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Production of human erythropoietin by chimeric chickens

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Abstract

The use of transgenic avian allows cost effective and safe production of pharmaceutical proteins. Here, we report the successful production of chimeric chickens expressing human erythropoietin (hEpo) using a high-titer retroviral vector. The hEpo expressed by transgenic hens accumulated abundantly in egg white and had *N*- and *O*-linked carbohydrates. While attachment of terminal sialic acid and galactose was incomplete, portions of *N*- and *O*-linked carbohydrates were present. *In vitro* biological activity of egg white–hEpo was comparable to that produced by recombinant CHO cells.

Keywords: Transgenic chicken; Retroviral vector; Erythropoietin; Carbohydrate

As an alternative method for producing large amounts of proteins, various livestock species have been used to generate transgenic animals [1–3]. Transgenic chickens have been established by gene transfer to embryo mediated by lentivirus [4] and retroviruses [5,6], and by transfection of non-viral DNA to ES cells [7]. Although several methods have been developed to manipulate chicken embryos or embryonic cells for establishment of transgenic chicken, model proteins such as GFP have been used in these experiments, with only a few reports on the production of pharmaceutical proteins directly applicable to diagnostics [6,7].

In the *N*-linked carbohydrate of IgG, the terminal sialic acid of the sugar moiety is *N*-acetyl-neuraminic acid in human, while almost all other vertebrates have *N*-glycoyl-neuraminic acid as terminal sialic acids [8]. Among the livestock that has been established as transgenic animals, only chickens contain solely the human-relevant *N*-acetyl-neuraminic acid as a terminal residue in *N*-linked carbohydrate [8]. In general, this fact gives an advantage to chicken as transgenic livestock. However, Zhu et al. reported recently that *N*-linked carbohydrate of an antibody produced and deposited in egg white by transgenic chicken does not contain terminal sialic acids [7]. Since the addition of terminal sialic acid profoundly affects the half-life of proteins in serum, incomplete sugar modification presently limits the usefulness of chicken transgenic technology.

In this regard, we examined the variation of the sugar moiety in human erythropoietin (hEpo) produced and accumulated in egg white of transgenic chicken in the present study. hEpo is a glycoprotein consisting of 166 amino acids with a calculated molecular weight of 18,399 that stimulates the proliferation and maturation of erythroid precursor cells [9]. In the molecule, there are three potential *N*-glycosylation and one *O*-glycosylation sites. Carbohydrate, especially terminal sialic acid, considerably affects the serum half-life and *in vivo* activity of hEpo [10]. To date, several groups have explored the possibility of producing hEpo in the milk of transgenic mammals [11–13], but the production level has been low. In the

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present study, we successfully used chimeric hens to produce hEpo in milligram quantities in egg white and analyzed the nature of sugar modification.

Materials and methods

Vector construction. hEpo cDNA sequence was amplified by PCR with the primers summarized in Supplementary Table 1. hEpo cDNA was ligated to the HindIII-digested retroviral plasmid vector pMSCV/GΔAscFvFc, which contains a single-chain FvFc cDNA [14]. Instead of the single-chain FvFc gene, hEpo cDNA was inserted in this plasmid construct to generate the vector plasmid pMSCV/GΔAhEpo(c). The plasmid pWHV8, which contains the WPRE sequence of Woodchuck hepatitis virus, was obtained from the ATCC (GenBank Accession No.: J04514). This sequence stabilizes mRNA [15]. The amplified DNA fragment containing WPRE sequence and pMSCV/GΔAhEpo(c) were digested with ClaI and ligated. This plasmid was designated as pMSCV/GΔAhEpo(c)W and is shown in Supplementary Fig. 1A.

Retroviral vector production and injection into chicken embryos. The packaging cell line GP293/MSCV/GΔAhEpo(c)W was established as reported previously [6]. Packaging cells were transfected with pVSV-G (Clontech), and virus particles were concentrated by centrifugation. Microinjection and culture of embryos were performed as reported [6].

Determination of transgene copy number by real-time PCR. Genomic DNA was isolated from organs, erythrocytes, and sperm using Mag extractor-genome-(Toyobo). Twenty nanogram of purified DNA was used for real-time PCR using LightCycler Faststart DNA Master Hybprobe (Roche). Primers and probes were designed inside a packaging signal region of mouse stem cell virus (Supplementary Table 1), which allows direct comparison of data with other transgenic chickens for calculation of copy number. As a copy number standard, erythrocytes from a G_2 transgenic chicken producing scFvFc [6] (one copy) and from non-transgenic chickens (zero copy) were used.

Detection and measurement of hEpo produced by chimeric chickens. hEpo samples from serum and egg white of chimeric chickens were separated on a 10% SDS–polyacrylamide gel. After transfer to a PVDF membrane (GE Healthcare), hEpo was detected with rabbit anti-human Epo antibody (R&D Systems) and goat anti-rabbit IgG-HRP (Santa Cruz Biotechnology) with recombinant hEpo produced by CHO cells (Epoetin Beta; EPOGIN, Chugai Pharmaceutical) as a control. For enzyme-linked immunosorbent assay (ELISA), anti-hEpo monoclonal antibody, purified from culture supernatant of a hybridoma (HB-8209, ATCC) using a protein A column, and rabbit anti-hEpo antibody were used. As a standard, egg white–hEpo, whose quantity was determined by a Recombigen EPO radioimmunoassay kit (Mitsubishi Kagaku Iatron) was used.

Partial purification of hEpo from serum and egg white. Egg white was mixed using a magnetic stirrer for 15 min at room temperature, and 5 volumes of deionized water were then added. After adjusting the pH of the solution to 5.0, the sample was mixed for 15 min followed by centrifugation at 5000 rpm at 4 °C for 10 min. The supernatant was recovered, pH was adjusted to 7.1, and the sample was stored at −80 °C until required. Serum and egg white samples were absorbed to Blue-sepharose (GE Healthcare) equilibrated with 50 mM Tris–HCl (pH 7.0), the resin was washed with the same buffer, and hEpo was eluted with 50 mM Tris–HCl (pH 7.0) containing 1 M NaCl.

Enzymatic release of carbohydrates. For removal of *N*-linked carbohydrate, CHO-, serum-, and egg white–hEpo samples were digested with 0.5 U of PNGase F (*N*-glycosidase from *Elizabethkingia meningoseptica*; Sigma–Aldrich) in 50 mM of sodium phosphate buffer (pH 7.5) containing 0.75% (v/v) Triton X-100 in a total volume of 50 μl at 37 °C for 90 min. For partial digestion, 0.05 U of PNGase F was used. For enzymatic removal of *O*-linked carbohydrate, hEpo samples were initially digested with 3 mU of Neuraminidase F (Marukin Bio) at 37 °C for 1.5 h, with 2.5 mU of *O*-glycosidase from *Streptococcus pneumoniae* (Sigma–Aldrich) in 50 mM of sodium phosphate buffer (pH 5.0) in a total volume of 20 μl and finally with PNGase F. For enzymatic removal of the terminal sialic acid, hEpo samples were digested with 3 mU of Neuraminidase F in 8 mM of sodium phosphate buffer (pH 7.0) in a total volume of 50 μl at 37 °C for 16 h. For enzymatic removal of the terminal galactose, hEpo samples were first digested with 2 mU of Neuraminidase F in 50 mM of sodium phosphate buffer (pH 5.5) in a total volume of 48 μl at 37 °C for 3 h, and then with 1 mU of β-galactosidase from *Streptococcus* 6646K (Seikagaku Kogyo) for 16 h. For all the experiments, samples corresponding to 24 IU of hEpo were used.

Lectin blotting. Untreated samples and samples treated with Neuraminidase F and β-galactosidase were electrophoresed, blotted to a PVDF membrane, and the terminal sugar residue was detected with a suitable lectin. α2–6 linked sialic acid was detected by a combination of *Sambucus sieboldiana* (SSA) lectin (Seikagaku Kogyo), mouse anti-SSA lectin antiserum, and goat anti-mouse IgG-HRP (Santa Cruz Biotechnology). For detection of α2–3 linked sialic acid, *Maackia amurensis* (MAM) lectin conjugated with biotin (Seikagaku Kogyo) was used. Terminal galactose was detected with RCA-120 lectin from *Ricinus communis* (Seikagaku Kogyo) and mouse anti-RCA lectin serum, as with the SSA lectin. For detection of terminal *N*-acetylglucosamine, the membrane was reacted to *Griffonia simplicifolia* (GS-II) lectin conjugated with biotin (EY Laboratories). Antisera against SSA and RCA lectins were obtained by immunization of BALB/c mice. After the detection, lectins were removed by treatment with a stripping buffer (250 mM glycine–HCl, pH 2.5 containing 1% SDS), and membranes were reprobed with anti-hEpo antibody.

Isoelectric focusing. An Ampholine PAG plate (pH 3.5–9.5, $T = 5\%$, $C = 3\%$; GE Healthcare) with an anode buffer (1 M H_3PO_4) and a cathode buffer (1 M NaOH) was used. After electrophoresis (500 V, 8 mA, 90 min, 10 °C), proteins were blotted onto a PVDF membrane and analyzed by Western blotting.

In vitro assay. Ba/F3 cells expressing murine erythropoietin receptor (kindly provided from Dr. Nagamune, The University of Tokyo) [16] were seeded at 1×10^4 cells/well in 96-well plates (IWAKI), and a series of diluted samples was added. After cultivation for 24 h, 18.5 kBq/well of [^{3}H-methyl] thymidine (GE Healthcare) was added, cells were cultured for an additional 24 h, and DNA was recovered on a glass microfiber filter (Whatman) using a cell harvester (Nunc). Isotope incorporation was measured by a liquid scintillation counter. Half maximal effective concentration (EC_{50}) was determined using GraphPad Prism (GraphPad Software).

Results

Production of chimeric chickens

The retroviral vector containing the hEpo expression cassette under control of the chicken β-actin promoter (Supplementary Fig. 1A) was introduced into the heart of each developing chicken embryo after incubation for 55 h at stages 13–15 of the staging of Hamburger and Hamilton [17]. Virus injection was attempted separately five times. In total, 90 embryos were injected with the virus preparations (titers of 3×10^8–5×10^{10} cfu/ml), and 24 embryos hatched (27%; Table 1).

Specificity of organs in gene delivery was studied with one male (#103) and one female (#117) chickens (Supplementary Fig. 1B). While the transgene was detected in a number of organs, the copy number differed between organs and between individuals. Since the virus was injected into the embryonic heart, the heart and erythrocytes showed a higher mosaic rate of 2.5–4.5 DNA copies per cell. It was noteworthy that at the stage of infection, primordial germ cells are migrating in blood stream [18], but the copy number in sperm was very low (Table 1). Male chickens with copy numbers exceeding 0.04 (theoretically