Recombinant proteins produced into yolk of genetically manipulated chickens are partly sialylated in *N*-glycan

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Abstract The transgenic chicken is a candidate for the production of biopharmaceutical proteins with several economic superiorities. In general, the addition of sialic acid at the terminal of *N*-glycan is important for the bioactivity of biopharmaceuticals including plasma half-life; however, sialic acid has not been detected in the *N*-glycan of proteins produced in the egg white of genetically manipulated chickens. In this study, the extracellular domain of the TNF receptor and single chain Fv fused to Fc (referred to

as TNFR/Fc and scFv/Fc, respectively) were purified from the egg yolk of genetically manipulated chickens and their sialylation in *N*-glycan was examined. In contrast to the glycan in egg white, yolk-derived proteins were partly sialylated. Lectin blot showed the existence of α 2,6-sialic acid on TNFR/Fc, which disappeared with the removal of *N*-glycan by PNGase. In scFv/Fc, up to 7 % of *N*-glycan contained sialic acid. Disialyl glycans, which were detected in serum-derived scFv/Fc in a previous study, were not found in the yolk sample. Ovarian follicular tissue, which surrounds growing yolk, expressed several neuraminidases, suggesting the partial truncation of glycan during the yolk transfer process from the blood.

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Introduction

Rapidly increasing demand for protein therapeutics has been encouraging the establishment of mass production platforms using transgenic animals. Chickens have several merits over mammals including their rapid maturation, ease of and small space requirement for breeding, and daily high protein production in eggs (Kamihira et al. 2004). Since many therapeutics are

glycoproteins that at least partly require *N*-glycosylation for their biological functions, the ability for *N*-glycosylation is a basic requirement for these production systems. Chickens essentially add *N*-acetylneuraminic acid as the terminal sialic acid of glycan; therefore, they have the potential to produce therapeutics with ‘humanized’ glycans, which may reduce undesired immune reactions (Raju et al. 2000). This is in contrast to glycans in mammalian species other than humans: *N*-glycolylneuraminic acid alone or both *N*-glycolyl- and *N*-acetylneuraminic acids have been added as the terminal residue of *N*-glycans. However, exogenously expressed antibodies and erythropoietin in the egg white of genetically manipulated chickens were shown to mainly possess the truncated forms of *N*-glycans without galactose and sialic acid (Kamihira et al. 2009; Kodama et al. 2008; Zhu et al. 2005), which was partly improved by the genetic introduction of the galactosyltransferase gene (Mizutani et al. 2012).

Newly hatched chickens are protected from pathogens by maternal antibodies (IgY, the counterpart of mammalian IgG and IgE) accumulated in the yolk. The uptake of yolk IgY into embryonic blood is mediated by transcytosis using the chicken yolk sac IgY receptor, which transports IgY through the endosome-mediated process (Tesar et al. 2008; West et al. 2004). On the other hand, the mechanism for transportation from the serum of the parental hen to the yolk is largely unknown, although selective accumulation of IgY and not IgA or IgM has been well known (Kowalczyk et al. 1985; Rose and Orlans 1981). In addition to endogenous chicken IgY, human and mouse IgGs, as well as Fc-fused proteins, injected into the blood stream of hens can be recovered from the yolk of laid eggs (Kawabe et al. 2006; Morrison et al. 2002). Due to this transport system, several recombinant proteins with the human Fc domain accumulated in the yolk of genetically manipulated chickens (Kamihira et al. 2005, 2009; Kyogoku et al. 2008; Penno et al. 2010): the single-chained Fv fragment fused to Fc (scFv/Fc), the extracellular domain of the TNF receptor fused to Fc (TNFR/Fc), and erythropoietin fused to Fc (EPO/Fc), as well as humanized antibody, were deposited in the yolk when they were expressed under the control of a ubiquitous promoter. Structural analysis of *N*-glycan on scFv/Fc purified from the serum revealed the considerable proportions of sialylated glycans (Kamihira et al. 2009), which

implies the potential to obtain sialylated Fc-fused proteins from the egg yolk of genetically manipulated chickens. In this study, we examined the sialylation of yolk-derived Fc-fused proteins.

Materials and methods

Reagents

Sambucus sieboldiana (SSA) and *Maackia amurensis* (MAM) lectins were purchased from Seikagaku Kogyo (Tokyo, Japan). Neuraminidase was purchased from Sanyo Fine (Osaka, Japan). PNGase (*N*-glycanase from *Elizabethkingia miricola*) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Horseradish peroxidase (HRP)-conjugated goat anti-human IgG and goat anti-mouse IgG antibodies were purchased from MP Biomedicals (Solon, OH, USA) and Santa Cruz Biotechnology (Santa Cruz, CA, USA), respectively. Goat anti-chicken IgY-biotin was purchased from Santa Cruz Biotechnology.

Analysis of sialylation in scFv/Fc from the yolk

The establishment and basic characteristics of genetically manipulated chickens that expressed scFv/Fc were described previously (Kamihira et al. 2005). These were generated by the injection of a concentrated retroviral vector into the heart of 2.5-day embryos. scFv/Fc was purified from the yolk of one of the highest producers in the first generation (G₀; #407, accumulating scFv/Fc at a concentration of 0.25–0.5 mg/mL in the yolk) as described previously (Kamihira et al. 2005). In brief, rProtein A Sepharose™ Fast Flow (GE Healthcare, Waukesha, WI, USA) was added to yolk that had been diluted 5 times with phosphate-buffered saline (PBS) and stirred overnight. The resin was packed in a column (φ10 mm × 65 mm) that was then extensively washed. scFv/Fc was eluted with 0.1 M glycine–HCl (pH 2.7), followed by neutralization with the addition of a seventh volume of 1 M Tris–HCl (pH 8.0). Purified protein was dialyzed against 10 mM ammonium acetate, and was subjected to glycosylation analysis with DEAE-HPLC to determine the charge of *N*-glycan after pyridylation labeling of the oligosaccharides obtained by hydrazinolysis (analyzed by Toray Research Center, Tokyo, Japan).

Detection of sialic acid in TNFR/Fc by lectin blot

The basic characteristics of the genetically manipulated chickens that expressed TNFR/Fc were described previously (Kyogoku et al. 2008). TNFR/Fc was purified from the yolk of G₀ #711, containing 30–60 µg/mL TNFR/Fc, in a similar manner to scFv/Fc, except that the sample was dialyzed against PBS. In some experiments, the purified TNFR/Fc protein (590 ng) was treated with 3 mU neuraminidase for 24 h at 37 °C in a 50 mM phosphate buffer (pH 6.0) to remove sialic acid. To remove *N*-glycan, 500 ng TNFR/Fc was treated with 0.24 U PNGase in a 50 mM phosphate buffer (pH 7.5) supplemented with 0.72 % (v/v) Triton X-100 at 37 °C for 20 h. An aliquot of the reaction mixture was further treated with 0.5 mU neuraminidase. Samples were analyzed by lectin blot as described previously (Kodama et al. 2008; Mizutani et al. 2012). In brief, samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by transfer to a polyvinylidene difluoride membrane. The existence of sialic acid was detected by the sequential incubation of the membrane with SSA lectin, anti-SSA mouse antiserum, anti-mouse IgG-HRP, and chemiluminescent reagent (ECL plus, GE Healthcare). After development, the membrane was incubated with 63 mM Tris-HCl (pH 6.7) containing 100 mM 2-mercaptoethanol and 2 % (w/v) SDS for 1 h at 60 °C to remove antibodies. The Fc domain was then detected by western blot using anti-human IgG-HRP.

Quantitative reverse transcription (RT)-PCR

A White Leghorn hen was dissected and total RNA was extracted from the ovarian follicular tissue and the liver by the illustra RNAspin Mini RNA Isolation kit (GE Healthcare). Total RNA was reverse transcribed by ReverTra Ace (Toyobo, Osaka, Japan) using oligo-dT as a primer. Real-time PCR was performed using LightCycler (Roche Diagnostics, Mannheim, Germany) in 20 µL reaction mixture containing 10 µL Platinum SYBR Green qPCR SuperMix-UDG (Life Technologies, Carlsbad, CA, USA), 750 nM of each primer, and 2 µL of sample DNA. LightCycler amplification involved a first denaturation at 95 °C for 120 s followed by amplification of the target DNA for 45 cycles (94 °C for 15 s, 57 °C for 30 s, and 72 °C for 15 s). The amount of each gene was determined with

LightCycler Software version 3.5 (Roche Diagnostics). Expression levels were normalized to that of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene. The primers used are as follows. Neuraminidase 1 (*NEU1*), direct: 5'-TGCTGAACATCCGCAACCAG-3' and reverse: 5'-TTCATACGCCCGTTCTGACAC-3'; neuraminidase 2 (*NEU2*), direct: 5'-CCTTGTTCTCTTGCTCTTTG-3' and reverse: 5'-ATCAGCAAAGGTGATTTTCAGAAG-3'; neuraminidase 3 (*NEU3*), direct: 5'-AGAGGAAGATTTTAGGGATGCG-3' and reverse: 5'-CACCCAGTTCCTTTCTACACC-3'; neuraminidase 4 (*NEU4*), direct: 5'-TGCTTTACTCACACCCAC-3' and reverse: 5'-CTCCATGTAAGCCAGGTCTG-3'; and GAPDH, direct: 5'-GGGCACGCCATCCTATC-3' and reverse: 5'-GTGAAGACACCAGTGACTCC-3'. Primers for neuraminidase 1–4 were designed based on NCBI sequence data (BG710400.1, XM_001231584, XM_428099, and XM_003641732, respectively).

Results

scFv/Fc produced in the yolk is partly sialylated

We previously reported the establishment of genetically manipulated chickens that produced scFv/Fc under the control of a ubiquitous actin promoter. High levels of scFv/Fc were detected in the serum and the yolk as well as in the egg white. Since yolk-derived scFv/Fc likely originated from the serum, we analyzed the sialylation pattern of recombinant protein that was deposited in the yolk of a genetically manipulated chicken and compared it with that in the serum. Staining with Coomassie Brilliant Blue (CBB) revealed that single major band corresponding to digested form of scFv/Fc was detected in the sample for sialylation analysis (Fig. 1, inset). Although yolk contained both intact and digested forms of scFv/Fc (Kamihira et al. 2005), first fraction eluted from Protein A column contained only the digested form. Anion exchange chromatography revealed that approximately 93 % of the *N*-glycans in scFv/Fc from the yolk were the neutral asialo-form, while 7 % were monosialylated glycans (Fig. 1). Yolk-derived scFv/Fc did not contain the disialyl forms, which were detected in serum-derived scFv/Fc (Kamihira et al. 2009). These results indicate that scFv/Fc recovered from the yolk was partly sialylated.

Fig. 1 Partial sialylation of scFv/Fc from the yolk of genetically manipulated chicken. Sialic acid analysis of *N*-glycan of scFv/Fc from the yolk. Excised and labeled *N*-glycan was analyzed by DEAE-HPLC. *Inset* CBB staining of the analyzed sample. SDS-PAGE gel was stained with 0.25 % CBB R-250 in water, 2-propanol and acetic acid (13:5:2) and destained with this solution without CBB

TNFR/Fc produced in the yolk is partly sialylated in *N*-glycan

We previously reported that TNFR/Fc accumulated in the yolk of genetically manipulated chickens (Kyogoku et al. 2008). Three and 10 putative sites have been identified in TNFR/Fc for the addition of *N*- and *O*-glycan, respectively. Thus, the glycosylation of yolk TNFR/Fc was studied. TNFR/Fc was partially purified from the yolk followed by analysis with lectin blot. The yolk sample from a wild-type chicken served as the control. As shown in Fig. 2a (right panel), the anti-Fc antibody detected several molecular species of TNFR/Fc in the yolk, as was previously reported (Kyogoku et al. 2008). The major band above 61 kD was broad (61–73 kD, and there appeared to be several protein bands), possibly by differential glycosylation (see below). There were several bands of smaller sizes (about 55 kD and a fainter band), which were yolk-specific because they were not observed in a serum sample (Kyogoku et al. 2008). The smaller size protein species may have been possibly generated by partial

Fig. 2 Sialylation of TNFR/Fc from the yolk of genetically manipulated chicken. **a** Staining of TNFR/Fc by SSA lectin. Yolk sample of normal chicken (WT) served as the control. GM, genetically manipulated chicken; *IgY; **the broad band above 61 kD that may contain TNFR/Fc and IgY. **b** Bands with SSA lectin disappeared with the neuraminidase treatment. *Left* lectin blot using SSA lectin, which recognizes α 2,6-sialic acid. *Right* TNFR/Fc was detected by the anti-Fc antibody

proteolysis. On the other hand, the antibody did not detect any protein in the wild-type chicken. SSA lectin, which is specific to α 2,6-sialic acid, showed the appearance of several bands in the yolk of the genetically manipulated chicken (Fig. 2a, left panel). A broad band above 61 kD (61–73 kD, indicated by **) and a 55 kD band were observed with SSA lectin. In addition, bands were observed with a molecular mass of 35 and 24 kD, which were not detected by the anti-Fc antibody (Fig. 2a, right panel). Several bands were also detected in the control yolk by SSA lectin. The sialylated protein that appeared in the control yolk was of a size that overlapped the upper (higher molecular mass) part of the broad 61 kD TNFR/Fc band in reducing SDS-PAGE

(indicated as * in Fig. 2a, left panel). The overlapping band seemed to hamper the detection of sialic acid on TNFR/Fc in the upper part of the 61 kD band by SSA lectin (Fig. 2a, left panel). Mobility was also close to that of TNFR/Fc under non-reducing condition (approximately 140 kD, data not shown). This suggested dimer formation of the protein. Western blot using a specific antibody identified the protein as IgY (data not shown), which formed the dimer structure. Although IgY is usually considered not to bind to Protein A/G, it was likely that a small portion of IgY, within the large amount of IgY in the yolk, weakly bound to Protein A-Sepharose and was copurified with TNFR/Fc. Contaminated IgY and TNFR/Fc could not be separated by stepwise elution with buffers of gradually increasing acidity (data not shown). Currently, we do not know which of Protein A and Sepharose bound to IgY; this will be important in purifying antibodies and Fc-fused proteins from yolk in the future. SSA detection of IgY appeared to be consistent with the report that IgY contains two *N*-glycans: one high mannose-type and one biantennary complex-type oligosaccharide, and 33 % of these oligosaccharides contained sialic acid (Suzuki and Lee 2004).

As shown in Fig. 2b (left panel), the neuraminidase treatment, which removes both α 2,6- and α 2,3-sialic acids, completely eliminated the SSA bands including the broad band above 61 kD and the 55 kD band in genetically manipulated chicken. The bands detected by the anti-Fc antibody in non-treated sample (broad band >61 and 55 kD) disappeared and four bands (65, 59, 50 and 44 kD) appeared after the neuraminidase treatment (Fig. 2b, right panel). The reduction in the molecular mass of TNFR/Fc after neuraminidase digestion suggested the existence of sialic acid in the smeared 61 kD and the 55 kD bands.

To confirm the sialylation of *N*-glycan, the protein was treated with PNGase, which removes *N*- and not *O*-glycan, with or without subsequent neuraminidase treatment, and was analyzed by lectin blot. The molecular mass of TNFR/Fc detected by the anti-Fc antibody was reduced by the removal of *N*-glycans in the absence of neuraminidase (Fig. 3, right panel), as reported previously (Kyogoku et al. 2008). On the other hand, the IgY band in the SSA lectin blot, with a molecular mass over 67 kD that was also detected in the wild-type chicken (Fig. 2a, left panel), exhibited the same molecular mass before and after the PNGase treatment (Fig. 3, left panel). The reason why PNGase

Fig. 3 Existence of α 2,6-sialic acid in the *N*-glycan of TNFR/Fc. TNFR/Fc from the yolk was sequentially digested by PNGase to remove *N*-glycan, and then by neuraminidase to remove sialic acid, and was analyzed by SSA lectin (left panel) and the anti-Fc antibody (right panel). Asterisks show the major TNFR/Fc species after the treatment with PNGase and neuraminidase

did not remove the *N*-glycan of IgY is not clear. IgY has a specific structure that lacks a flexible hinge region; thus, it is possible that this difference may have affected the reactivity of PNGase. As shown in Fig. 3 (left panel), after the removal of *N*-glycan by the PNGase treatment, SSA lectin did not bind to the TNFR/Fc bands, which were detected by the anti-Fc antibody. This result suggests the existence of α 2,6-sialic acid in the *N*-glycan of TNFR/Fc in the smeared 61 kD and the 55 kD bands. We could not analyze the existence of α 2,6-sialic acid after *O*-glycan removal, because *O*-glycanase requires the pre-depletion of terminal sialic acid to remove *O*-glycan. As long as we tried, α 2,3-sialic acid was not detected by specific MAM lectin in the TNFR/Fc sample (data not shown). Since RT-PCR analysis showed that α 2,3-sialyltransferase was expressed in several tissues in the chicken including the liver (data not shown), and the reactivity of commercially available MAM lectin seemed to be relatively low, we could not rule out the possibility that TNFR/Fc from the yolk contained α 2,3-sialic acid in *N*-glycan.

By neuraminidase treatment following PNGase digestion, the molecular mass was reduced (indicated as * in Fig. 3, right panel). This suggested the existence of sialic acid in *O*-glycan. The most common structure of sialic acid in *O*-glycan is α 2,3-form (Fukuda 2002), which was consistent with the lack of the SSA band for TNFR/Fc after PNGase digestion.

Expression of genes that putatively modify glycans in ovarian follicular tissue

We previously analyzed the glycan structure of serum-derived scFv/Fc and found that more than 10 % of glycan had sialic acid, including more than 7 % of the disialyl form (Kamihira et al. 2009). However, the sialylation of scFv/Fc in the yolk was reduced as shown in Fig. 1. The lower amount of sialic acid in the yolk implies the possibility that the ovarian follicular tissue surrounding the developing oocytes may process *N*-glycan, such as the removal of sialic acid, during transfer from the serum to the yolk. Four neuraminidases have been identified in mammals: those that reside in mainly lysosomes (NEU1), in the cytoplasm (NEU2), in the membranes (NEU3), and in mitochondria/lysosomes (NEU4) (Monti et al. 2010). Four homologous sequences were found in the chicken nucleotide database, though only the enzymatic activity of NEU3 has been confirmed (Giacopuzzi et al. 2011) and the NEU1 orthologue was just reported as the EST fragment (Giacopuzzi et al. 2011). To identify the possible mechanism for the reduced levels of sialic acid in the yolk, ovarian follicular tissue was harvested and subjected to RT-PCR analysis (Fig. 4). Among the four putative neuraminidases, *NEU3* was expressed in ovarian follicular tissue at a similar level to that in the liver. *NEU3* localizes to caveolae, the membrane domain that mediates some types of endocytosis in mammals. However, *NEU3* was highly specific for ganglioside; thus, it is unlikely that *NEU3* was involved in the modification of scFv/Fc. Ovarian follicular tissue expressed substantial level of *NEU1*, approximately 15 % of that in the liver. *NEU1* is ubiquitously but differentially expressed in various cell types and its preferred substrates are both α 2,3- and α 2,6-sialyl linkage on glycoproteins, and the distribution of *NEU1* is not restricted to lysosomes: it was detected on the cell surfaces of various cell types (Hinek et al. 2006; Liang et al. 2006). These are consistent with the notion that *NEU1* decreased the sialic acid content of scFv/Fc during transfer to the yolk. The expression levels of *NEU2*, which mainly digests sialic acid on ganglioside, but less efficiently on glycoproteins, with a preference for α 2,3-sialyl linkage, and *NEU4* with a wide substrate specificity were expressed in ovarian follicular tissue at a low level. These results suggest that ovarian follicular tissue may have the machinery to modify protein

Fig. 4 Expression analysis of the putative neuraminidase genes. Expression in the ovarian follicular tissue was compared with that in the liver by RT-PCR. Expression levels were normalized to that of GAPDH. White bar liver; black bar ovarian follicular tissue

glycosylation during transfer to the yolk and one possible candidate may be *NEU1*, although careful analysis is required in the future.

Discussion

Antibodies and Fc-fused proteins can accumulate in the egg yolk of transgenic chickens since they can be transported to the egg yolk from the serum. In this study, we showed that recombinant proteins produced in the yolk of genetically manipulated chickens were partly sialylated. This is in clear contrast to the protein from the egg white. To our knowledge, this is the first indication that recombinant proteins containing the sialylated *N*-glycan can be recovered from the eggs of genetically manipulated chickens. Recently, several proteins were fused with the Fc domain of human IgG to make delivery into the blood by inhalation possible (Bitonti and Dumont 2006; Bitonti et al. 2004; Czajkowsky et al. 2012; Lee et al. 2007; Low et al. 2005; Vallee et al. 2012). These drugs can be

transported into the blood via the neonatal Fc receptor expressed on lung epithelial cells. In general, serum proteins require sialylation to extend plasma half-life. For example, EPO/Fc that had more sialic acid exhibited a longer plasma half-life and higher in vivo activity (Im et al. 2011). On the other hand, sialylation in Fc does not affect the plasma half-life of the antibody, while the sialylation of IgG Fc was reported to enhance biological activity: sialic acid with an α 2,6 linkage on Fc enhanced the anti-inflammatory activity of human IgG in intravenous immunoglobulin (IVIG) therapy (Anthony et al. 2008). These findings suggested the importance of the sialylation of drug proteins. Therefore, the yolk of transgenic chickens could be a source of sialylated proteins, although further improvements are necessary.

We previously reported that the molecular mass of serum-derived TNFR/Fc was higher than that of yolk, and that this difference disappeared after the removal of *N*- and *O*-glycans (Kyogoku et al. 2008). In this study, we showed that the extent of sialylation of scFv/Fc obtained from the serum of genetically manipulated chickens (Kamihira et al. 2009) was different from that of the yolk (Fig. 1). One possible reason for reduced sialylation in the yolk is the trimming of glycans during transfer to the yolk. To this end, we studied the expression of neuraminidases in ovarian follicular tissue since scFv/Fc may be transported to the yolk through those cells. Among them, NEU1 may catalyze desialylation. On the other hand, we cannot rule out the possibility that desialylation may occur in the yolk as has been observed with the proteolysis of vitellogenin by cathepsin D in the yolk (Deeley et al. 1975; Retzek et al. 1992). Further study is required to clarify this point.

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