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Aggregated human serum immunoglobulin A1 induced by neuraminidase treatment had a lower number of *O*-linked sugar chains on the hinge portion

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Abstract

A part of human serum immunoglobulin A1(IgA1) was aggregated by treatment with neuraminidase. Aggregated IgA1 was separated from non-aggregated IgA1 by gel permeation chromatography. The prepared asialo-hinge glycopeptide (asialo-HGP) from both IgA1 subfractions was treated with β-galactosidase to determine the number of *O*-linked sugar chains attached on the hinge region. Removal of the galactose residue from asialo-HGP resulted in the HPLC separation of three major peaks. MALDI-TOFMS analysis of the glycopeptides also indicated the presence of three HGP components with three, four and five *N*-acetylgalactosamine (GalNAc) residues, respectively. Comparison of their relative content among the glycopeptide components showed a higher content of the HGP component with a lower number of GalNAc residues on aggregated IgA1. Thus, asialo-HGP prepared from aggregated IgA1 induced by neuraminidase treatment had an incomplete core structure of *O*-linked oligosaccharides. Especially, the result suggested that the reduced number of the attached *O*-linked oligosaccharides on IgA1 take part in phenomena such as self-aggregation of asialo-IgA1.

Keywords: Immunoglobulins; Neuraminidase

1. Introduction

Human serum IgA1 is one of the most exceptional glycoproteins among the serum glycoproteins because it has *O*-linked oligosaccharides in its hinge portion in addition to the *N*-linked carbohydrate chains in its structure [1–3]. In our previous report, the glycoform of the *O*-linked oligosaccharide of the IgA1 subclass from a healthy control and IgA1 myeloma patients was analyzed. Three glycoforms for IgA1 from myeloma patients and only one glycoform from healthy individuals were found [4]. However, IgA1 from a healthy individual could be further fractionated depending on its different affinity toward jacalin and its different heat stability. These subfractions had mutually different glycoforms of the *O*-linked oligosaccharide. Polymerized IgA1 was

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abundant in asialo-Galβ1,3GalNAc and heat-stable IgA1 subfraction was abundant in a sialylated disaccharide [5]. These lines of fact suggested the possibility that the incomplete structure of the hinge *O*-linked oligosaccharide was one of the causes of the production of such a polymerized IgA1.

IgA nephropathy is a common disease characterized by predominant IgA deposits in the renal mesangium. It is well known that IgA1 among two subclasses, IgA1 and IgA2, is a dominant deposit in glomeruli during IgA nephropathy [6,7]. The most prominent structural difference between the IgA1 and IgA2 subclasses was the duplicated proline-rich hinge portion and the characteristic *O*-linked oligosaccharide chains on the IgA1 hinge portion. There are many reports on the presence of an incompletely glycosylated *O*-linked oligosaccharide(s) on the IgA1 hinge region in some of the IgA nephropathy patients [8–18].

In this report, the number of *O*-linked sugar chains on the aggregated IgA1 induced by its neuraminidase treatment was examined by HPLC and MALDI-TOFMS analysis as summarized in Fig. 1.

2. Experimental

2.1. Reagents and chemicals

The following compounds and materials were commercially obtained: Normal human serum was from China Newtech Development and Trade Corp. and was stored at −20°C. PD-10 and Sephacryl S-300 were from Pharmacia Biotech (Uppsala, Sweden); jacalin-agarose was from Vector Laboratories, (Burlingame, CA, USA); 4-vinyl pyridine was from Aldrich (Milwaukee, WI, USA); and neuraminidase from *Streptococcus 6646K* was from Seikagaku (Tokyo, Japan). β-galactosidase from bovine testes and trypsin were from Sigma (St. Louis, MO, USA).

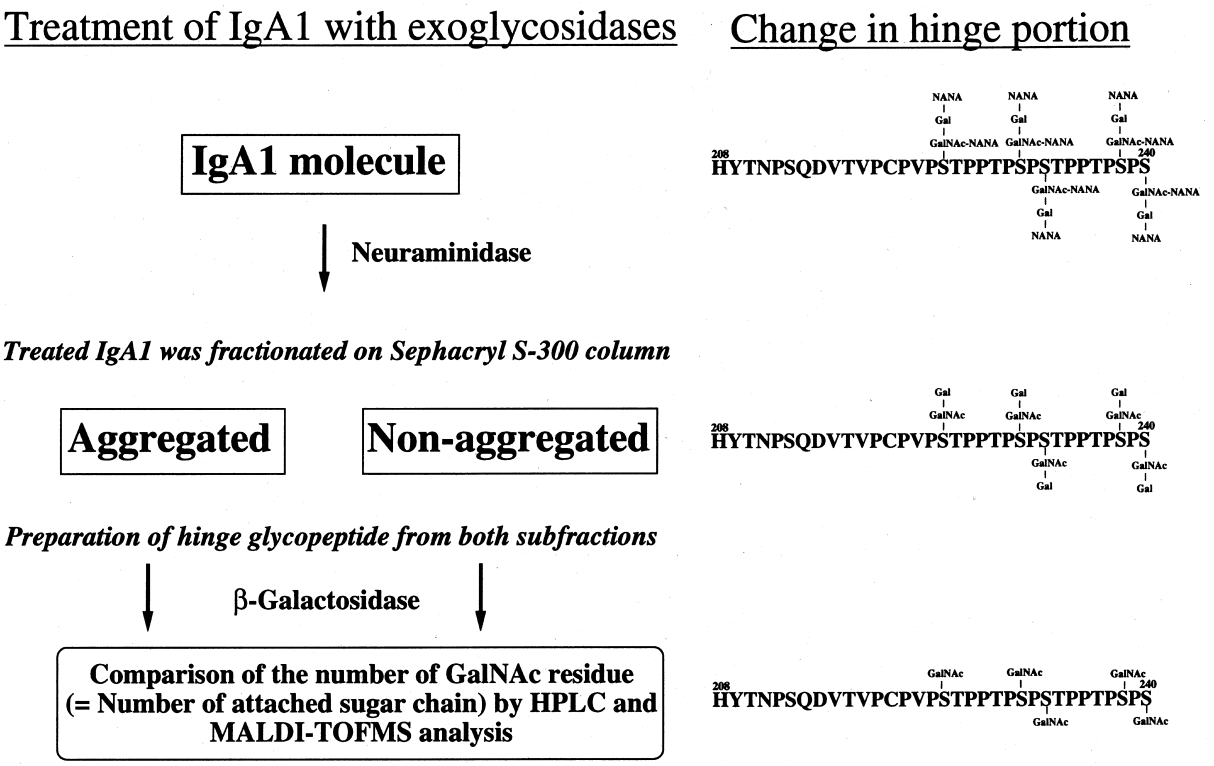

Fig. 1. Flow chart for counting the number of sugar chains of the 'aggregated' and 'non-aggregated' IgA1. NANA, Gal and GalNAc in the figure indicated N-acetylneuraminic acid, galactose and *N*-acetylgalactosamine residue, respectively.

2.2. Preparation of IgA1 by jacalin-agarose affinity chromatography

If not stated, the jacalin-agarose affinity chromatography was carried out at room temperature. Ten milliliters of serum were applied to the jacalin column (10 ml) and washed with 0.1 *M* Tris–HCl buffer, pH 7.6, containing 0.02% sodium azide. The thoroughly washed column was first eluted with 0.8 *M* glucose (50 ml) and then with 0.8 *M* galactose (50 ml) in the above buffer. Separation between aggregated IgA1 and non-aggregated IgA1 was carried out by Sephacryl S-300 column chromatography (62×1.5 cm I.D.) equilibrated with 50 m*M* ammonium bicarbonate. Purified monomeric IgA1 was used for further analysis.

2.3. Preparation of aggregated IgA1 and non-aggregated IgA1 induced by neuraminidase treatment

Monomeric IgA1 (20 mg) was incubated with 50 mU of neuraminidase in 50 m*M* sodium acetate buffer pH 5.0 at room temperature overnight. The reaction mixture was applied to a Sephacryl S-300 column and separated into aggregated and non-aggregated IgA1. Each IgA1 subfraction was lyophilized and rechromatographed by the same column. About 5 mg of aggregated IgA1 and 9.5 mg of non-aggregated IgA1 were obtained.

2.4. Preparation of S-pyridylethylated α1 chain from IgA1

About 2 mg of IgA1 was first desalted into water using a PD-10 column and lyophilized. The sample was dissolved in 1 ml of 0.4 *M* Tris–HCl buffer, pH 8.6, containing 6 *M* guanidine-HCl and 0.2 *M* EDTA. To dissociate the disulfide linkage, 10.8 μl of dithiothreitol solution (200 mg/ml) was added and stirred. After heating at 50°C for 4 h, 3.2 μl of 4-vinyl pyridine was added and the reaction mixture was allowed to stand for 90 min at room temperature. The reaction was terminated by the addition of 100 μl of 2.0 *M* formic acid.

The α-chain dissociated as described above was fractionated by HPLC using a Cosmosil 5C4-300 column (Nacalai Tesque, 150×4.6 mm I.D.) equipped with Shimadzu LC-4A. Elution was carried out by a linear gradient for 60 min from 10% to 90% acetonitrile in 0.1% trifluoroacetic acid (TFA). The material eluted at the peak position around 30 min was collected and concentrated.

2.5. Preparation of hinge glycopeptide from a trypsin digest of the heavy chain

About 0.5 mg of heavy chain was dissolved in 160 μl of 50 m*M* Tris-HCl buffer, pH 8.0, containing 2.0 *M* urea. Twenty microliters of trypsin solution (10 μg trypsin/20 μl of the above buffer) and 20 μl of 0.1 *M* $CaCl_2$ were added, and the reaction mixture was incubated overnight at 37°C. The trypsin digest was made up to 1ml by adding 0.8 ml of 0.175 *M* Tris-HCl buffer, pH 7.6. The sample was applied to a jacalin agarose column (2 ml), and the passed fraction was eluted with 6 ml of the above buffer. After further washing the column with 6 ml of the buffer, the hinge glycopeptide fraction was eluted with 6 ml of 0.1 *M* melibiose in the buffer. Purification of the glycopeptide by HPLC was carried out using a Cosmosil 5C18-300 column (150×4.6 mm I.D.). Elution was carried out using a linear gradient for 60 min from 0% to 90% acetonitrile in 0.1% TFA. Detection was performed by monitoring UV absorbance at 220 nm. The material eluted at the peak position of the hinge glycopeptide around 23 min was collected and concentrated.

2.6. Treatment of glycopeptide with β-galactosidase

Purified glycopeptide was dissolved in 30 μl of 0.2 *M* acetate buffer, pH 5.0, and 20 μl of β-galactosidase from bovine testes (1.0 U/ml) was added to the sample. The reaction mixture was incubated overnight at room temperature. Fractionation of degalactosylated glycopeptide was carried out by HPLC using Cosmosil 5C18-300 (150×4.6 mm I.D.) under the same conditions described above. The purified glycopeptide was analyzed by MALDI-TOFMS.

2.7. MALDI-TOFMS analysis of glycosidase-treated hinge glycopeptide

The hinge glycopeptide was analyzed by MALDI-TOFMS in the negative ion mode to suppress the production of the adduct ion. The mass spectrometer used in this work was a Finnigan Lasermat (Finnigan MAT, Hemel Hempstead, UK). The sample solution (0.5 μl) was mixed with 0.5 μl α-cyano-4-hydroxy cinnamic acid (10 mg/ml in 70% acetonitrile–30% water). The mixture (1 μl) was applied to the sample target and allowed to air-dry prior to MALDI-TOFMS analysis. The error of the mass spectra was less than 0.3%.

3. Results and discussion

As previously reported, a comparative study of heat aggregated IgA1 and heat stable IgA1 indicated that the heat-stable IgA1 contained a much higher amount of the sialylated Galβ1,3GalNAc on the hinge portion [5]. It was also reported that a part of IgA1 prepared from normal human serum was aggregated by the neuraminidase treatment [18]. In order to examine the role of sialic acids attached to *O*-linked sugar chains on the hinge portion, aggregated and non-aggregated IgA1 from neuraminidase-treated IgA1 were separately purified (Figs. 2 and 3). Hinge glycopeptide was prepared from each IgA1 subfraction and followed by treatment with β-galactosidase as previously reported [19]. HPLC analysis of asialo-, agalacto-HGP indicated the presence of three components, A, B and C (Fig. 4). MALDI-TOFMS analysis showed that the three components corresponded to the 33mer peptide, HYTNPSQDVTVPCPVPSTPPTPSPSTPPTPSPS, having five, four and three GalNAc residues (Figs. 5 and 6). Comparing the relative contents of the three components between aggregated IgA1 and non-aggregated IgA1, aggregated IgA1 had a lower number of *O*-linked sugar chains on the hinge portion (Fig. 7). The result coincided with the results of experiments counting the non-substituted GalNAc residues on the hinge portion [20].

As we reported before, glycosylation failure of the hinge portion of IgA1 will be related to the cause of the self aggregation of IgA1, and this may related the

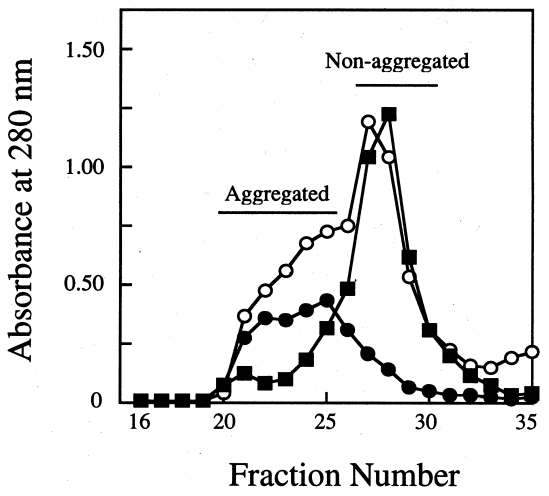

Fig. 2. Sephacryl S-300 column chromatography of neuraminidase-treated IgA1. Separated monomeric IgA1 was treated with neuraminidase and then fractionated on the column (open circles). Aggregated and non-aggregated IgA1 fractions indicated by the bar were separately collected and were rechromatographed (Aggregated IgA1; solid circles: Non-aggregated IgA1; solid squares). Purified subfractions were subjected to further analysis.

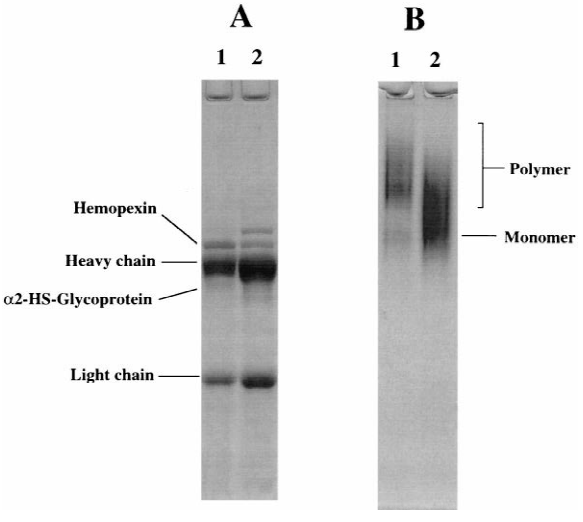

Fig. 3. Polyacrylamide gel electrophoresis of IgA1 subfractions. SDS-PAGE (A) under reduced condition and native PAGE (B) of IgA1 subfractions were carried out using the ready-made 5–20% gradient gel and the 3–10% gradient gel (PAGEL SPG-520L and PAGEL NPG-310L, Atto Corp., Tokyo, Japan), respectively. A sample containing 2–5 μg in 5 μl was applied to each well and electrophoresis was carried out at a constant current of 20 mA. The gel was stained by Coomassie Brilliant Blue R-250. A-1and B-1; Aggregated IgA1, A-2 and B-2; Non-aggregated IgA1

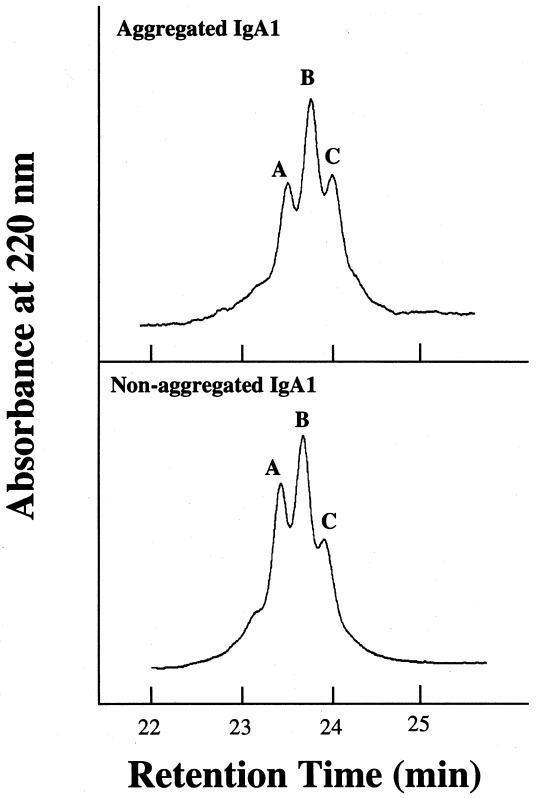

Fig. 4. HPLC profile of asialo-, agalacto-HGP prepared from aggregated and non-aggregated IgA1 subfractions. Asialo-, agalacto-HGPs prepared from aggregated and non-aggregated IgA1 subfractions were fractionated on HPLC using a Cosmosil 5C4-300 column. Peaks A, B and C corresponded to the 33mer peptide HYTNPSQDVTVPCPVPSTPPTPSPSTPPTPSPS having five, four and three GalNAc residues, respectively [19].

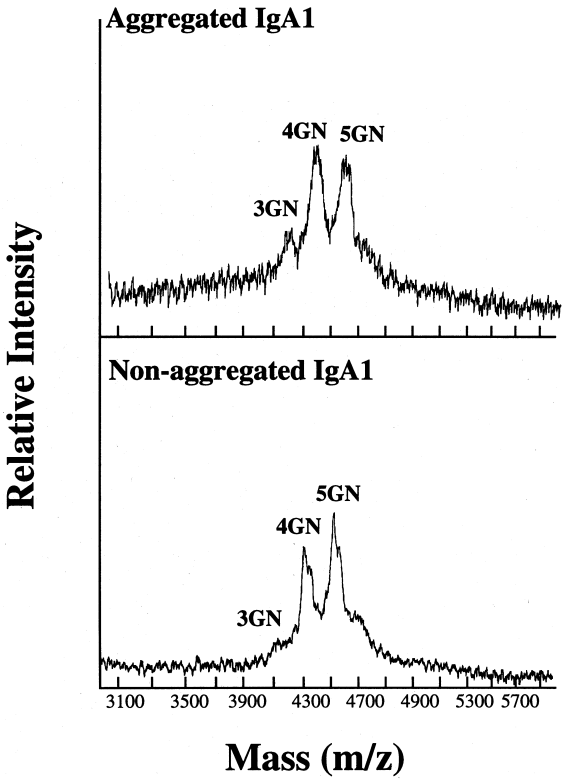

Fig. 5. MALDI-TOFMS analysis of asialo-, agalacto-HGP prepared from aggregated and non-aggregated IgA1 subfractions. Asialo-, agalacto-HGPs prepared from aggregated and non-aggregated IgA1 subfractions were analyzed by MALDI-TOFMS. 3GN, 4GN and 5GN in the figure indicate the 33mer peptide having three, four and five GalNAc residues, respectively.

cause of IgA nephropathy [13–18]. Saulsbury [21] also reported the increment in asialo-*O*-linked sugar chains of IgA1 in children with Henoch-Schonlein purpura.

Although the detailed mechanism of the self aggregation was not clear, the results obtained here indicated the production of aggregated IgA1 by neuraminidase treatment and the tendency of aggregation of IgA1 molecules having a lower number of *O*-linked oligosaccharides on the hinge portion. Generally speaking, a sugar chain on the glycoprotein plays the role of stabilization of the protein portion. Especially, clustered sialic acid in mucin-type glycoprotein functions to form an extended structure. The innermost *N*-acetylgalactosamine residue must also be available to maintain the peptide conformation [22]. Therefore, truncating the oligosaccharides should lessen the stability of the IgA1 molecule as shown in these results. Probably, the removal of sialic acid from the IgA1 will first destabilize the IgA1 molecule and a lower number of attached *O*-linked oligosaccharides will accelerate the aggregation. Evidence was reported for involvement of the IgA1 hinge glycopeptide in the IgA1–IgA1 interaction in IgA nephropathy [15,18]. Also, it was suggested that the IgA1 prepared from an IgA nephropathy patient has a lower number of sugar chains than that from a healthy control [17]. However, it is still not clear whether the mechanism

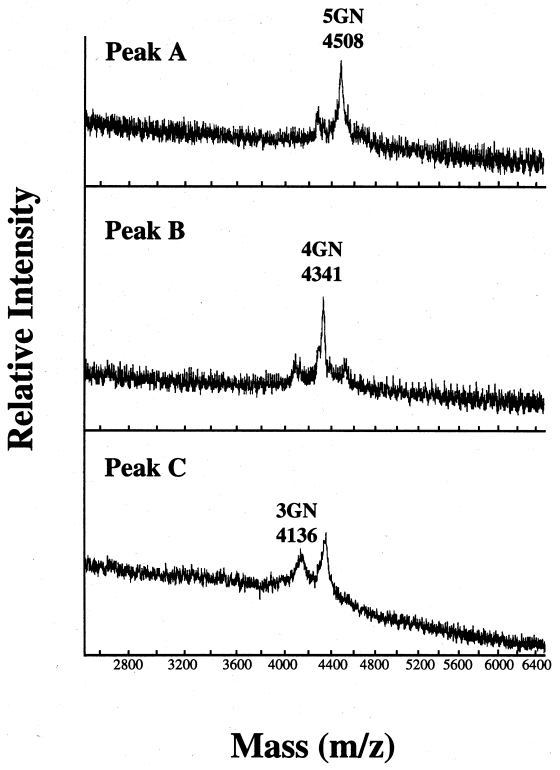

Fig. 6. MALDI-TOFMS analysis of subfractionated peaks A, B and C on HPLC. Peaks A, B and C from aggregated IgA1 in the Fig. 2 were analyzed by MALDI-TOFMS. Each mass number indicated on the peak was somewhat larger than the calculated mass number of the glycopeptides, 4491 for A, 4288 for B and 4080 for C.

of aggregation induced by the removal of sialic acid was the same as that for IgA1–IgA1 interaction in the IgA nephropathy patients.

In this report, it was found that the asialo-, agalacto-HGPs could be separated from each other by HPLC, dependent upon their GalNAc content. Recently, Mattu et al. [23] reported that the *O*-linked sugar chain was attached not only to a serine residue but also to a threonine residue on the hinge portion of the recombinant IgA1 molecule. Thus, the method developed here would be useful to analyze the details of the attachment site of GalNAc residues on the hinge portion of IgA1 from a healthy control and an IgA nephropathy patient.

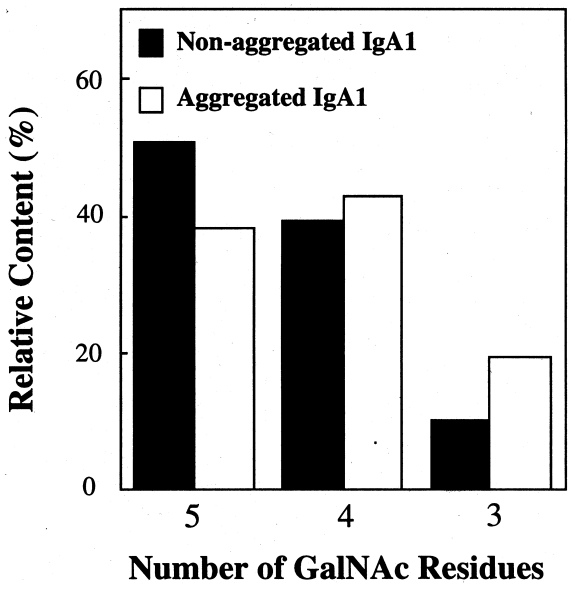

Fig. 7. Comparison of the relative content of three HGP components having different numbers of GalNAc residues prepared from aggregated IgA1 and non-aggregated IgA1. The relative content of three HGP components having different numbers of GalNAc residues was compared between aggregated IgA1 and non-aggregated IgA1.

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