Establishment and analysis of genetically manipulated chickens producing pharmaceutical Fc-fusion proteins

Fc 領域を含む医薬品タンパク質を生産する遺伝子導入鳥類の作製と解析

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Chapter 1

General introduction

The development of recombinant DNA technology has made it possible to genetically modify various organisms. The use of such technology greatly promotes both basic and applied research. For example, the role of each gene product was investigated *in vivo* using transgenic and gene-knockout mice in basic research, and several useful proteins including pharmaceuticals have been produced using this technology. Among host organisms, *Escherichia coli* has been used for the production of simple peptide/proteins because they are cost-effective. However, *E. coli* cannot be used for the production of complex proteins such as hormones because they lack most post-translational modifications. Thus, several pharmaceutical products such as hormones and antibodies are currently produced in mammalian cells, particularly CHO cells. CHO cells are superior to *E. coli* in several ways, including the existence of post-translational modifications such as glycosylation and the ability to support proper protein folding and assembly. Although the cost-effectiveness and productivity in CHO cells have been greatly improved, further improvement is still under vigorous investigation (1).

Genetically modified animals could provide an alternative production system offering high productivity, cost-effectiveness, and the ease of scaling up. Several proteins have been produced in transgenic mammals because

they have high protein productivity in milk. For example, human serum albumin and anti-thrombin-III were produced in the milk of transgenic cows and goats, respectively. Because a chicken lays approximately 330 eggs/year, a large quantity of pharmaceutical proteins can be obtained if they are accumulated in eggs. Chickens offer several advantages compared with the other genetically modified animals, such as short period for sexual maturation and small space requirement for breeding. However, the gene transfer technique in chickens has not been as well developed compared with that in mammals because of several reasons. First, the microinjection in a fertilized chicken egg is more difficult than that in mammalian ones because large yolk prevents microscopic observation of embryos. Second, a fertilized egg when laid already contains approximately 60,000 cells. Thus, the only way to obtain a single-cell stage fertilized egg is by sacrificing a hen. Third, the injection of plasmid DNA has induced only transient expression of target genes in varios studies (2-8).

Recently, our group and others have reported the production of genetically manipulated chickens using retroviral vectors. We have used a replication-defective pantropic retroviral vector, which was originally developed for gene therapy. We produced genetically manipulated chickens by injecting the concentrated virus into the heart of an embryo, which had been incubated for 55 h (stage 14-15) (9, 10). Using this strategy, we succeeded in producing genetically manipulated chickens, expressing anti-prion single-chain Fv fragment fused to Fc (scFv/Fc), antibodies, and erythropoietin (9, 11, 12). In these studies, we used a ubiquitous actin

promoter to express the target genes and succeeded in the accumulation of target protein into eggs. In case of anti-prion scFv/Fc, the amount of antibody in the egg white was as high as 200 mg/egg, which is a considerably higher value than previously reported for proteins produced by transgenic chickens (9).

Inflammation is an important immune response against pathogens; however, undesired or strong inflammation can cause tissue damage and various diseases such as rheumatoid arthritis, certain allergies, and cardiovascular diseases. Inflammation is mediated by several molecules such as reactive oxygen and nitrogen species as well as inflammatory cytokines. Among these mediators, tumor necrosis factor-α (TNF-α) plays an important role to induce inflammation, and several drugs against TNF-α have been developed for clinical use. For example, anti-TNF-α antibody (infliximab) was developed as a drug for Crohn's disease and is currently also used for rheumatoid arthritis and psoriasis. Moreover, soluble TNF-α receptor (TNFR) is effective in neutralization of TNF-α by working as a decoy that binds TNF-α. For example, the hybrid protein (TNFR/Fc, etanercept), in which the extracellular domain of TNF-α receptor type II is fused with the Fc domain of IgG to prolong its half-life in blood because of binding neonatal Fc receptor, is now used for therapy of rheumatoid arthritis and juvenile idiopathic arthritis as Enbrel (13, 14).

In chapter 2, we selected TNFR/Fc as a model protein and the recombinant protein was produced by genetically manipulated chickens. Concentrated retroviral vector expressing TNFR/Fc under the control of

actin promoter was injected into chicken embryos; eggs containing these embryos successfully hatched. These chickens produced TNFR/Fc in the blood. Because Fc-fusion proteins are transferred from the blood into yolk, we could obtain TNFR/Fc from the egg yolk after sexual maturation. I also calacterized TNFR/Fc produced in the yolk.

Recently, the structures of glycans that are attached to pharmaceutical proteins have drawn considerable attention. It is known that glycan structures can affect the activity of pharmaceuticals. In general, lack of terminal sialic acid residue reduces the blood half-life of serum proteins. In addition, IgG having α2,6-sialic acid linkage on its glycan moiety exhibits increased anti-inflammatory activity in intravenous immunoglobulin (IVIG) therapy (15). Therefore, sialylation of Fc-fused proteins may affect their biological activity.

In chapter 3, we analyzed the glycans of the yolk-derived recombinant proteins. In the analysis, we focused on the sialic acid residue in the *N*-linked glycan of anti-prion scFv/Fc and TNFR/Fc, which were purified from the yolk.

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Chapter 2

Production of recombinant tumor necrosis factor receptor/Fc fusion protein by genetically manipulated chickens

Introduction

To date, transgenic livestock animals such as goat and sheep have been generated for the production of recombinant proteins in milk (2-4). Chickens and quails have also attracted considerable attention as alternative animals as the host for transgenic bioreactors, since they have a relatively short breeding time for sexual maturation (6, 16). We and other research groups have reported the production of recombinant proteins using genetically manipulated avian species (7-9, 17, 18). In most cases, recombinant proteins were accumulated in egg white, since the recovery of exogenous proteins from yolk has several obstacles.

Newly hatched chickens are protected from pathogens by maternal antibodies (IgY) accumulated into yolk from maternal blood during yolk formation, which is mediated by Fc receptors on the ovum (19-23). By using this mechanism, the process by which specific antibodies are collected from

yolk after the immunization of hens with particular antigens has been widely carried out. In addition, Morrison *et al.* reported that human and mouse IgGs injected into the blood stream of hens can be collected from yolk of laid eggs (24). This suggests that mammalian IgG can also be transported to and accumulated in yolk through a mechanism similar to that of IgY accumulation. We also reported the difference in the yolk transport among subclasses of IgGs and Fc-fusion proteins (25).

Tumor necrosis factor-a (TNF-α) has various cytological effects and causes autoimmune and inflammatory diseases (26, 27). Two types of TNF-α receptors (TNFRs) with molecular mass of 55 kD and 75 kD involved in the TNF-α signaling pathway are known (28). The extracelluar domains of the two receptors are homologous and have been produced as chimeric proteins with the Fc region of IgG for therapeutic use (27, 29-31). Although the extracellular domain of TNFRs alone can bind to TNF-α and block the interaction between TNF-α and cell surface TNFRs (32), the fused proteins exhibit stronger activity because the truncated form of the receptor is unstable *in vivo* (29). Now, the fused protein of TNFR II and Fc is used for the treatment of inflammatory diseases such as rheumatoid arthritis, psoriatic arthritis, ankylosing spondylitis and psoriasis (33).

In this study, we describe the production of a recombinant fusion protein of TNFR II and Fc region of human IgG1 (TNFR/Fc) in yolk of genetically manipulated chickens.

Materials and Methods

Reagents

Human TNF-α was purchased from ProSpec-Tany TechnoGene (Rehovot, Israel). Recombinant human TNFR/Fc produced by mouse myeloma NSO cells was purchased from R&D Systems (Minneapolis, MN, USA). This protein molecule contains 5 residues of additional amino acids as a linker, and a histidine tag (6 residues) at the C-terminal, compared with the TNFR/Fc produced in this study.

Retrovirus vector construction and virus production

We constructed two types of plasmids, pMSCV/GDA-TNFR/Fc and pQMSCV/GDA-TNFR/FcW for the production of replication-defective retroviral vectors derived from mouse stem cell virus (MSCV) (34, 35). pMSCV/GDA-TNFR/Fc was based on the pMSCVneo plasmid (Clontech, Mountain View, CA, USA), while pQMSCV/GDA-TNFR/FcW was based on the Q vector system (Clontech) (35, 36). The construction of pMSCV/GDA-TNFR/Fc was described in our previous report (35). Briefly, the scFv-Fc gene of the pMSCV/GDAscFvFc plasmid (9, 18) was replaced with the extracellular domain of the human TNFR2 gene and the Fc gene of the human IgG1. pQMSCV/GDA-TNFR/FcW was constructed by transferring the entire provirus region of pMSCV/GDA-TNFR/Fc except for a portion of the 5' long terminal repeat (LTR) to pQCXIX and then inserting a posttranscriptional regulation element derived from Woodchuck hepatitis

virus (WPRE), which can stabilize mRNA (37), just downstream of the TNFR/Fc coding sequence (35). In these constructs, TNFR/Fc is expressed under the control of the chicken b-actin promoter. Schematic drawings of the retroviral vectors are shown in Fig. 2-1.

VSV-G pseudotyped pantropic retroviral vectors were produced by a similar method as described in our previous studies (9, 38). Briefly, the virus producer cell lines were established by infecting the viral vector transiently produced using pMSCV/GDA-TNFR/Fc into a retroviral packaging cell line GP293 (Clontech) and successive cloning with the aid of limiting dilution. The viral particles were produced by the transfection of a VSV-G expression vector plasmid pVSV-G (Clontech) into the virus-producing cells using a lipofection reagent (Lipofectamine 2000; Invitrogen, Carlsbad, CA). For pQMSCV/GDA-TNFR/FcW, virus particles were produced by transient cotransfection of the virus vector plasmid and pVSV-G to GP293 cells. The culture medium containing viral particles was concentrated by ultracentrifugation in both cases. The viral titer was determined with NIH3T3 cells and indicated as infectious unit (IU).

Microinjection of viral vectors into avian embryos and embryo culture

The embryo culture was carried out using the method of Perry (39) with some modifications as essentially described in our previous report (40). After the laid fertilized eggs (White Leghorn) were incubated for 50-55 h at 37.8˚C with rocking (stage 14-15 according to Hamburger and Hamilton [10]), the

viral solution was injected into the heart of developing embryos (1.5-3.5 ml per embryo) as described previously (9, 38). All the experiments were performed according to the ethical guidelines for animal experimentation of Nagoya University.

Measurement of TNFR/Fc concentration

The TNFR/Fc concentration was determined by enzyme-linked immunosorbent assay (ELISA). An ELISA plate was coated with goat anti-human IgG (MP Biomedicals, Solon, OH, USA), and the protein was detected with goat anti-human IgG antibody conjugated with peroxidase (POD) (MP Biomedicals) using *o*-phenylenediamine as the substrate. The TNFR/Fc concentration was determined by making calibration curves using commercially available purified Fc or TNFR/Fc from NSO cells as the standard.

Western blot analysis

Samples were electrophoresed on a 7.5% polyacrylamide gel and transferred onto a polyvinylidene fluoride (PVDF) membrane (Hybond-P; Amersham Biosciences, Piscataway, NJ, USA). TNFR/Fc was detected with POD-conjugated goat anti-human IgG antibody (MP Biomedicals) followed by the detection of POD activity based on chemiluminescence reaction (Western Lightning Chemiluminescence Reagent Plus; Perkin Elmer Life Sciences, Boston, MA, USA).

Purification of TNFR/Fc

The egg yolk was diluted 5 times with phosphate- buffered saline (PBS) and centrifuged at 10,000 x *g* repeatedly until no precipitate was observed after centrifugation. The supernatant was passed through a protein A column (rProtein A Sepharose Fast Flow; Amersham Biosciences) equilibrated with PBS, and absorbed TNFR/Fc was eluted using 0.1 M glycine-HCl buffer (pH 2.7) followed by immediate neutralization with 1 M Tris-HCl buffer (pH 8.0). Serum-derived TNFR/Fc was purified by a similar method.

Enzymatic release of *N*- and *O*-linked carbohydrates

TNFR/Fc in the samples (0.4 mg as TNFR/Fc) was digested with PNGase (*N*-glycanase from *Chryseobacterium* sp., Sigma-Aldrich, St Louis, MO, USA), which specifically digests *N*-linked glycans, in 50 mM sodium phosphate buffer (pH 7.5) containing 0.75 % Triton X-100 for 90 min at 37˚C. A portion (0.2 μg) of the samples was further digested with 3 mU of Neuraminidase F (Marukin-Bio, Kyoto) at 37˚C for 1.5 h in 50 mM sodium phosphate buffer (pH 5.0), then 2.5 mIU of *O*-glycosidase derived from *Streptococcus pneumoniae* (Sigma-Aldrich) was added, followed by incubation for an additional 18 h at 37˚C to remove *O*-linked carbohydrates.

In vitro bioassay

The bioactivity of TNFR/Fc was measured using L929 cells on the basis of neutralizing activity toward the cytotoxic effect of TNF-α (41). L929 cells

were obtained from the Riken BioResource Center (Tsukuba) and maintained in MEM (Invitrogen) supplemented with 10% horse serum, 40 mg/ml penicillin and 45 mg/ml streptomycin. Cells (4×10^4 cells/well) were cultured in a 96-well microtiter plate for 3 days and the medium was changed to a fresh one containing 2 mg/ml actinomycin D, 0.16 ng/ml TNF-α and serially diluted TNFR/Fc samples. After incubation for 18 h, the viable cell number was measured using a cell counting kit (Cell Counting Kit-8; Dojin, Kumamoto) according to the supplier's instruction. Before the experiments, it was confirmed that the observed value of OD_{450} is proportional to the viable cell number (data not shown).

Results

Generation of chickens expressing TNFR/Fc

As reported previously, transgene expression was suppressed when the retroviral vector was introduced to avian embryos at the blastodermal stage (9, 18, 38). Therefore, the viral vector containing a TNFR/Fc expression cassette was introduced into the heart of developing embryos after 55 h of incubation. With this procedure, the viral vector was efficiently delivered to the entire embryo through circulating blood stream without the suppression of transgene expression (9, 18). Table 2-1. summarizes the experiments of retroviral injection into chicken embryos. Chicken embryos injected with the viral vectors steadily hatched. The average hatchabilities were 15% and 44% for pMSCV/GDA-TNFR/Fc and pQMSCV/GDA-TNFR/FcW, respectively. The values are comparable to those in previous studies with different vector construct (9). After sexual maturation, the copy numbers in sperm DNA of several male chickens (nos. 414, 601 and 708 in Table 2-2.) were determined by real-time PCR as reported previously (9). The average copy number was quite low, less than 0.005 for all the chickens tested. Judging from the results of our previous study (9) and the copy number in this study, it is estimated that we need to analyze more than 500 offspring to obtain a full transgenic progeny. Thus, we did not carry out mating experiments.

The TNFR/Fc concentration in the serum of manipulated chickens is shown in Table 2-2. Chickens generated in the second injection experiment for pMSCV/GDA-TNFR/Fc (Table 2-1.) produced the highest levels of the

target protein: 200-350 mg/ml TNFR/Fc was detected in the serum. Several male and female chickens were selected for analyzing the stability of the transgene expression. As shown in Figure 2-2., these chickens stably produced TNFR/Fc in the serum for 6 months. For hens, the accumulation of TNFR/Fc in the yolk and egg white was also studied after sexual maturation (Table 2-2.). In all the hens tested, the TNFR/Fc concentration in the yolk was much higher than that in the egg white. The highest value in the yolk was 25 mg/ml (no. 314) and the protein was stably produced over 6 months (Fig. 2-2.). The expression levels in the yolk were 10-30% of those in serum, which is slightly less than that for recombinant scFv-Fc in our previous study (9, 18), and for endogenous IgY (42). These results indicated that a portion of the TNFR/Fc produced in the serum of genetically manipulated chickens was transported to the yolk, as reported in our previous study, where TNFR/Fc was injected into the vein of hens (25).

Characterization of TNFR/Fc produced by chickens

Then, the TNFR/Fc was characterized after partial purification from yolk and serum using a protein A column. Figure 2-3. shows the results of Western blotting. The TNFR/Fc derived from NSO cells and that produced by manipulated chickens showed broad bands indicating that the proteins are modified with polysaccharide chains. The apparent molecular weight of the TNFR/Fc derived from NSO cells and the serum of a manipulated chicken was approximately 70 kD. However, the band corresponding to TNFR/Fc from the yolk was slightly smaller. This suggests a difference in

posttranslational modification between the samples. In addition, a smaller band with a molecular mass of 55 kD was detected in the yolk sample.

To further characterize the glycosylation pattern of TNFR/Fc in the different samples, the carbohydrates of the proteins were enzymatically removed, and the changes in the molecular weight were analyzed by Western blotting (Fig. 2-4.). There are two possible *N*-glycosylation sites in the extracellular domain of TNFR and one in the Fc region, and 10 putative *O*-glycosylation sites in the extracellular domain of TNFR. Upon removal of the *N*-linked carbohydrate, the NSO- and serum-derived samples yielded two bands, and the major band of the NSO-derived TNFR/Fc had a lower molecular weight than that of the serum TNFR/Fc. On the other hand, the complete removal of carbohydrates by the digestion with *N*- and *O*-specific glucanases yielded a single band. These results suggested that the TNFR/Fc samples derived from NSO cells and the serum of a manipulated chicken were heterogeneous in terms of their glycosylation modification. Although both TNFR/Fc samples gave a single band by SDS-PAGE after the complete removal of carbohydrates, the mobilities of these two proteins were still different. This difference (2 kD) seems to be bigger than that estimated from the variation in amino acid residues in the linker and tag sequences (1 kD). The reason is currently unknown but we cannot rule out the possibility that other modifications such as phosphorylation or partial proteolysis occurred in either sample. For the sample from yolk, three bands with molecular mass of 56, 49 and 44 kD were observed by the complete removal of *N*- and *O*-linked carbohydrates. This indicates that proteolytic digestion occurred in

more than two different positions.

We then analyzed the *in vitro* biological activity of TNFR/Fc on the basis of an inhibitory effect of TNF-α-mediated apoptosis in L929 cells. As shown in Figure 2-5., the serum-derived TNFR/Fc showed strong activity almost comparable to that of commercially available TNFR/Fc, whereas the yolk-derived TNFR/Fc showed much lower activity: the concentration that gave the half-maximum value of the inhibitory effect was approximately 4 ng/ml for NSO- and serum-derived TNFR/Fcs under this experimental condition, while that for yolk-derived TNFR/Fc was almost ten times higher (40 ng/ml). This could be partly explained by the existence of degraded products in the yolk sample and overestimation of the amounts of intact TNFR/Fc, because the ELISA used in this study cannot discriminate the degradation products. However, judging from the intensity of each band shown in Figures. 2-3. and 2-4., this overestimation only partly contributes to the decreased specific activity of the yolk-derived TNFR/Fc. Although *N*-glycosylation patterns of the serum, and NSO cells derived TNFR/Fc were different (Fig. 2-4), *in vitro* bioactivity of them were almost the same (Fig. 2-5. and data not shown). This suggested that the *in vitro* neuralization activety is independent on glycosylation as was reported with type I TNFR fused to Fc (43) and reduced bioactivity of the yolk sample can not be explained by the difference in the sugar modification. In addition, we cannot rule out the possibility that the difference in protein structure and sugar modification reduces the stability of the protein, which may result in the loss of biological activity.

Discussion

In this study, we described the generation of genetically manipulated chickens producing TNFR/Fc, which is a promising drug for inflammatory diseases. The production of TNFR/Fc in the serum and yolk of manipulated chickens was stable for six months. Although we could detect a certain level of transgene expression in the oviduct of manipulated chickens by RT-PCR (data not shown), the accumulation of the protein in the egg white was quite low compared with that in the yolk. Thus, the reason for the apparent failure in the production in egg white is unclear. Nevertheless, our results clearly indicate the possibility for recovery of Fc-fused proteins from the yolk of genetically manipulated chickens.

Previously, we reported a difference in the transport efficiency of human and mouse IgG subclasses and Fc-fusion proteins into chicken eggs, when these proteins were injected into the vein of hens (25). Fc-fusion proteins, such as EphB1, CTLA4, TNFR and scFv fused with the Fc region of human IgG, were transported and accumulated into the yolk from blood, although the transport efficiencies were different among the proteins. Similarly, the TNFR/Fc that accumulated in the yolk in this study is probably transported from serum. In the case of endogenous IgY, the physical and biological characteristics of the protein in the serum and yolk are similar (20). However, the present study showed that the transported protein accumulated in the yolk is not a direct reflection of that in serum. First, small Fc-containing proteins corresponding to partially degraded species were detected in the

yolk sample by Western blotting. A similar degradation was observed in our previous study: while most human IgGs and Fc-fusion proteins were transported intact into the yolk, a major part of the transported scFv-Fc proteins in the yolk was digested between the scFv and Fc around the hinge region of Fc (25).

Second, the apparent molecular weights of the intact form of TNFR/Fc derived from chicken serum and yolk were different. One possible explanation for this difference is in posttranslational modification such as glycosylation modification. After the *N*- and *O*-glucanase digestion of the samples, the largest bands of the TNFR/Fc derived from the serum and yolk exhibited the same mobilies on SDS-PAGE, suggesting that the difference in the mobilities is due to the difference in the glycosylation pattern in addition to partial degradation. Whether the selective transport depending on the structure of carbohydrate chain or the modification during yolk formation is responsible for the generation of differences in the glycosylation pattern of the protein still remains to be studied. In addition, yolk specific Fc species with smaller molecular mass (55 kDa in Fig. 2-3.) was observed. Similar yolk specific band possibly a degradation product was observed with scFv/Fc accumulated in the yolk (9). Thus, it is possible that exogeneously expressed proteins that accumulate in yolk may partly cleaved. By now, the precise mechanism has not be clear, but it is noteworthy that a major yolk protein vitellogenin is cleaved by cathepsin. In order to produce intact proteins in the yolk, precise mechanism of the degradation need to be clarified.

In this study, we could not obtain transgenic progenies. To obtain

transgenic descendants efficiently, further improvement of transgenic technology for chickens is essential. One of the candidates for this may be the use of isolated primordial germ cells, which differentiate to sperm or oocyte. We are now trying to establish transgenic technology using primordial germ cells.

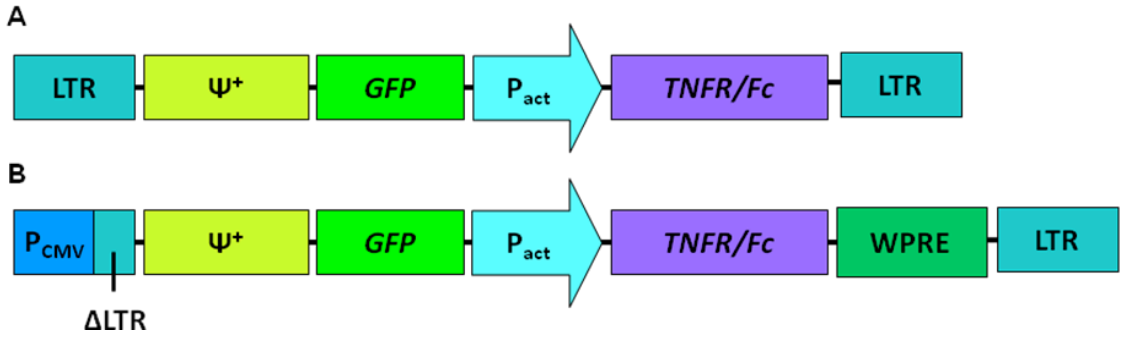

FIG. 2-1. Structure of retroviral vector plasmid. Although a part of the 3' LTR was deleted in the original Q-vector, the vector used in this study had an intact 3' LTR. Thus, in the form of integrated provirus, the only difference between the two vectors is the existence of the WPRE sequence. (A) Schematic representation of pMSCV/GΔATNFR/Fc vector. (B) Schematic representation of pQMSCV/GΔATNFR/Fc vector. LTR, Long terminal repeat; DLTR, truncated LTR; Ψ^+, packaging signal derived from MSCV; GFP, green fluorescent protein gene; P_{Act}, chicken ß-actin promoter; P_{CMV}, cytomegalovirus immediate early enhancer-promoter; TNFR/Fc, extracellular domain of human TNF receptor 2 fused with Fc region of human IgG1; WPRE, Woodchuck hepatitis virus posttranscriptional regulation element.

Table 2-1. Retroviral injection into chicken embryos

Vector construct	Expt no.	Viral titer (IU/ml)	Number of embryos		Hatchability (%)
			Injected	Hatched	
pMSCV/GΔATNFR/Fc	1	1.5×10^{7}	20	4	20
	2	1.8×10^{8}	19	2	11
pQMSCV/GΔATNFR/FcW	1	6.6×10^{9}	21	12	57
	2	5.3×10^{7}	24	7	29
	3	1.7×10^{9}	12	5	42
	4	3.0×10^{8}	13	7	54
Total			109	37	34

Table 2-2. Expression levels of TNFR/Fc in chickens

Vector construct	Expt no.	Chicken number	Sex	TNFR/Fc concentration (μg/ml)		
				Serum	Yolk	White
pMSCV/GΔATNFR/Fc	1	204	F	11.0±4.6	1.0±0.5	0.2±0.2
		205	F	12.4±2.6	1.2±1.0	0.2±0.0
		207	M	9.8±3.1	-[c]	-[c]
		218	F	4.1±3.1	0.7±0.5	ND[d]
	2	301	-[a]	374[b]		
		314	F	230±53.7	25.4±3.6	1.4±0.7
pQMSCV/GΔATNFR/FcW	1	404	M	69.3±19.7	-[c]	-[c]
		407	M	85.1±12.2	-[c]	-[c]
		410	M	81.1±9.8	-[c]	-[c]
		414	M	106±19.4	-[c]	-[c]
	2	513	F	9.8±2.9	2.2±1.4	ND[d]
		514	F	10.8±1.7	0.5	ND[d]
		521	M	8.9±1.0	-[c]	-[c]
	3	601	M	24.7±9.6	-[c]	-[c]
		608	F	17.8±2.9	5.3±2.9	0.2±0.1
		610	M	7.0±2.6	-[c]	-[c]
	4	708	M	8.0±1.3	-[c]	-[c]
		709	F	34.8±12.9	10.6±3.6	0.7±0.5
		711	F	37.4±4.1	11.0±3.1	1.4±0.5
		712	F	20.4±3.8	4.8±1.2	0.2±0.2

[a] Died before sex typing.

[b] The concentration was determined only once because the chicken died.

[c] Not applicable.

[d] Not detected.

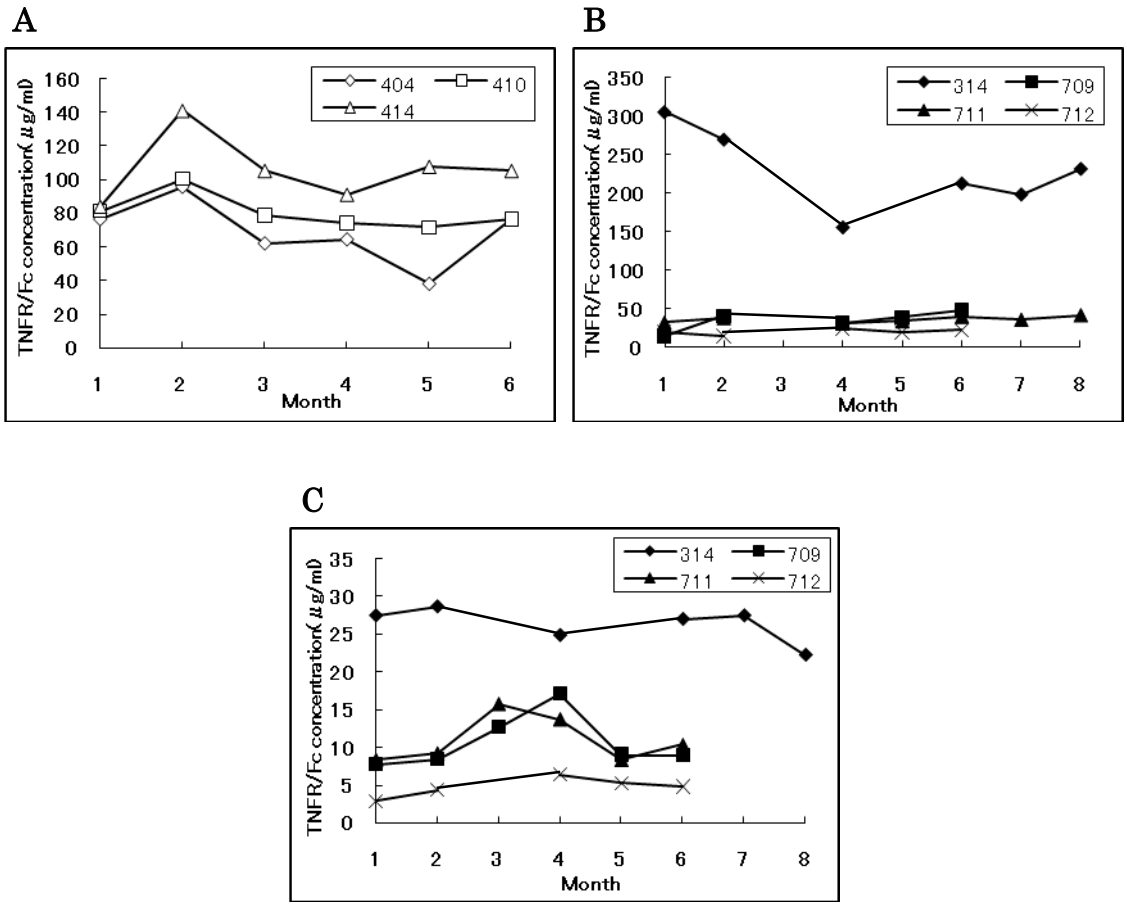

FIG. 2-2. Long-term (6 months) expression of TNFR/Fc by chickens. (A) Expression level of TNFR/Fc in serum (male). (B) Expression level of TNFR/Fc in serum (female). (C) Accumulation of TNFR/Fc in the yolk. Closed diamond, 314; closed squares, 709; closed triangles, 711; cross, 712, open diamond, 404; open squares, 410; open triangles, 414. Data extended to 8 months for 314 (serum and yolk) and 711 (serum) are also shown.

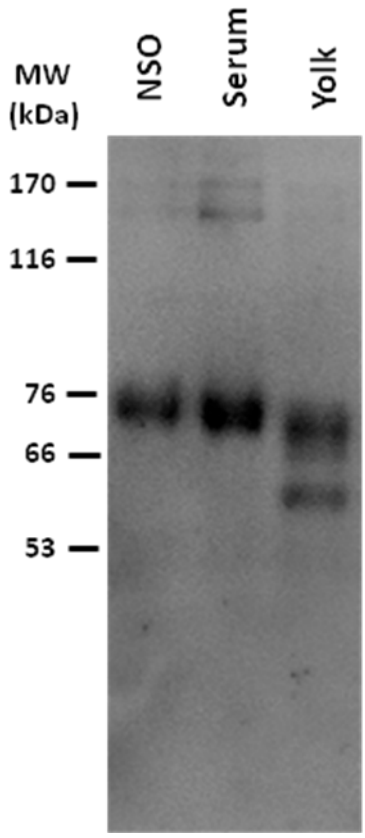

FIG. 2-3. Western blotting of TNFR/Fc proteins. TNFR/Fc proteins in the samples were detected using anti-Fc antibody.

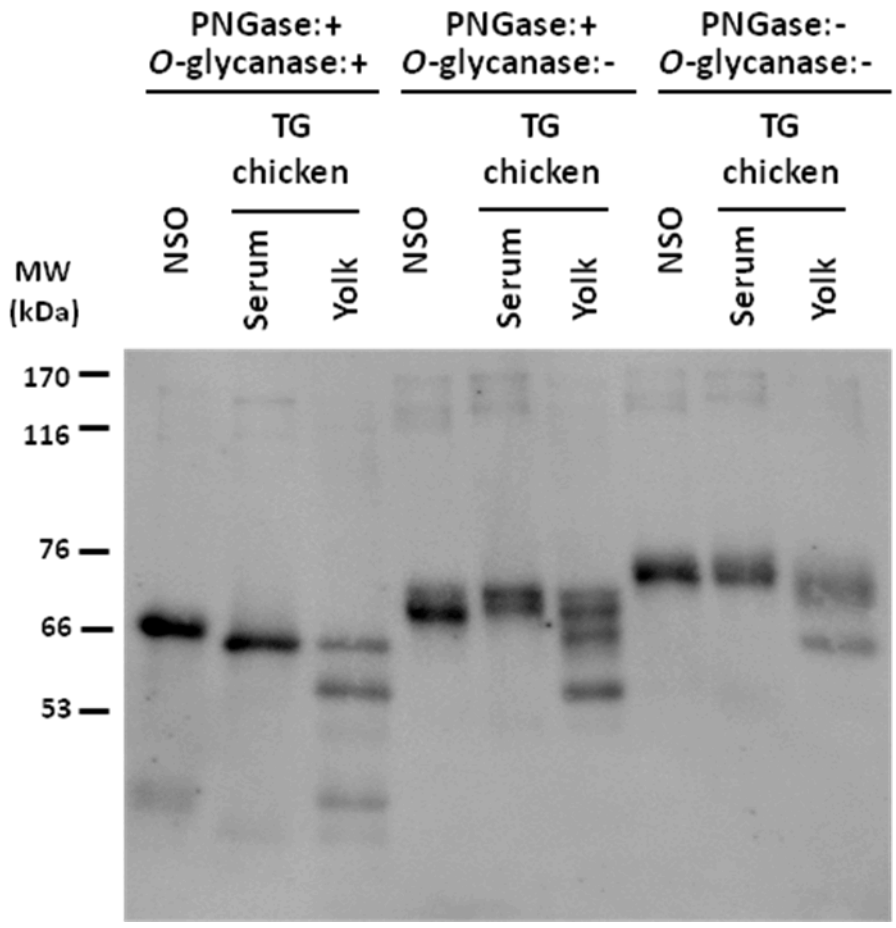

FIG. 2-4. Analysis of *N*- and *O*-linked carbohydrates in TNFR/Fc proteins by glycanase treatment. Each sample was either mock-treated or digested with glycanases and analyzed by Western blotting.

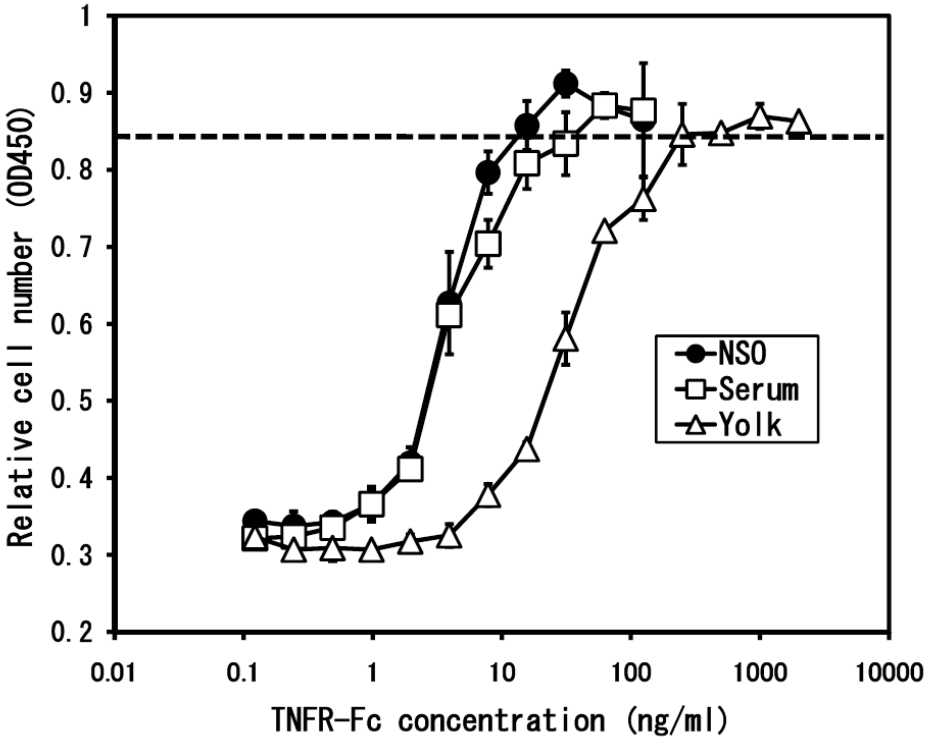

FIG. 2-5. *In vitro* bioactivity of TNFR/Fc. L929 cells were cultured in the presence of TNF-α. Indicated amounts of TNFR/Fc were added at the beginning of the culture. Broken line indicates the value without TNF-α. Closed circles, NSO; open squares, serum; open triangles, yolk. The concentrations of TNFR/Fc that yielded a half-maximum inhibition were estimated to be 4, 4 and 40 ng/ml for NSO-, serum- and yolk-derived TNFR/Fc, respectively.

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Chapter 3

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

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 (44). 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 (45). 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 (11, 12, 17), which was partly improved by the genetic introduction of the galactosyltransferase gene (46).

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 meditated by transcytosis using the chicken yolk sac IgY receptor, which transports IgY through the endosome-mediated process (47, 48). 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 (23, 49). 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 (24, 25). Due to this transport system, several recombinant proteins with the human Fc domain accumulated in the yolk of genetically manipulated chickens (9, 11, 50, 51): 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 (11), 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 (9). 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 (G_0; #407, accumulating scFv/Fc at a concentration of 0.25-0.5 mg/ml in the yolk) as described previously (9). 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 pyridylamination 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 (50). TNFR/Fc was purified from the yolk of G_0 #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 reaction mixture was further treated with 0.5 mU neuraminidase. Samples were analyzed by lectin blot as described previously (12, 46). 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’-CCTTGTTCTCTTGCCTCTTTG-3’ and reverse: 5’-ATCAGCAAAGGTGATTTTCAGAAG-3’; neuraminidase 3 (*NEU3*), direct: 5’-AGAGGAAGATTTTAGGGATGCG-3’ and reverse: 5’-CACCCAGTTCCTTTCTACACC-3’; and neuraminidase 4 (*NEU4*), direct: 5’-TGCTTTACTCACACCCCAC-3’ and reverse: 5’-CTCCATGTAAGCCAGGTCTG-3’; and GAPDH, direct: 5’-GGGCACGCCATCACTATC-3’ and reverse: 5’-GTGAAGACACCAGTGGACTCC-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 scFvFc was detected in the sample for sialylation analysis (Fig. 3-1., inset). Although yolk contained both intact and digested forms of scFvFc (9), first fraction eluted from Protin A column contained only 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. 3-1.). Yolk-derived scFv/Fc did not contain the disialyl forms, which were detected in serum-derived scFv/Fc (11). These results indicate that scFv/Fc recovered from the yolk was partly sialylated.

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 (50). 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 Figure 3-2. (A, right panel), the anti-Fc antibody detected several molecular species of TNFR/Fc in the yolk, as was previously reported (50). The major band above 61 kD was broad (and there appeared to be several protein bands), possibly by differential glycosylation (see below). There were several bands of smaller sizes (about 55 and 46 kD), which were yolk-specific because they were not observed in a serum sample (50). The smaller size protein species may have been possibly generated by partial proteolysis. On the other hand, the antibody did not detect any protein in the wild-type chicken. SSA lectin, which is specific to α2,6-sialic acid, showed the appearance of several bands in the yolk of the genetically manipulated chicken (Fig. 3-2. A, left panel). A broad band above 61 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. 3-2. A, 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. 3-2. A, 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. 3-2. A,

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 (52).

As shown in Figure 3-2. (B, left panel), the neuraminidase treatment, which removes both α2,6- and α2,3-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 kD and 55 kD) disappeared and four bands (65, 59, 50 and 44 kD) appeared after the neuraminidase treatment (Fig. 3-2. B, 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. 3-3., right panel), as reported previously (50). 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. 3-2, A, left panel), exhibited the same molecular mass before and after the PNGase treatment (Fig. 3-3., left panel). The reason why PNGase 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 Figure 3-3. (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 α2,6-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 α2,6-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, α2,3-sialic acid was not detected by specific MAM lectin in the TNFR/Fc sample (data not shown). Since RT-PCR analysis showed that α2,3-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 α2,3-sialic acid

in *N*-glycan.

By neuraminidase treatment following PNGase digestion, the molecular mass was reduced (indicated as * in Fig. 3-3., right panel). This suggested the existence of sialic acid in *O*-glycan. The most common structure of sialic acid in *O*-glycan is α2,3-form (53), 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 (11). However, the sialylation of scFv/Fc in the yolk was reduced as shown in Figure 3-1. 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) (54). Four homologous sequences were found in the chicken nucleotide database, though only the enzymatic activity of NEU3 has been confirmed (55) and the NEU1 orthologue was just reported as the EST fragment (55). 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. 3-4). 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 (54); 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 α2,3- and α2,6-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 (56, 57). 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 α2,3-sialyl linkage, and *NEU4* with a wide substrate specificity (54) were expressed in ovarian follicular tissue at a low level. These results suggest that ovarian follicular tissue may have the machinery to modify protein 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 (58-63). 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 (64). 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 α2,6 linkage on Fc enhanced the anti-inflammatory activity of human IgG in intravenous immunoglobulin (IVIG) therapy (15). 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 (50). In this study, we showed that the extent of sialylation of scFv/Fc obtained from the serum of genetically manipulated chickens (11) was different from that of the yolk (Fig. 3-1.). 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 (65, 66). Further study is required to clarify this point.

Recently, TNFR/hyFc having hybrid Fc (hyFc) has been produced (67, 68). Hybrid Fc contains CH2 and CH3 domains of IgG4 and the flexible linker domain of IgD. Therefore, TNFR/hyFc can suppress antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC). Thus, a functionally superior protein has been developed on the basis of the Fc-fusion protein TNFR/Fc. We hope that transgenic technology can support these kinds of newly developed TNFR/Fc derivatives.

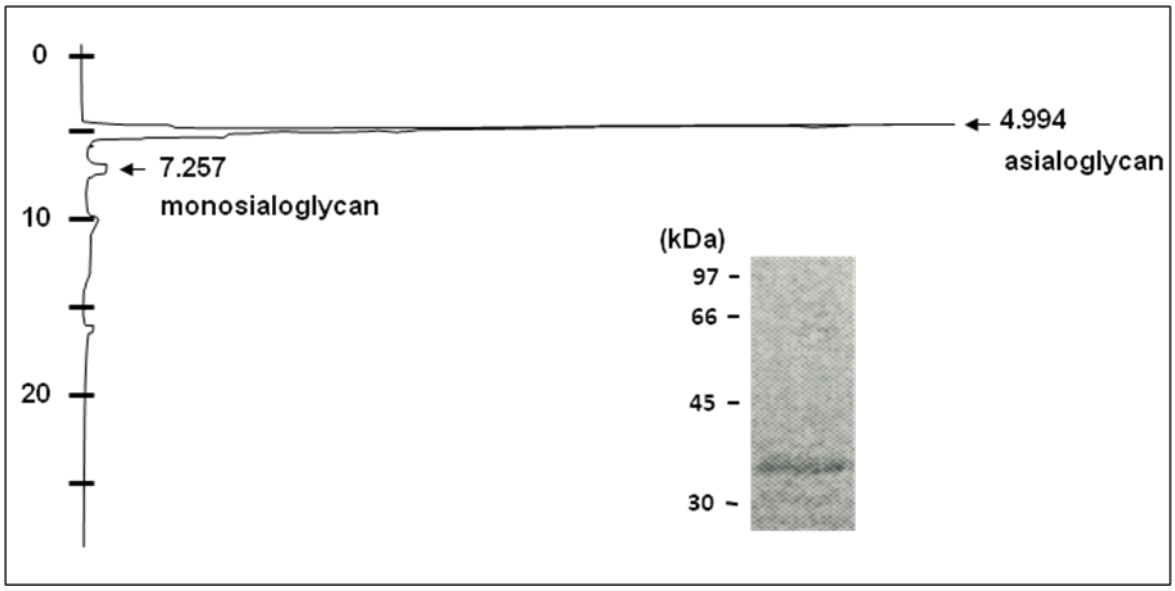

Fig. 3-1. Partial sialylation of scFv/Fc from the yolk of genetically manipulated chicken. Sialic acid analysis of *N*-glycan of scFv/Fc from the yolk. Excised and labeled *N*-glycan was analyzed by DEAE-HPLC. (Inset) CBB staining of the analyzed sample. SDS-PAGE gel was stained with 0.25 % CBB R-250 in water, 2-propanol and acetic acid (13:5:2) and destained with this solution without CBB.

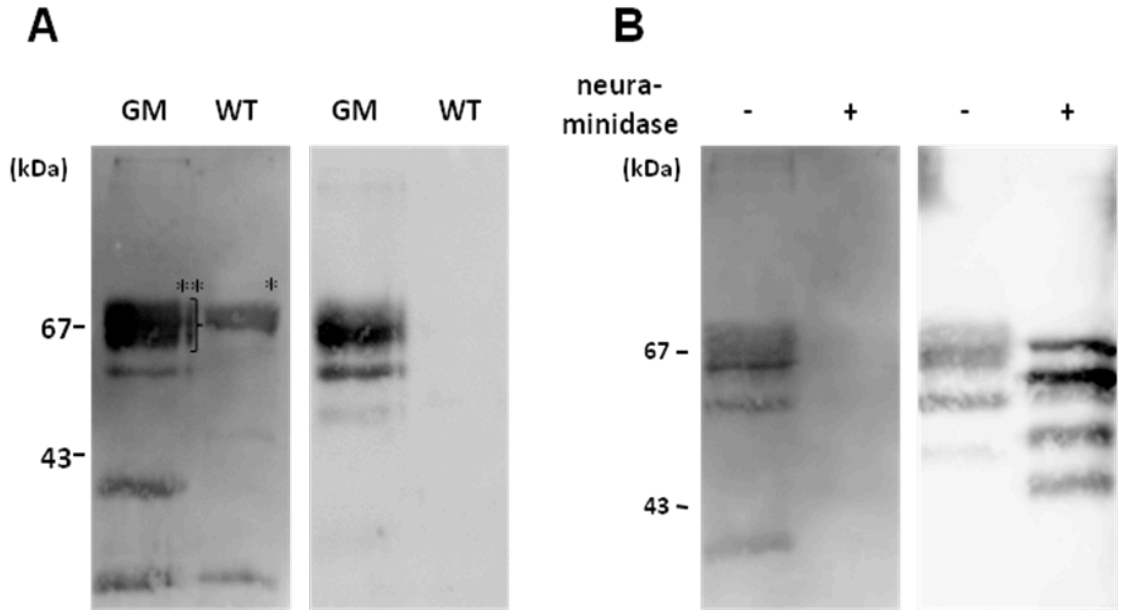

Fig. 3-2. Sialylation of TNFR/Fc from the yolk of genetically manipulated chicken. (A) Staining of TNFR/Fc by SSA lectin. Yolk sample of normal chicken (WT) served as the control. GM, genetically manipulated chicken; *, IgY; **, the broad band above 61 kD that may contain TNFR/Fc and IgY. (B) Bands with SSA lectin disappeared with the neuraminidase treatment. (A, left and B, left) Lectin blot using SSA lectin, which recognizes α2,6-sialic acid. (A, right and B, right) TNFR/Fc was detected by the anti-Fc antibody

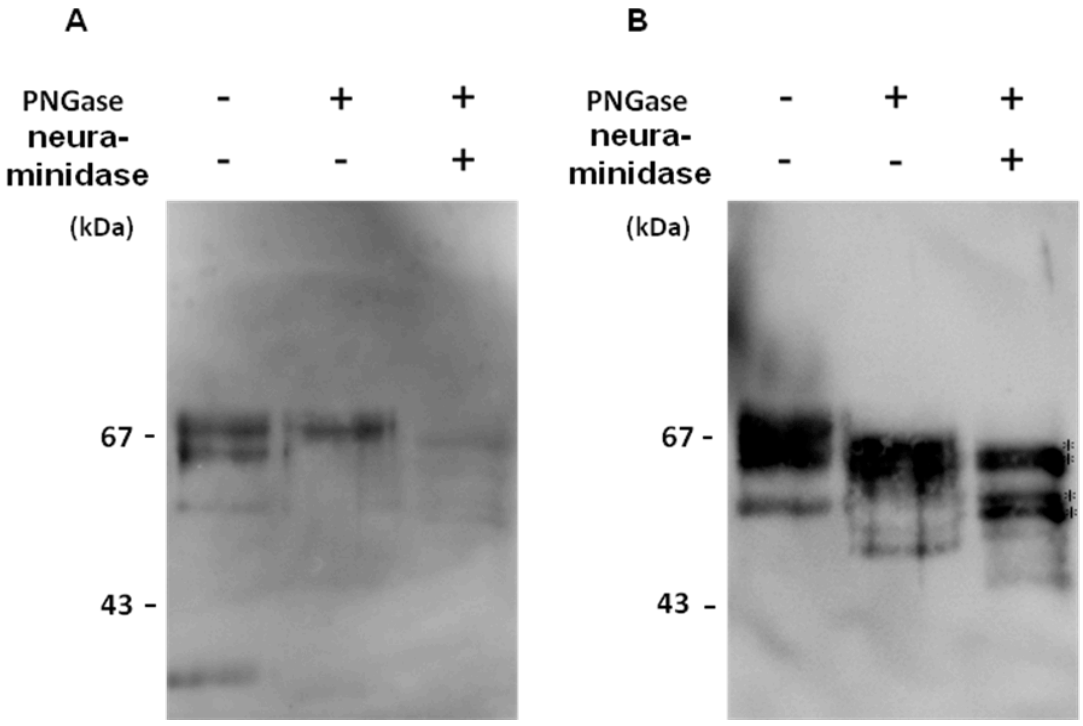

Fig. 3-3. Existence of α2,6-sialic acid in the *N*-glycan of TNFR/Fc. TNFR/Fc from the yolk was sequentially digested by PNGase to remove *N*-glycan, and then by neuraminidase to remove sialic acid, and was analyzed by SSA lectin (left panel) and the anti-Fc antibody (right panel). Asterisks show the major TNFR/Fc species after the treatment with PNGase and neuraminidase

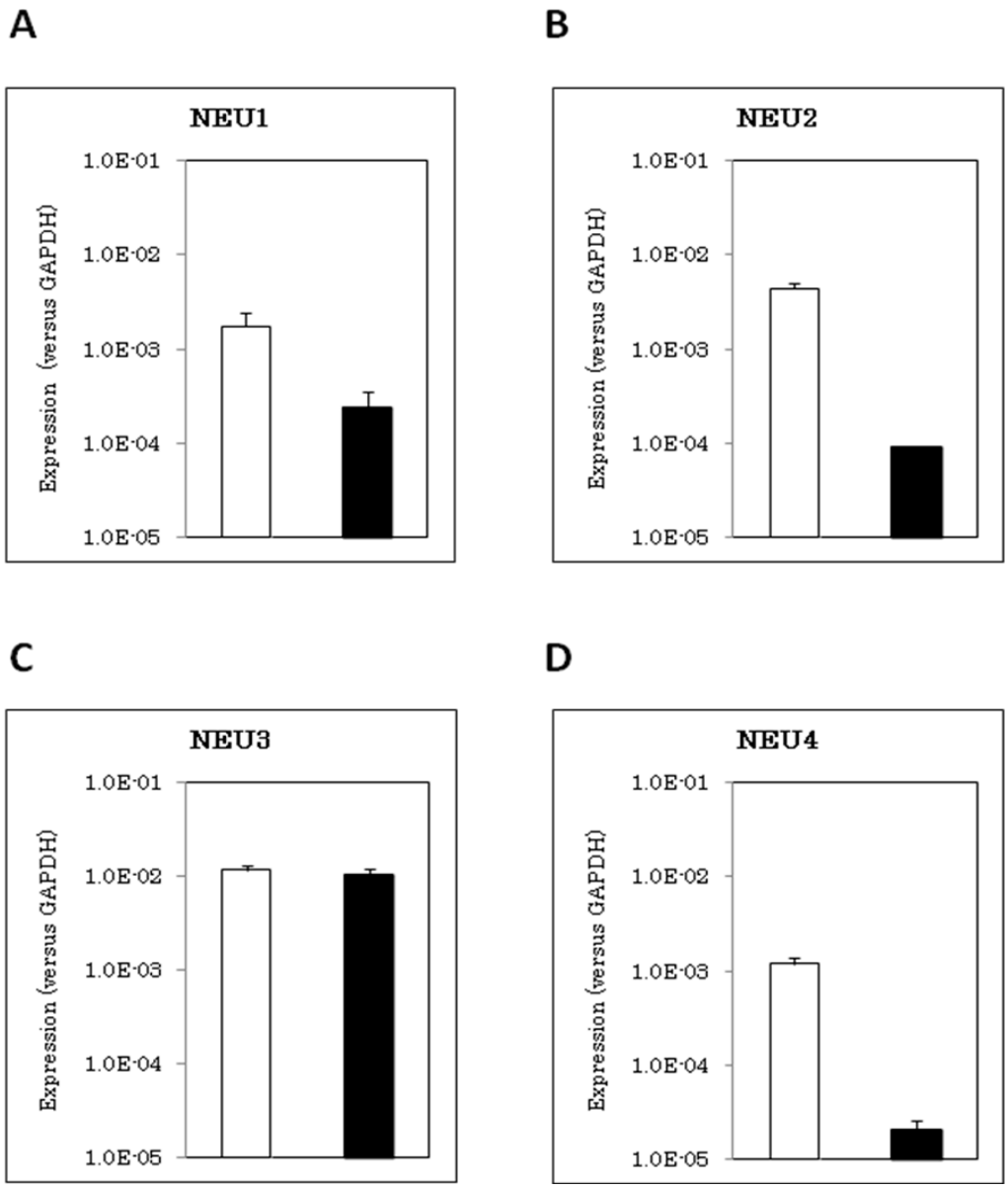

Fig. 3-4. Expression analysis of the putative neuraminidase genes. Expression in the ovarian follicular tissue was compared with that in the liver by RT-PCR. Expression levels were normalized to that of GAPDH. white bar, liver; black bar, ovarian follicular tissue

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Chapter 4

Concluding remarks

In recent years, various pharmaceutical proteins have been produced using recombinant DNA technology. Complex proteins such as antibodies are mainly produced using CHO cells because these cells support glycan modification of the exogenously expressed proteins making them suitable for human use. However, they have limitations including higher production cost. Thus, we try to establish genetically manipulated chickens as an alternative system for the production of useful proteins.

In chapter 2, we reported the generation of genetically manipulated chickens that produce TNFR/Fc. TNFR/Fc was expressed in various tissues under the control of actin promoter and was accumulated in serum and egg, particularly in the yolk. TNFR/Fc expression was stable for more than 6 months. Similar to TNFR/Fc derived from NSO cells, *N*- and *O*-linked glycans were also detected in TNFR/Fc derived from the serum and yolk. Furthermore, TNFR/Fc derived from the serum or yolk showed neutralization activity against TNF-α. These results indicate the benefits of using genetically manipulated chickens for the production of biologically active TNFR/Fc.

In chapter 3, the *N*-linked glycans of the yolk-derived recombinant proteins anti-prion scFv/Fc or TNFR/Fc, which were expressed in genetically manipulated chickens, were investigated. Sialic acid was added through an

α2,6-linkage to the terminal of *N*-linked glycan of yolk-derived anti-prion scFv/Fc. The ratio of sialylated glycans was lower than that of serum-derived anti-prion scFv/Fc. α2,6-sialic acid was also added to the *N*-linked glycan of yolk-derived TNFR/Fc. Our results suggested that α2,3-sialic acid was also added to *O*-linked glycan. Taken together, it appears that scFv/Fc or Fc-fusion proteins were accumulated in the yolk of genetically manipulated chickens in a partially sialylated form.

In summary, these observations suggest the potential benefits of using genetically manipulated chickens for the production of pharmaceutical proteins.

Publication list

Publications for this thesis

Kyogoku, K., **Yoshida, K**., Watanabe, H., Yamashita, T., Kawabe, Y., Motono, M., Nishijima, K., Kamihira, M.,and Iijima, S.: Production of recombinant tumor necrosis factor receptor/Fc fusion protein by genetically manipulated chickens. J. Biosci. Bioeng., 105, 454-459 (2008).

Yoshida, K., Okuzaki, Y., Nishijima, K., Watanabe, H., Kyogoku, K., Yamashita, T., Kawabe, Y., Motono, M., Kamihira, M., and Iijima, S.: Recombinant proteins produced into yolk in genetically manipulated chickens are partly sialylated in *N*-linked glycans. Cytotechnology, (2013).

Other publications

Kodama, D., Nishimiya, D., Iwata, K., Yamaguchi, K., **Yoshida, K**., Kawabe, Y., Motono, M., Watanabe, H., Yamashita, T., Nishijima, K., and other 2 authors: Production of human erythropoietin by chimeric chickens. Biochem. Biophys. Res. Commun., 367, 834-839 (2008).

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