N-Glycosylation Profiles of Chicken Immunoglobulin Y Glycoproteins Expressed by Different Production Vehicles

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Abstract

Immunoglobulin Y (IgY), abundant in egg yolk, is widely used as an immunochemical reagent and has been recently appreciated as a potential therapeutic tool. The Fc portion of IgY conserves an N-glycosylation site at Asn407, which is structurally equivalent to conserved glycosylation sites of other Ig classes in mammals. Despite such similarities, IgYs from chicken serum and egg yolk have been shown to be distinct glycoforms, best exemplified by the high incidence of monoglucosylated high mannose-type oligosaccharides, which are rarely found in mammalian glycoproteins. To gain further insight into the glycosylation properties of IgY, we report a comparative N-glycosylation profiling of recombinant chicken IgYs that have identical amino acid sequences in their variable regions but are expressed by different production vehicles. The N-linked oligosaccharides cleaved from these IgYs were subjected to multidimensional HPLC mapping in conjunction with mass spectrometric analyses. N-glycosylation profiles of the IgYs showed obviously different patterns depending on the production vehicle. While IgY expressed by chicken hybridoma cells retained the premature high mannose-type oligosaccharides, IgY expressed by CHO cells experienced extensive N-glycan processing and displayed high antenary oligosaccharides. Significant differences were also observed in the levels of core fucosylation and biecting N-acetylglucosaminylation and in sialyl linkage types among the IgYs expressed by different vehicles. Despite such differences, the recombinant IgY antibodies commonly expressed considerable populations of monoglucosylated glycoforms, suggesting that this glycosylation feature is, to some extent, associated with the distinctive quaternary structure of IgY-Fc, which, at least partially, masks the Asn407 N-glycans and sequesters them from chaperone mechanisms in the endoplasmic reticulum. We suggest that the timing and efficiency of N-glycan processing and the quaternary structure formation of IgY are considerably different from those of IgE and depend on the vehicles used for recombinant IgY production, resulting in varying incidence of monoglucosylated species.

Keywords: Immunoglobulin Y; N-glycosylation; HPLC mapping; Production vehicle; Chicken hybridoma; Monoglucosylated high mannose-type oligosaccharide

Introduction

Immunoglobulin Y (IgY) is a major antibody class in birds and reptiles. Because of their abundance in chicken egg yolk, IgYs are being used not only as immunochemical reagents but also as potential therapeutic tools [1-4]. Like mammalian IgE, avian IgY possesses one additional domain in each heavy chain instead of the flexible hinge region. N-glycosylation profiles of IgY glycoproteins isolated from hen and Japanese quail egg yolks have been studied extensively and the results showed that IgYs exhibit complex-type oligosaccharides as well as high mannose-type oligosaccharides [5,6]. Interestingly, in each case, more than half of the high mannose-type oligosaccharides (approximately 30% of total N-glycans) were identified as monoglucosylated forms, which are rarely found in mammalian glycoproteins. Chicken IgY has two conserved N-glycosylation sites in the constant region, Asn308 in the Cγ2 domain and Asn407 in the Cγ3 domain; the latter is structurally equivalent to the conserved N-glycosylation site Asn297 in the Cγ2 domain of mammalian IgG, which expresses biantennary complex-type oligosaccharides exclusively. Site-specific N-glycosylation profiling of IgY isolated from chicken serum has revealed that Asn308 expresses exclusively complex-type N-glycans, whereas Asn407 expresses only high mannose-type oligosaccharides [7].

This distinctive glycosylation pattern at the conserved N-glycosylation site has been attributed to differences in molecular structure between IgY and IgG, i.e., the Cγ3 domain versus the hinge region. Human serum IgE has been reported to express only high mannose-type oligosaccharides at the conserved N-glycosylation site (Asn394) [8]; however, monoglucosylation has not been detected in the IgE oligosaccharides, suggesting that the characteristic N-glycosylation profile of IgY cannot be ascribed simply to the existence of the additional domains. This raises the possibility that the efficiency of the N-glycan processing pathways in antibody-producing cells differs between birds and mammals.

To address this issue, we performed N-glycosylation profiling of chicken monoclonal IgY glycoproteins that have identical amino acid sequences in their variable regions but are expressed by different production vehicles, i.e., chicken hybridoma, HEK293T, and CHO cells, in comparison with polyglcylc IgY isolated from egg yolk.

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Materials and Methods

Materials

Materials used for the experiments were purchased from the sources indicated below. N-glycosidase F from Flavobacterium meningosepticum was purchased from BioLabs Inc. (Ipswich, MA), N-acetyl-β-D-galactosaminidase from jack bean was from Seikagaku Kogyo Co. (Tokyo, Japan), a-sialidase from Arthrobacter ureafaciens was from Nacalai Tesque (Kyoto, Japan), a2-3 sialidase from Salmonella typhimurium was from TAKARA Bio Inc. (Otsu, Japan), and trypsin and chymotrypsin were from Sigma Chemical Co. (St. Louis, MO). The pyridyldiamino (PA) derivatives of isomaltol-oligosaccharides 4-21 (indicating the degree of polymerization of glucose residues) and the PA derivatives of N-linked oligosaccharides (code nos. M5.1, M7.1, M7.2, M7.6, M8.1, M8.2, M8.3, M8.4, M9.1, M9.2, 210.1, 210.3, 210.4, 211.1, 211.2, 211.4, 310.11, 310.18, 1A1-200.3, 1A1-200.4, 1A1-210.4, 1A2-210.4, 1A3-210.4, 1A1-211.3, 1A1-211.4, 2A1-200.4, 2A3-200.4, 2A4-210.4, 2A1-211.4, 2A1-300.8, 3A3-300.8, 3A2-300.8, 3A2-310.8, 4A1-300.22, and 4A2-300.22 in GALAXY(http://www. glycoanalysis.info/galaxy2/ENG/index.jsp) [9] were purchased from Seikagaku Kogyo Co. and GLYENCE Co. (Nagoya, Japan).

Preparation of IgY glycoproteins

Egg: IgY was prepared from chicken egg yolk using the Eggcellent Chicken IgY Purification Kit (Pierce Biotechnology, Rockland, IL) according to the manufacturer’s instructions.

Chicken hybridoma cells: The chicken hybridoma (clone 3E1; MBL, Nagoya, Japan) was produced by fusion of chicken B-cell line MUHI with chicken splenocytes immunized with recombinant Fc fusion SynCAM [10]. The hybridoma cells were cultivated in Iscove’s modified Dulbecco’s Medium (IMDM) (Sigma) containing 15% fetal calf serum (FCS), 4 mM L-glutamine, 50 µg/mL gentamycin, and 50 µg/mL kanamycin. Monoclonal IgY was purified from the supernatant of confluent cells on an immunoadsorbent column obtained by covalently linking rabbit anti-chicken IgY-F(ab)2 to cyanogen bromide-activated Sepharose (GE Healthcare, Piscataway, NJ).

Mammalian cell lines: Total RNAs were isolated from the chicken hybridoma 3E1 using the RNeasy Mini Kit (QUIGEN, Valencia, CA). cDNAs were subsequently synthesized from the total RNA using a GeneRacer Kit (Invitrogen, Carlsbad, CA). DNA fragments encoding the IgY light chain and variable region of the heavy chain were amplified from the cDNA template by PCR using following primer sets: 5′-AATAGCGGCCGCACTATGATGCTGGGTCTCTCTCTC-3′ and 5′-AATATTATTTATTCTTGCCGACTGCTCTCTCAG-3′ for the light chain; 5′-AATAGCGGCCGCACTATGATGCCCACAGC-3′ and 5′-AATAGCGGCCGCACTATGATGCTCCAC-3′ for the variable region of the heavy chain.

The amplified light chain PCR fragment was subcloned into the pQClIP vector (Clontech, Palo Alto, CA) with NotI and PciI. The amplified PCR fragment of the heavy-chain variable region was subcloned with NotI and BsiWI into the pQC×IP vector (Clontech, Palo Alto, CA) with NotI and PacI.

HEK293T and CHO cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) (Sigma) containing 10% FCS and Nutrient Mixture F-12 Ham (Sigma) containing 10% FCS, respectively. Both cell lines were co-transfected with the two plasmids using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. After 48 h of culture, when the cells were at the confluent stage of growth, the media were collected and dialyzed in 40 mM phosphate buffer containing 150 mM NaCl (pH 7.5). Recombinant IgY antibodies possessing the myc epitope followed by the hexahistidine tag were expressed in the media and purified with a TALON column (Clontech) according to the manufacturer’s instructions.

Glycosylation profiling by HPLC mapping method

Experimental procedures, including chromatographic conditions, glycosidase treatments, and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS), were as described previously [11-14]. The purified IgY was proteolyzed with chymotrypsin-trypsin mixture and further digested with N-glycosidase F to release N-glycans. After removal of the peptides, the reducing ends of the N-glycans were derivatized with 2-aminopyridine (Wako, Osaka, Japan). The PA derivatives of the N-linked oligosaccharides were separated by HPLC on a TSK-gel diethylaminooethyl (DEAE)-5PW column (Tosoh, Tokyo, Japan) according to their sialic acid content. The fractionated PA-N-glycans were then individually separated and identified sequentially on a TSK-gel Amide-80 column (Tosoh) and on a Shim-pack HRC-tetradecyl silica (ODS) column (Shimadzu, Kyoto, Japan). The identification of N-glycan structures was based on their elution positions from these three types of columns in comparison with PA-glycans in the GALAXY database [9]. The N-glycan structures were confirmed by co-chromatography with standard PA-oligosaccharides and MALDI-TOF-MS analyses with an AXIMA-CFR MALDI-TOF MS spectrometer (Shimadzu). The structures of PA-glycans thus far not registered in GALAXY were characterized by exoglycosidase treatments and mass spectrometric techniques.
Results and Discussion

N-glycosylation profiling of a panel of IgYs was conducted using multidimensional HPLC in conjunction with mass spectrometric methods. In this method, N-glycans were released by N-glycosidase F, labeled with 2-aminopyridine, and separated on a DEAE anion-exchange column according to the degree of sialylation (Figure 1). The fractions corresponding to mono-, di-, tri-, and tetrasialyl PA-N-glycans as well as neutral PA-N-glycans were individually separated on an amide-silica column. Figure 2 compares elution profiles of these fractions of monoclonal IgY glycoproteins produced in chicken hybridoma, mammalian HEK293T and CHO cells along with polyclonal IgY isolated from egg yolk. Individual fractions separated on the amide-silica column were further applied onto an ODS column to record the elution times on the two types of columns. The elution time was represented by glucose units (GU) on columns that were calibrated with a PA-derivatized isomalto-oligosaccharide mixture. The PA-oligosaccharides were identified on the basis of coincidence of the elution data with those in the GALAXY database [9]. The PA-oligosaccharides thus far not registered in GALAXY were identified by trimming with various exoglycosidases. The structure and incidence of N-glycans corresponding to each HPLC peak are summarized in Table 1 (Data included as supplementary).

Egg yolk

Consistent with a previous study [6], the N-glycans of polyclonal IgY from egg yolk were classified primarily into two types: monoglucosylated high mannose-type oligosaccharides (30.7%) and biantennary complex-type oligosaccharides (neutral, 20.6%; monosialyl, 31.5%; disialyl, 11.9%) (Figure 3). Major portions of the complex-type oligosaccharides contained the bisecting GlcNAc and the core fucose residues.

Chicken hybridoma cells

In the monoclonal IgY expressed by chicken hybridoma cells, the ratio of high mannose-type and complex-type oligosaccharides was approximately 1:1. Considering that this monoclonal antibody possesses no N-glycosylation sites in the variable region (unpublished data), this result is consistent with the conclusions of the previous study [7]: complex-type and high mannose-type oligosaccharides are exclusively exhibited at Asn308 and Asn407, respectively, in chicken serum IgY. Monoglycosylated high mannose-type oligosaccharides

Figure 2: N-glycosylation profiles of the fractions separated by the DEAE column, on an amide column. N1-10, M1-4, D1-3, T1-2, and Te1-2 denote the fractions of neutral, monosialyl, disialyl, trisialyl, and tetrasialyl oligosaccharides, respectively, each of which were numbered in order of elution time. Asterisks indicate fractions containing no detectable PA-oligosaccharide.
accounted for approximately 50% of the total high mannose-type oligosaccharides (Figure 3). This level is lower than that in egg yolk IgY (approximately 90%) or serum IgY (approximately 70%) in chicken, but obviously much higher than that observed in mammalian glycoproteins in general. The complex-type N-glycans were exclusively biantennary oligosaccharides, most of which were sialylated primarily through α2-6 linkages and contained the bisecting GlcNAc residue and the core fucose residue.

HEK293T cells

The N-glycosylation profile of the monoclonal IgY expressed by HEK293T cells showed a similar incidence and monoglucosylation level of high mannose-type oligosaccharides as that of IgY from chicken hybridoma: 21.6% monoglucosylated high mannose-type oligosaccharides of the total N-glycans (Figure 3). By contrast, profiles of the complex-type oligosaccharides were significantly different between these IgYs. The HEK293T cell-derived IgY was less sialylated (28.1% of the total N-glycans) than the IgY from chicken hybridoma (47.8%) and contained a higher content of α2-3 sialyl groups. Occurrence of the bisecting GlcNAc residue was much less pronounced (6.5%) and high antennary structures including trisialyl and tetrasialyl triantennary oligosaccharides emerged. Such high antennary glycans have been identified in recombinant human acetylcholinesterase expressed by HEK293 cells [15].

CHO cells

Although the glycosylation profile of monoclonal IgY expressed by CHO cells differed the most from that of egg yolk IgY, it still had a significant quantity of monoglucosylated high mannose-type oligosaccharides (16.5% of the total N-glycans) (Figure 3). The ratio of high mannose-type to complex-type oligosaccharides was approximately 2:7, suggesting that N-glycan processing was more extensive even at the Asn407 glycosylation site. High antennary complex-type oligosaccharides had more sialylation (mostly with NeuAc) through both α2-3 and α2-6 linkages, with a predominance of the former and bisecting GlcNAc was not detected.

Thus, monoclonal IgY antibodies exhibited different N-glycosylation profiles depending on the production vehicle. While IgY expressed by the chicken hybridoma cells retained the premature high mannose-type oligosaccharides (presumably at Asn407), IgY expressed by CHO cells experienced extensive N-glycan processing and displayed high-antennary oligosaccharides. The major sialyl linkages were α2-6 in chicken hybridoma cells and α2-3 in CHO cells. Most of the complex-type N-glycans in the IgY expressed by chicken hybridoma cells possessed the bisecting GlcNAc residue, whereas the IgY expressed by CHO cells lacked this modification. The fucosylation level of IgY was higher in chicken hybridoma cells than in CHO cells. In HEK293T cells, all these tendencies were in between those of the chicken hybridoma cells and the CHO cells.

These cell-specific modifications of the IgY complex-type oligosaccharides are consistent with the previous reports. Namely, major sialyl N-glycans share α2-3 linkage and no bisecting GlcNAc residue is displayed in glycoproteins produced by CHO cells [16-19]. By contrast, N-glycans possessing α2-6 sialic acid and those carrying bisecting GlcNAc residues have been identified in chicken serum IgY, while no α2-3 sialyl group has been found in its N-glycans [7]. In general, hallmarks of the cell-specific glycosylation are considered to result from variations in transcript expression of the corresponding biosynthetic enzymes. Indeed, in mouse liver, N-glycans containing core α1-6 Fuc residue were of lower abundance in comparison with those expressed in other tissues, in correlation with ~10-fold lower

Figure 3: Molar ratios of specific N-glycan forms to total N-linked oligosaccharides derived from egg yolk IgY and monoclonal IgYs expressed by different production vehicles.
transcript level for the α1-6 fucosyltransferase FUT8 in liver as compared with kidney, testis, and brain [20]. It has also been reported that CHO cells exhibit little expression of the α2-6 sialyltransferase STEGaII resulting in low incidence of α2-6 sialylation of N-glycans [21]. The predominant α2-6 sialylation in chicken IgY-producing cells may be ascribed to increase of α2-3 sialyltransferase and/or decrease of α2-6 sialyltransferase expression levels in these cells.

Conversely, the monoclonal IgY derived from the three production vehicles did share common N-glycosylation features. The most pronounced characteristic was high incidences of monoglucosylated high mannose-type oligosaccharides, suggesting that this glycosylation feature is, to some extent, associated with the distinctive quaternary structure of IgY-Fc, even though structurally homologous IgE does not exhibit such monoglucosylated glycoforms [8]. Site-specific N-glycosylation profiling of chicken serum IgY indicates that the monoglucosylated glycans are displayed exclusively at Asn407 [7].

Cumulative evidence indicates that high mannose-type oligosaccharides interact with a series of intracellular lectins and thereby govern the fates of their carrier proteins in cells [22-25]. In particular, monoglucosylated high mannose-type oligosaccharides serve as tags that are displayed on nascent polypeptides and recognized by the molecular chaperones calnexin and calreticulin during the folding process in the endoplasmic reticulum (ER) [26]. The terminal glucose residue is removed by glucosidase II but is reproduced by UDP-glucose:glycoprotein glucosyltransferase, which operates as a folding sensor acting on only glycoproteins that have yet to fold correctly. X-ray crystallographic data indicated that the N-glycans attached to Asn407 are, at least partially, packed between the two Cai domains, although no interpretable electron density was provided for the outer branches of the carbohydrate moieties [27]. Hence, the accessibility of these ER enzymes and chaperones could be a determining factor in the high incidence of the monoglucosylated species of IgY. Therefore, it is possible that the timing and efficiency of N-glycan processing and quaternary structure formation of IgY in the ER are considerably different from those of IgE, despite the structural similarities of IgY and IgE. The varying incidences of the monoglucosylated IgY glycoforms suggest that these factors also depend on the vehicles used for recombinant IgY production. The N-glycosylation profiling data presented herein can provide a guide for choosing an appropriate vehicle for the expression of IgYs and other glycoproteins for immunological and therapeutic purposes.

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