ESCs) may enable the mass production of recombinant proteins from chicken oviduct and egg white [20,21]. Indeed, the safety for human use of the recombinant proteins is an issue due to the use of viruses [22,23]. These limitations have been overcome by the development of primordial germ cell (PGC)-mediated transgenic technology and non-viral vector systems, such as *piggyBac* transposition. The use of transgenic PGCs enhanced the efficiency of protein production using germline transgenic chicken [24,25], and this technological advancement will enable the development of chicken bioreactors [26,27].

Chicken bioreactor systems allow post-translational modification (PTM) of the recombinant protein,for example, *N*-glycan species terminated by high mannose with a core afucosylated form [28]. A therapeutic monoclonal antibody (mAb) produced from egg white of somatic chimeric transgenic chicken reportedly has similar *N*-glycan profiles [21], and the U.S. Food and Drug Administration has approved a recombinant protein-based drug (Kanuma; sebelipase alfa) produced by transgenic hens, in which *N*-glycan is terminated by the high-mannose form, which facilitates transport of therapeutic enzymes into the target cells [29]. Therefore, the *N*-glycosylation of recombinant proteins produced in chickens can be used to enhance the efficacy of therapeutic proteins for use in humans. Indeed, *N*-glycosylation of the CH2 domain is an important PTM of therapeutic antibodies because its stabilisation of the Fc structure plays a role in activation of immune-effector functions, including ADCC and CDC [27,28,30,31]. The lack of the α1,6-core fucose *N*-glycan in the Fc region dramatically increases ADCC activity by enhancing the binding affinity of human IgG1 to Fcγ receptor IIIa (FcγRIIIa) on immune cells [32–35]. Antibodies with a high level of *N*-linked high-mannose species also show enhanced ADCC activity and increased FcγRIIIa-binding affinity [36]. Moreover, the increase of terminal galactose in *N*-glycan content is correlated with higher CDC activity and C1q-binding affinity but does not significantly affect ADCC activity [30,37]. Therefore, characterisation of the *N*-glycan species in the Fc region is critical for the production of therapeutic antibodies that rely on ADCC and CDC for their mode of action. Therefore, we anticipated that an anti-cancer antibody produced in transgenic chickens would show high ADCC and CDC activities.

In this study, germline transgenic chickens were used to enhance the efficacy of therapeutic antibodies by modulating the *N*-glycosylation profile. We produced an anti-CD20 human-mouse chimeric mAb rituximab as a representative anti-cancer antibody from transgenic chickens. Next, we purified the chickenised CD20 mAb (cCD20 mAb) from egg-whites of transgenic chickens of different genotypes and performed quantitative and qualitative analyses, including of the *N*-glycan structures. We further explored the biofunctional activity of the cCD20 mAb from transgenic chickens compared with that of rituximab.

2. Materials and methods

2.1. Experimental animals and animal care

The care and experimental use of chickens was approved by the Institute of Laboratory Animal Resources, Seoul National University (SNU-150827-1). Chickens were maintained according to a standard management program at the University Animal Farm, Seoul National University, Korea. The procedures for animal management, reproduction, and embryo manipulation adhered to the standard operating protocols of our laboratory.

2.2. Construction of cCD20 mAb expression vector

Codons of the anti-CD20 mAb gene were optimised for expression in the hen using the *Gallus gallus* codon database (https://www.kazusa.or.jp/codon). A codon-optimised cCD20 mAb gene consisting of a chicken lysozyme signal peptide sequence with a VL + hIg kappa region and VH hIgG constant region, consecutively conjugated with internal ribosome entry site sequences (Fig. 1a), was synthesised by Bioneer Company (Daejeon, Korea). This cassette was ligated into the *piggyBac*, which contained 3.5 kb of the chicken OV promoter and 1.6 kb of the 3′-UTR, including the poly-A tail sequence. This vector was based on the *piggyBac* OVcEGF vector from our previous study [27].

2.3. Transfection and G418-selection of PGCs and transplantation of donor germ cells into recipient embryos

A male PGC line was established using day-6 (stage 28) WL embryonic gonads and was maintained in knockout Dulbecco's Modified Eagle's Medium (KO-DMEM) (Invitrogen, Life Technologies, Carlsbad, CA, USA) supplemented with 20% (v/v) foetal bovine serum (Invitrogen, Life Technologies), 2% (v/v) chicken serum (Sigma-Aldrich, St. Louis, MO, USA), 1 × nucleoside mix (EMD Millipore, Temecula, CA, USA), 2 mM L-glutamine, 1 × nonessential amino acid mix, 2-ME, 10 mM sodium pyruvate, and 1 × antibiotic-antimycotic mix (Invitrogen, Life Technologies). Human basic fibroblast growth factor (10 ng/mL; Koma Biotech, Seoul, Korea) was used to activate PGC proliferation. PGCs were cultured at 37 °C in an atmosphere of 5% (v/v) CO_2 and at 60–70% relative humidity. Cultured PGCs were subcultured onto mitomycin-inactivated layers of mouse embryonic fibroblasts at 5- to 6- day intervals via gentle pipetting (in the absence of any enzyme treatment). The cCD20 mAb expression vector and CAGG-PBase (pCyL43) were co-transfected into the PGC line via lipofection using the Lipofectamine 2000 reagent (Invitrogen, Life Technologies). At a day after transfection, G418 (to 300 μg/mL) was added to culture medium to enable the selection of transfected PGCs. Selection required up to 3 weeks to complete. All transfection and selection procedures followed those established in our previous reports [26,27].

To inject selected transfected PGCs into recipient embryos, we made a small window on the pointed end of each recipient KO egg at Hamburger and Hamilton (HH) stages 14–17, and microinjected via micropipette a 2 μL aliquot containing at least 3000 PGCs into the dorsal aorta of the recipient embryo. Each egg window was sealed with paraffin film, and the eggs were incubated with the pointed end down prior to further screening and eventual hatching.