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Production of chimeric monoclonal antibodies by genetically manipulated chickens

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ABSTRACT

Genetically manipulated chickens producing chimeric monoclonal antibodies were generated by injecting retroviral vectors encoding genes for the heavy and light chains of antibodies into developing embryos. The transgene was detected in all chickens that hatched, and they stably produced the chimeric antibodies in their serum. After sexual maturation, the antibodies were also produced in eggs laid by the manipulated hens. The stable antibody production was observed both in egg white and yolk throughout the breeding period. The chimeric antibodies produced by the chickens were properly assembled and exhibited antigen-binding activities. Furthermore, we characterized the structures of the *N*-linked oligosaccharide chains added to the Fc-region of the recombinant antibodies produced in the serum, egg white and yolk of the chickens.

1. Introduction

Since Köhler and Milstein (1975) established the hybridoma technique, murine monoclonal antibodies have become routine laboratory tools. Although monoclonal antibodies have been recognized as potentially excellent pharmaceuticals for various diseases, murine antibodies are limited for therapeutic uses due to antigenicity of the antibodies. Significant progress in recombinant DNA technology has provided the solution to this problem and enabled the production of chimeric, humanized and fully human antibodies to a desired antigen (Kipriyanov and Little, 1999). Therapeutic antibodies can potentially be used for various diseases such as cancer, autoimmune disorders, and viral infections. A large number of therapeutic antibodies are currently available in the market (Brekke and Sandlie, 2003; Reichert and Valge-Archer, 2007).

The commercial production of recombinant antibodies is performed at present by animal cell culture in tank-type bioreactors using Chinese hamster ovary (CHO) cells and mouse myeloma, NS0, cells. Although such cells can produce recombinant antibodies with proper folding and glycosylation (Roque et al., 2004; Wurm, 2004), the production costs of recombinant proteins by animal cell culture are very high (Dyck et al., 2003; Chadd and Chamow, 2001). Furthermore, in the case of therapeutic antibodies, patients are repeatedly administrated with the antibodies at high doses of several hundreds of milligrams at once. Thus a cheaper, but no less effective, procedure is required for the production of therapeutic antibodies.

As an alternative method for large-scale production of therapeutic antibodies, transgenic livestock animals (Limonta et al., 1995; Rudolph, 1999; Pollock et al., 1999) and plants (Zeitlin et al., 1998; Ma et al., 1998) have attracted a great deal of attention. It was reported that whole antibodies were expressed at concentrations of up to 14 mg/ml in the milk of transgenic goats (Pollock et al., 1999). However, the generation of transgenic livestock animals is an inefficient process because it requires large and long-term financial support (Whitelaw, 2004). Another issue that should be considered in the production of recombinant proteins using livestock animals is the presence of infectious diseases such as prions.

On the other hand, chickens have several advantages as transgenic bioreactors for the production of recombinant proteins, including high production yield of egg proteins, ease of breeding, and proper post-translational modifications (Ivarie, 2003; Sang, 2004; Lillico et al., 2005). In addition, developing chicken embryos have been used for production of human vaccines, and specific pathogen free (SPF) species of chicken are available. In this regard, a variety of methods for generating genetically manipulated (GM) chickens have been reported (Love et al., 1994; Mizuarai et al., 2001; Harvey et al., 2002; Mozdziak et al., 2003; McGrew et al., 2004; Kamihira et al., 2005; Zhu et al., 2005; Van de Lavoir et al., 2006). However, the biopharmaceuticals produced by GM chickens have been restricted to relatively small proteins with simple structures

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(Rapp et al., 2003; Lillico et al., 2007). In recent years, our group reported the generation of GM chickens and quails producing Fc-fused single chain antibody fragments fused with the Fc-region of an antibody (scFv-Fc) (Kamihira et al., 2005; Kawabe et al., 2006a), human erythropoietin (Kodama et al., 2008) and the Fc-fused extracellular domain of TNF-α receptor (Kyogoku et al., 2008), using replication-defective retroviral vectors for gene transfer. It was found that maximal expression was observed throughout chicken embryos when concentrated retroviral vectors were injected into the heart of developing embryos after 55 h incubation. Transgenic progeny could be generated by mating the GM chicken with wild-type chickens, and they also produced the recombinant protein stably (Kamihira et al., 2005).

In the present study, we attempted to generate GM chickens producing whole antibodies using retroviral vectors in which the bicistronic expression of the genes for the heavy (H-) and light (L-) chains of chimeric anti-CD2 and anti-prion peptide (PrP) monoclonal antibodies was induced under the control of a constitutive chicken β-actin promoter. The expression levels of antibodies in the serum and eggs of GM chickens were measured throughout the breeding period. The characteristics of antibodies produced by the GM chickens, such as antigen-binding activity and structures of oligosaccharide chains, were also analyzed.

2. Materials and methods

2.1. Vector construction

The construction of plasmids, pMSCV/ΔALIH (CD2) and pMSCV/ΔALIH (PrP), which encode expression cassettes of chimeric anti-CD2 and anti-prion monoclonal antibodies, respectively, and which are designed for the production of retroviral vectors based on a mouse stem cell virus (MSCV) (Hawley et al., 1994), was basically as described in our previous studies (Ono et al., 2003; Hotta et al., 2004). The genes for H- and L-chains of the antibodies were bicistronically expressed using an internal ribosomal entry sequence (IRES) derived from encephalomyocarditis virus (EMCV) (Ghattas et al., 1991) under the control of chicken β-actin promoter. The genes for variable regions (V_H and V_L) derived from the expression plasmids of mouse anti-CD2 (Hotta et al., 2004) and chicken anti-prion (Nakamura et al., 2000; Ono et al., 2003) monoclonal antibodies were obtained by PCR. The genes for constant regions of human antibody ($hC_{\gamma 1}$ and hC_{κ}) were obtained from human myeloma IM-9 cells (JCRB Cell Bank, Tokyo, Japan) producing human IgG1. The chimeric H-chain (V_H and $hC_{\gamma 1}$) and L-chain (V_L and hC_{κ}) genes were inserted into the plasmid pMSCV (Clontech, Palo Alto, CA, USA). A schematic drawing of the structure of retroviral vector is shown in Fig. 1.

2.2. Retroviral vector production and injection into chicken embryos

Production of VSV-G pseudotyped pantropic retroviral vectors, microinjection of viral solution into developing chicken (White leghorn) embryos, and culture of manipulated embryos were all performed as described in our previous studies (Kamihira et al., 2005; Kawabe et al., 2006a). The concentrated viral solution, with a titer of 9×10^8 (CD2) or 4×10^8 (PrP) infectious unit (IU) per ml, was injected into the heart of chicken embryos after incubation of about 55 h. The injection volume was 2.0–3.0 μl per embryo.

2.3. Southern blotting

Genomic DNA extracted from the blood of GM chickens using a genomic DNA preparation kit (MagExtractor; Toyobo, Osaka, Japan) was digested with XbaI. The digested DNA was electrophoresed on a 0.8% agarose gel and then transferred to a nylon membrane (Hybond-XL; GE healthcare, Buckinghamshire, UK). The L-chain probe was prepared from the pMSCV/ΔALIH (CD2) plasmid by digestion with SalI and labeled with ^{32}P (BcaBEST Labeling kit; Takara, Japan). The GFP probe was prepared from the pMSCV/ΔALIH (PrP) plasmid by digestion with NotI and labeled using a commercially available kit (ECL Direct Nucleic Acid Labeling and Detection System; GE healthcare). Hybridized signals were detected by autoradiography or using a kit based on chemiluminescence (ECL detection system; GE healthcare) for anti-CD2 or anti-PrP, respectively.

2.4. Measurement of antibody concentration

A solid-phase enzyme-linked immunosorbent assay (sandwich ELISA) was performed to measure the antibody concentration in the serum, egg white and yolk of the GM chickens, as descried previously (Hotta et al., 2004; Kamihira et al., 2005). Rabbit anti-human IgG (Fc specific) (Organon Teknika, Durham, NC, USA) was used as the primary antibody. Rabbit anti-human IgG antibodies conjugated with peroxidase (Organon Teknika) were used as the secondary antibodies and the substrate *o*-phenylenediamine were used for detection. The antibody concentration was quantified using a human Fc fragment (Jackson ImmunoResearch, West Grove, PA, USA) or chimeric antibodies purified from egg white as described below.

2.5. Western blot analysis

For Western blot analysis, samples produced in the serum, egg white and yolk were diluted 5-fold with phosphate buffered saline (PBS). After removing insoluble matters by centrifugation (20,000 × *g*, 10 min, 4 °C), a small but sufficient amount of protein-A Sepharose beads (rProtein A Sepharose™ Fast Flow; GE healthcare) were added to the solution to capture the antibodies and incubated overnight at 4 °C. After washing 5-times with PBS, the beads were suspended in SDS-sample buffer with or without 2-mercaptoethanol and boiled for 5 min. The samples were electrophoresed on 10% or 7.5% SDS-polyacrylamide gels (reducing or non-reducing conditions, respectively) and transferred onto a polyvinylidene fluoride (PVDF) membrane (GE healthcare). The PVDF membrane was then immersed in a blocking solution containing 5% skimmed milk and 0.05% Tween 20 in PBS. After washing with PBS containing 0.05% Tween 20, the membrane was incu-

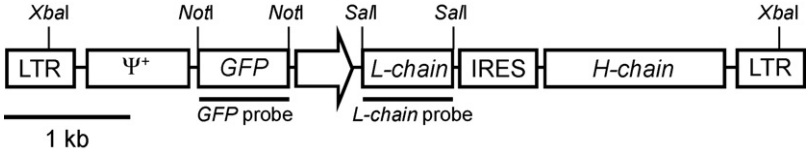

Fig. 1. Structure of the retroviral vector for the production of chimeric monoclonal antibodies. LTR, long terminal repeat; Ψ+, virus packaging signal sequence; GFP, green fluorescent protein gene; Pact, chicken β-actin promoter; L-chain, antibody light chain gene; IRES, internal ribosomal entry site sequence from EMCV; H-chain, antibody heavy chain gene. Locations of probes (GFP and L-chain probes) for Southern blot analysis are also indicated.