Kidney International, Vol. 59 (2001), pp. 1077–1085

CLINICAL NEPHROLOGY – EPIDEMIOLOGY – CLINICAL TRIALS

Mass spectrometry proves under-*O*-glycosylation of glomerular IgA1 in IgA nephropathy

YOSHIYUKI HIKI, HIROKO ODANI, MAMI TAKAHASHI, YOSHINARI YASUDA, AKEYO NISHIMOTO, HITOO IWASE, TORU SHINZATO, YUTAKA KOBAYASHI, and KENJI MAEDA

Department of Medicine, Daiko Medical Center, Nagoya University, Nagoya, Aichi, and Departments of Biochemistry and Medicine, Kitasato University, Sagamihara, Kanagawa, Japan

Mass spectrometry proves under-*O*-glycosylation of glomerular IgA1 in IgA nephropathy.

Background. The IgA1 molecule, which is predominantly deposited in glomeruli in IgA nephropathy (IgAN), is a unique serum glycoprotein because it has *O*-glycan side chains in its hinge region. Our study was conducted to investigate the *O*-glycan structure in the glomerular IgA1 in IgAN.

Methods. The IgA1 was separated from 290 renal biopsy specimens of 278 IgAN patients and from four serum IgA1 samples (IgAN, 2; control, 2). The variety of *O*-glycan glycoform was determined by estimating the precise molecular weights of the IgA1 hinge glycopeptides using matrix-assisted laser desorption ionization time of flight mass spectrometry.

Results. The peak distribution of IgA1 hinge glycopeptides clearly shifted to lesser molecular weights in both glomerular and serum IgA1 in IgAN compared with the serum IgA1 of controls. In the five major peaks of IgA1 hinge glycopeptides in each sample, the numbers of carbohydrates composing *O*-glycans (GalNAc, Gal, and NANA) in the deposited and serum IgA1 in IgAN patients were significantly fewer than those in the serum IgA1 in the control groups.

Conclusion. The *O*-glycan side chains in the hinge of the glomerular IgA1 were highly underglycosylated in IgAN. These results indicate that the decreased sialylation and galactosylation of the IgA1 hinge glycopeptides play a crucial role in its glomerular deposition in IgAN.

The human IgA1 molecule, which is known to be predominantly deposited in glomeruli in IgA nephropathy (IgAN) [1], is one of the few serum glycoproteins because it possesses *O*-linked oligosaccharide (*O*-glycan) side chains in its hinge region [2, 3]. The majority of serum proteins are glycoproteins, and the sugar side chains linking to proteins are usually *N*-linked oligosaccharides (*N*-glycans). The fundamental structure of the *O*-glycans is the linkage between the α-anomeric carbon atom in *N*-acetylgalactosamine (GalNAc) and the hydroxy group of serine or threonine [GalNAcα1-*O*-Ser(Thr)].

The structural variety of the sugar side chains in glycoproteins is widely observed under physiological conditions. The phenomenon is called "the microheterogeneity of carbohydrate" [4]. As shown in Figure 1, the GalNAc residue in the glycans of the human IgA1 hinge may be extended with β1,3-linked galactose (Galβ1,3GalNAc). Also, sialic acid [(*N*-acetylneuraminic acid (NANA)] could bind to Gal with α2,3 and/or to GalNAc with α2,6 linkage. Therefore, there could be six varieties of *O*-glycans structures in the hinge region of IgA1 (Fig. 1A).

The IgA1 hinge region located between the Fab and Fc region has a proline-rich sequence (PSTPPTPSP STPPTPSPS) [5]. Within this sequence, there are nine potential *O*-glycosylation sites (5 Ser and 4 Thr) in each hinge peptide, causing a variable number of *O*-glycan side chains. According to a study by Mattu et al, *O*-glycans were located at Thr^{228}, Ser^{230}, and Ser^{232}, while *O*-glycan sites at Thr^{225} and Thr^{236} were partially occupied in the hinge peptide (Fig. 1B) [3].

There have been several reports analyzing the structural varieties of *O*-glycans in the hinge region of serum IgA1 in IgAN [6–9]. Mestecky et al [6], Allen, Harper, and Feehally [7], and Tomana et al [8] found the increased binding of GalNAc-specific lectins to serum IgA1 in IgAN, suggesting the undergalactosylation of the IgA1 hinge glycopeptides. However, the precise structural definition of the defect has proved technically difficult and remains unconfirmed. Recently, Allen et al precisely analyzed the *O*-glycan structure of serum IgA1 using fluorophore-assisted carbohydrate electrophoresis (FACE) and demonstrated that the IgA1 *O*-glycan chains were truncated with increased terminal GalNAc in IgAN [10].

However, almost all of the IgA1 molecules analyzed in the previous studies were obtained from serum because of the technical limits of the sensitivity. To the

Key words: serum glycoprotein, IgA1 hinge glycomerular deposition, *O*-linked oligosaccharide, IgA nephropathy.

Received for publication June 8, 2000
and in revised form August 24, 2000
Accepted for publication September 14, 2000

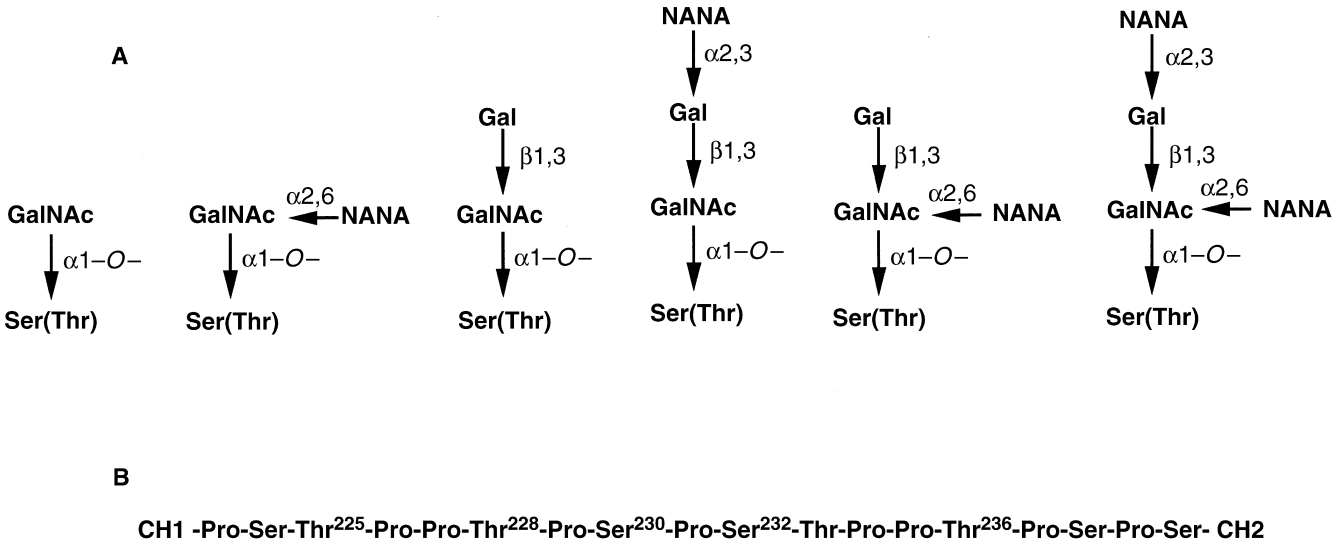

Fig. 1. (*A*) Possible structure of *O*-glycans in IgA1 hinge region. There could be the six varieties of *O*-glycans structure in the hinge region of IgA1. (*B*) Amino acid sequence of IgA1 hinge peptide. *O*-glycans could be located at Thr^{228}, Ser^{230}, and Ser^{232}, while *O*-glycan sites at Thr^{225} and Thr^{236} are partially occupied in the hinge peptide [3].

best of our knowledge, there have been only two studies investigating the *O*-glycan structure of deposited IgA1 in the glomeruli of IgAN patients (abstract; Allen et al, *J Am Soc Nephrol* 10:506A, 1999) [9]. Both of them were analyzed by lectin binding.

In this study, we attempted to purify the deposited IgA1 in the glomeruli isolated from the renal biopsy specimens of IgAN patients and to analyze the precise structure of *O*-glycans in the hinge region of the IgA1 using matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOFMS). From our previous study [11], it was expected that analyses using MALDI-TOFMS make it possible to overcome the problem of analyzing such a small amount of IgA.

METHODS

Materials

The following materials were purchased from the sources indicated: PD-10 from Pharmacia Biotech AB (Uppsala, Sweden); 4-vinyl pyridine and matrix for MALDI-TOFMS (2,5-dihydroxybenzoic acid (DHB), 5-methoxysalicylic acid) from Aldrich Chem. Co., Ltd. (Milwaukee, WI, USA); Jacalin-agarose from Vector Laboratories, Inc. (Burlingame, CA, USA); Goat anti-human IgA antibody (Organon Teknika Corp. (West Chester, PA, USA); and trypsin from Sigma Chemical Co. (St. Louis, MO, USA).

Biological materials

The 290 renal biopsy specimens obtained from 278 patients with IgAN for routine examination in the renal unit of the Kitasato University Hospital from 1988 to 1997 were the subjects of this study. Informed consent had been obtained from each patient when they underwent the renal biopsy. The mean age at the time of the renal biopsy was 36.8 ± 13.4 years old; 148 of them were male, and 130 were female. The specimens (approximate size of 2 to 3 mm^3) that were the remains of the routine immunofluorescence (IF) study for diagnosis had been stored at −80°C in OCT medium (Miles Lab., Elkhart, IN, USA). IF patterns of each sample evaluated by Y.H. and Y.K. at the time of the renal biopsy are listed in Table 1.

Table 1. Distribution of immunofluorescence pattern of 290 analyzed renal specimens (%)

	−	+	++	+++
IgA	0 (0%)	93 (32.1)	157 (54.1)	40 (13.8)
IgG	231 (79.7)	53 (18.3)	5 (1.7)	1 (0.3)
IgM	81 (27.9)	207 (71.4)	2 (0.7)	0 (0)
C3	4 (1.4)	202 (69.7)	79 (27.2)	5 (1.7)
C4	266 (91.7)	24 (8.3)	0 (0)	0 (0)
C1q	236 (81.4)	53 (18.3)	1 (0.3)	0 (0)
Fibrinogen	93 (32.1)	162 (55.9)	32 (11.0)	3 (1.0)

Four serum samples were also subjects of this study. Each sample was composed of sera pooled from the following individuals: IgAN sample (1), four biopsy-proven IgAN patients; IgAN (2), five IgAN patients; control (1), four nephrotic patients with other primary glomerulonephritis (GN) (1 membranous nephropathy), 1 lipoid nephrosis, and 2 focal glomerulosclerosis); control (2), seven non-nephrotic patients with other primary GN (2 non-IgAN, 3 diabetic nephropathy, 2 crescentic GN). IgA was negative in all patients in the control group on IF.

Isolation of glomeruli from renal biopsy specimens

The isolation procedure is based on the methods of Westberg and Michael [12]. Each of the frozen renal biopsy specimens in OCT compound was put together in a beaker containing 100 mL of cold phosphate-buffered saline [0.01 mol/L phosphate buffer, pH 7.4, 0.15 mol/L NaCl (PBS)].

The pooled specimens in PBS were then transferred to a 100 mesh stainless steel sieve (pore size 140 μ). The tissue was forced through the sieve by moderate pressure using the bottom of a metal spoon along with repeated washing with cold PBS (300 mL). The pressing was continued for about 10 minutes; the material remaining on the screen was discarded. The sieved suspension was then poured through a 60 mesh (pore size 230 μ) sieve, which retained a few of the large fragments, and finally through a 250 mesh sieve (61 μ opening). The material retained on the fine sieve was then extensively washed with 300 mL of cold PBS.

This material was washed into a beaker, transferred to a 50 mL plastic centrifuge tube, and centrifuged at $120 \times g$ at 4°C for 10 minutes. A sample of the sediment was examined by phase-contrast microscopy, and the number of nonglomerular fragments per 100 glomeruli was counted. The total number of isolated glomeruli was also calculated. It was found that 88% of the fragments were isolated glomeruli.

Elution of IgA from isolated glomeruli

To elute the deposited IgA, the pellet of the isolated glomeruli was suspended in 10 mL of PBS solution containing 6 mol/L urea and was incubated for 90 minutes at 37°C with shaking. The solution was then gently centrifuged ($120 \times g$ for 10 min). The supernatant was dialyzed against PBS overnight at 4°C.

Sample preparation of IgA

Isolation of eluted IgA from glomeruli. To perform 50% ammonium sulfate precipitation, the dialyzed supernatant containing deposited IgA was mixed with an equal volume of saturated ammonium sulfate. After dissolving the precipitate with 5 mL of PBS, the solution was applied to the anti-IgA antibody coupled activated sepharose 4 B column (1 mL). After the column was thoroughly washed with PBS, IgA was eluted with 2 mol/L urea. The solution was then applied on a PD 10 (5 mL) column to delete the salts and lyophilized.

Purification of serum IgA. Approximately 5 mL of serum from each subject were also mixed with an equal volume of saturated ammonium sulfate, and the precipitate obtained was dissolved, dialyzed against PBS, and applied to the Jacalin-agarose column (3 mL). After the column was thoroughly washed with PBS, it was first eluted with 0.8 mol/L glucose and then with 0.1 mol/L melibiose as previously described [13]. The absorbance of the eluate at 280 nm was read to detect the protein. Nonspecifically bound materials were excluded by washing with the glucose solution. The IgA1 fractions obtained by the stepwise elution with melibiose were dialyzed against distilled water and lyophilized. In one serum sample (IgAN 1), IgA was isolated by using the antihuman IgA column (3 mL) similar to the isolation of the glomerular IgA.

Preparation of IgA1 hinge glycopeptides

These procedures were performed according to our previous studies [11, 13].

Preparation of S-pyridylethylated α chain from separated IgA. The isolated IgA samples were dissolved in 500 μL of 0.4 mol/L Tris-HCl buffer, pH 8.6, containing 6 mol/L guanidine-HCl and 0.2 mol/L ethylenediaminetetraacetic acid (EDTA). To dissociate the disulfide linkage, dithiothreitol solution (200 mg/mL) was added and stirred (serum IgA, 5.0 μL/IgA mg; deposited IgA, 10 μL/sample). After heating at 50°C for four hours, 1.6 μL/IgA mg (deposited IgA, 10 μL/sample) of 4-vinyl pyridine was added, and the reaction mixture was allowed to stand for 90 minutes at room temperature. The reaction was terminated by the addition of 50 μL of 2 mol/L formic acid, and the mixture was then dialyzed overnight against distilled water at 4°C.

Preparation of IgA1 hinge glycopeptides from trypsin digest of IgA1 molecules. The dissociated and S-pyridylethylated IgA1 molecules were dissolved in 160 μL of 50 mmol/L Tris-HCl buffer, pH 8.0, containing 2 mol/L urea. Twenty microliters of trypsin solution (10 μg trypsin/20 μL of the previously mentioned buffer) and 20 μL of 0.1 mol/L $CaCl_2$ were added, and the reaction mixture was incubated overnight at 37°C. The trypsin digest was made up to 2 mL by adding PBS, pH 7.4. The sample was applied to a Jacalin-agarose column (2 mL), and the passed fraction was eluted with 20 mL of the previously mentioned buffer. After further washing the column with 20 mL of 0.8 mol/L glucose, the α1 hinge glycopeptide fraction was eluted with 6 mL of 0.1 mol/L melibiose in the buffer. In our previous study, it was established by the Edman degradation method that the following 33mer IgA1 fragments containing IgA1 hinge glycopeptides were isolated by these procedures [11] HYTNPSQDVTVPCPV*PSTPPTPSPSTPPTPSPS*, where the italicized sequence indicates hinge peptide core.

Purification of the glycopeptides by high-performance liquid chromatography (HPLC) was carried out using a Cosmosil 5C18-300 column (Nacalai Tesque, 4.6 × 150 mm). Elution was carried out using a linear gradient for 60 minutes from 0 to 90% acetonitrile in 0.1% trifluoracetic acid (TFA) with a 0.5 mL/min flow rate. Detection was performed by monitoring the ultraviolet absorp-

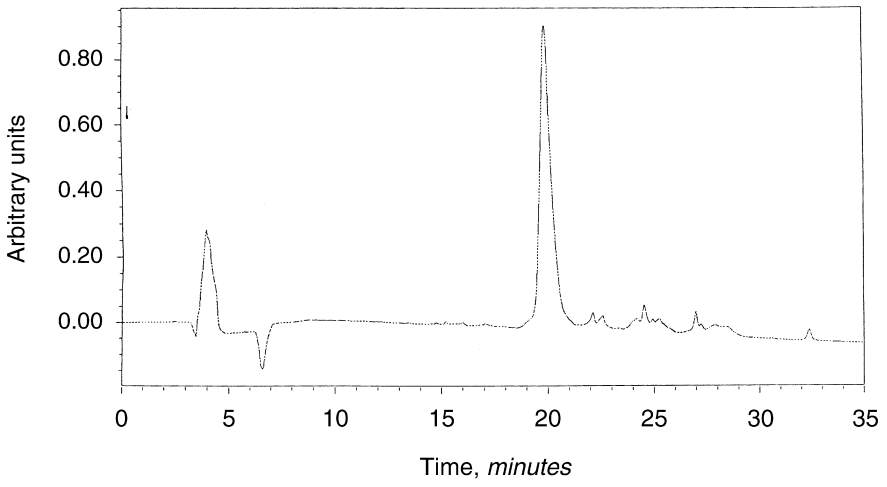

Fig. 2. High pressure liquid chromatography (HPLC) chromatogram of isolated 33mer IgA1 fragments containing hinge glycopeptides. A definite peak composed of the IgA1 hinge glycopeptides is observed around 20 minutes in all serum samples.

tion at 220 nm. Under these chromatographic conditions, the definite peak composed of the IgA1 hinge glycopeptides was observed around 20 minutes in all the serum samples (Fig. 2). The material eluted at the peak position of the hinge was collected and lyophilized. As expected, the content of the isolated hinge of glomerular IgA1 was so small that no definite peak was observed on the HPLC chromatogram. Therefore, the 19- to 21-minute fractions were obtained and lyophilized in the same manner.

MALDI-TOFMS analyses

The isolated hinge glycopeptides were analyzed by MALDI-TOFMS in the positive ion mode to suppress the production of the adduct ion. The mass spectrometer used in this work was a Voyager-DE (PerSeptive Biosystems, Framingham, MA, USA). The matrix used was a mixture of saturated solution of 2,5-dihydroxy benzoic acid (DHB) and 5-methoxy salicylic acid in the ratio of 9:1 in 50% acetonitrile and 0.1% TFA/H_2O. The conditions of the mass spectrometry were as follows: accelerating voltage of 20,000 and grid voltage of 94.0%; 256 shots were then summed.

The calibration of m/z was performed using adrenocorticoid hormone (ACTH; m/z, 3661) and bovine insulin (5737).

Data analyses

The distribution of the MALDI-TOFMS peaks was compared among the five samples. The molecular size (m/z) of each peak was estimated, and the structure of the carbohydrate side chains in the IgA1 hinge (number of x, y, and z in the formula shown later in this article) was determined by referring to the corresponding theoretical molecular weights calculated from the numbers of GalNAc, Gal, or sialic acid (NANA) according to the following formula: molecular weight = 33mer IgA1 fragment containing the hinge peptide (33mer HP) + x GalNAc + y Gal + z NANA. In this formula, the molecular weights of each factor were as follows: 33mer HP, 3475.7; GalNAc, 203.2, Gal, 162.2; and NANA, 291.3. The combination number of x, y, and z was determined from the molecular weights.

Each peak in the mass spectrum was regarded as the corresponding glycoform of the hinge, if the measured molecular weight of the peak was consistent with the theoretical molecular weight of the hinge structure within a 0.5% error.

To perform the further analyses, the largest five peaks in each of the five IgA1 samples (total 25 peaks) were selected. Since eight peaks overlapped among the samples, 17 peaks were studied. The peaks are underlined in the Table 2, and labeled alphabetically (a) to (e) in asialo-type and (1) to (12) in sialo-type structure (Fig. 3 and Table 2). The number of GalNAc, Gal, and NANA of the 17 major peaks was compared among the glomerular IgA (Glom. IgA) and serum IgA of IgAN (SN IgA) and that of the controls (SC IgA) using Mann–Whitney's U-test. As both serum groups had two samples, the analysis numbers were 5 in Glom. IgA and 10 in the serum groups.

It was regarded as statistically significant if the P value was less than 0.05.

RESULTS

Figure 3 shows the actual mass spectra of the IgA1 hinge in the five samples. It was obvious that the distribution of the peaks of glomerular IgA and of the IgAN groups was shifted to a lower molecular weight range compared with that of the control groups.

All of the assigned peaks in each IgA group are listed in Table 2. Among the 17 major peaks that were the largest five peaks in any of the five IgA1 samples (underlined in Table 2), it was noted that four of the five main

Table 2. Distribution of assigned peaks of IgA1 hinge glycopeptides

	Glycoform			Theoretical	Measured molecular wt of each peak				
						Serum IgA1			
	x	y	z	molecular wt	Dep.IgA1	IgAN(1)	IgAN(2)	Cont.(1)	Cont.(2)
Asialo-type									
	3	2	0	4410	4389	4409	4404	–	–
	3	3	0	4572	–	4573	–	–	–
(a)	4	0	0	4289	4287	–	–	–	–
(b)	4	2	0	4613	4602	4612	4608	–	–
(c)	4	3	0	4775	–	4774	4768	4771	4769
(d)	4	4	0	4937	4911	4936	4929	4931	4931
	5	3	0	4978	–	4992	4974	4975	4975
(e)	6	2	0	5019	5015	–	–	–	–
Sialo-type									
	3	2	1	4701	–	4702	4692	–	–
(1)	3	3	1	4863	–	4862	–	–	–
(2)	3	3	2	5154	5152	5152	5147	–	–
(3)	4	2	1	4904	4885	4904	4900	–	–
(4)	4	2	2	5195	–	–	5191	–	–
(5)	4	3	1	5066	5070	5065	5058	5063	5062
(6)	4	3	2	5358	5364	5357	5351	5356	5350
	4	3	3	5649	–	–	–	–	5665
(7)	4	4	1	5228	–	5227	5221	5224	5224
	4	4	2	5520	–	5518	5515	5514	5512
	4	4	4	6102	6100	–	–	–	–
	5	2	1	5107	–	5107	5101	–	–
	5	3	1	5270	–	5271	5265	5267	5265
(8)	5	3	2	5561	5562	5562	5554	5576	5576
(9)	5	3	3	5852	–	5854	–	5867	5866
	5	4	1	5432	–	–	–	5416	5412
(10)	5	4	2	5723	–	–	–	5739	5736
(11)	5	4	3	6014	–	–	–	6030	6028
	5	4	4	6305	–	–	–	6323	6316
	5	5	3	6176	–	–	–	6158	6158
	6	3	2	5764	–	–	–	5783	5777
(12)	6	3	3	6055	–	–	–	6073	6067
	6	4	1	5635	5632	–	5643	5624	5618
	6	4	2	5926	–	–	–	5910	5912
	6	4	3	6217	6218	–	–	–	–
	6	5	3	6380	–	–	–	6369	6360
	6	6	2	6250	–	–	–	6277	6275

Minus signs mean not determined. Molecular weight = 33mer hinge peptide + x GalNAc + y Gal + z NANA = 3475.7 + 203.2 x + 162.2 y + 291.3 z. The observed peaks in every group of IgA1 are listed. In the 17 major peaks underlined, note that four of the five main peaks (peaks a, b, d and e) are asialo-type in the glomerular IgA1 hinge glycopeptides.

(a–e): Assigned major peaks of asialo-type hinge in each group.

(1–12): Assigned major peaks of sialo-type hinge.

peaks (peak a, b, d, and e) were asialo-type ($Z = 0$) in the glomerular IgA1 hinge glycopeptides. Figure 4 shows a comparison of the number of GalNAc, Gal, and sialic acid residues of the major peaks among the three IgA groups. The number of GalNAc was significantly decreased in the SN IgA group (mean ± SD; 3.8 ± 0.4) compared with that of the SC IgA group (5.1 ± 0.6, $P < 0.001$). The Gal content of Glom. IgA (2.0 ± 1.4) was significantly lower than that of SC IgA (3.4 ± 0.5, $P < 0.05$). The most striking difference was found in sialic acid, which was significantly decreased in Glom. IgA (0.2 ± 0.4) compared with those of both the SN (1.2 ± 0.8, $P < 0.05$) and SC IgA groups (2.4 ± 0.7, $P < 0.005$). The number of SN IgA was also significantly smaller than that of SC IgA ($P < 0.01$). Overall, numbers of carbohydrates composing *O*-glycans in deposited and serum IgA1 in IgAN were fewer than those in serum IgA1 in the control group.

Urea was used for isolating deposited IgA from glomeruli. Since the effect of the treatment on the glycoform of the glom. IgA1 hinge was considerable, the *O*-glycan glycoform was compared between urea-treated and non-treated IgA1 hinge glycopeptides. There was no significant difference between them (data not shown).

DISCUSSION

IgA nephropathy is defined as mesangial proliferative glomerulonephritis with the deposition of IgA and C3 in the mesangium [14]. Therefore, circulating IgA immune complexes are thought to be involved in the pathogenesis of IgAN [15]. However, since deposited IgA appears to

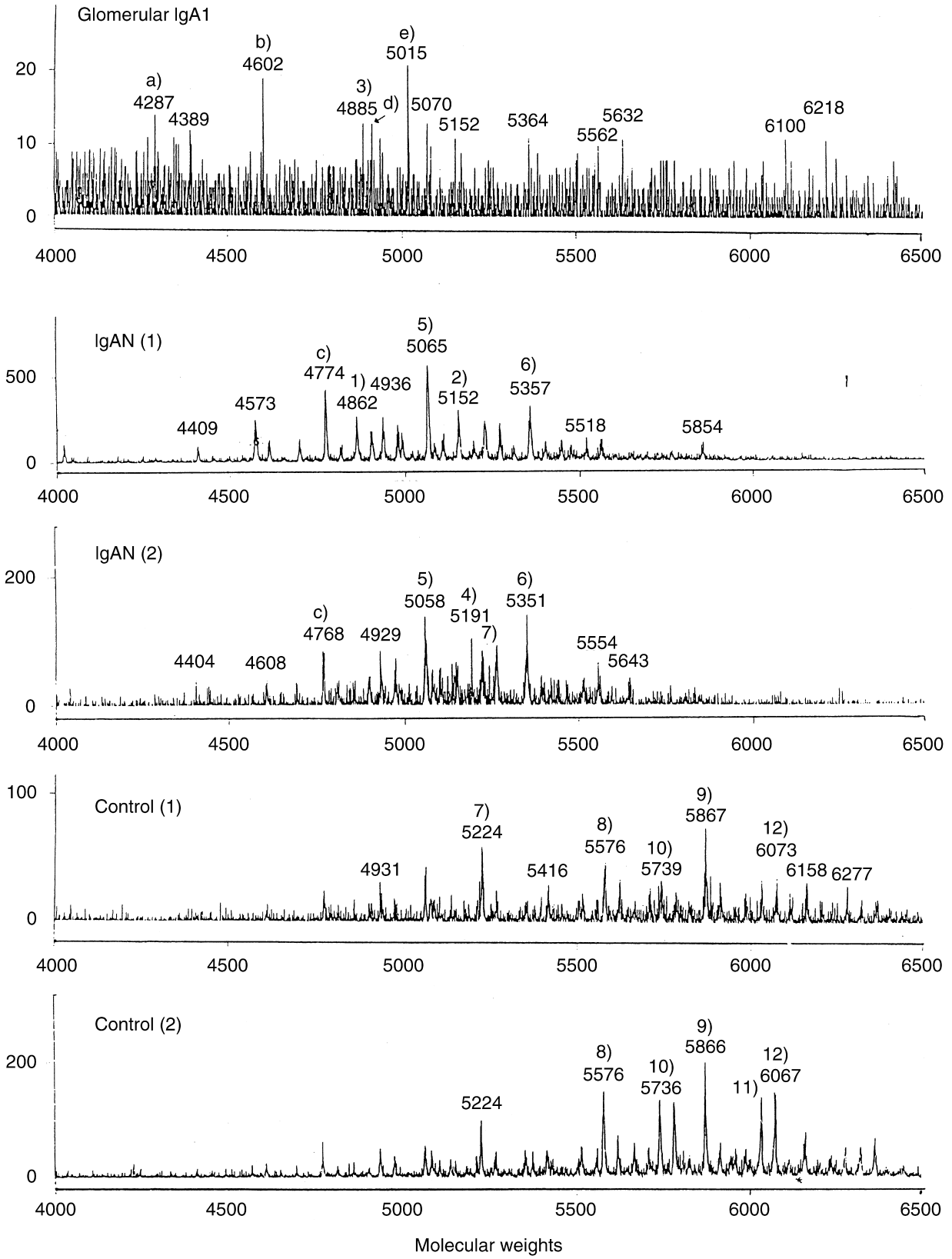

Fig. 3. Mass spectra of IgA1 hinge in five samples. The distribution of the peaks of glomerular IgA1 and IgA1 of the IgAN groups (upper 3 graphs) shifts to a molecular weight lower than in control groups (lower 2 graphs).

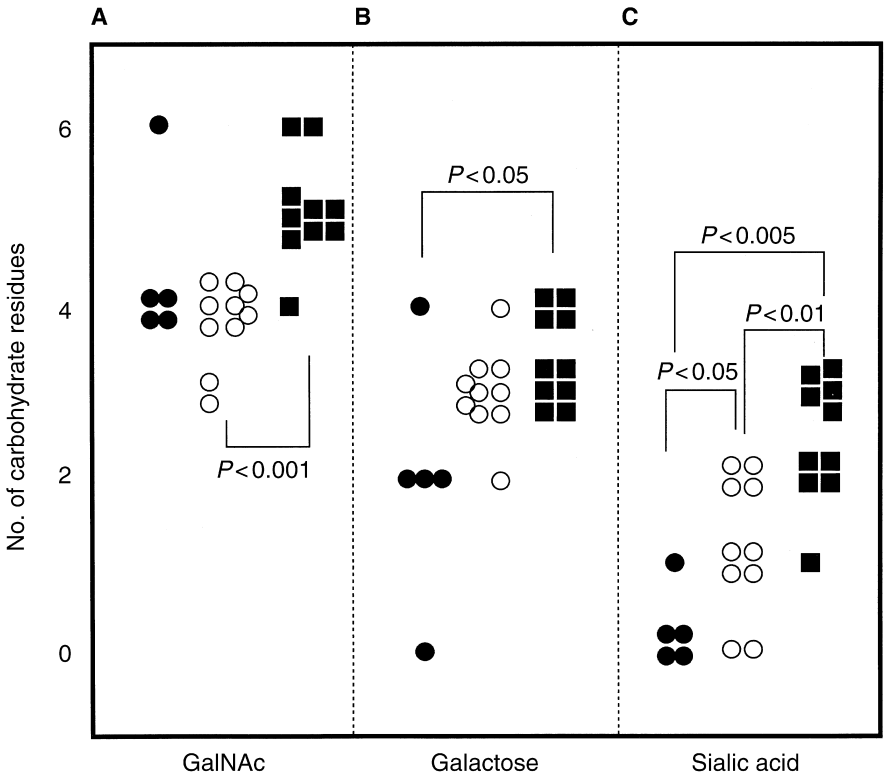

Fig. 4. Numbers of GalNAc, Gal, and sialic acid (NANA) residues in five main peaks in each group. (*A*; number of GalNAc) The mean numbers (± SD) of glomerular IgA1 (Glom. IgA; ●), serum IgA1 of IgAN (SN IgA; ○), and serum IgA1 of control (SC IgA; ■) groups are 4.4 ± 0.9, 3.8 ± 0.4, and 5.1 ± 0.6, respectively. The number is significantly decreased in SN IgA group compared with that of SC IgA group. (*B*; Gal) The mean numbers (± SD) of Glom., SN, and SC IgA groups are 2.0 ± 1.4, 3.0 ± 0.5, and 3.4 ± 0.5, respectively. In Glom. IgA, the number is significantly fewer than that of SC IgA. (*C*; sialic acid) The mean numbers (± SD) of Glom., SN, and SC IgA groups are 0.2 ± 0.4, 1.2 ± 0.8, and 2.4 ± 0.7, respectively. The content is significantly decreased in Glom. IgA group compared with those of both SN and SC IgA groups. The number of the SA IgA group is also significantly smaller than that of the SC IgA. The overall numbers of carbohydrates (GalNAc, Gal, and sialic acid) in the deposited and serum IgA1 in IgAN patients are fewer than those in the serum IgA1 in the control groups.

be polyclonal, displaying no apparent antigen restriction, we have suggested that physicochemical abnormalities of IgA might be ascribed to its glomerular deposition in a nonimmunologic manner [16–19].

In this study, we present the first information of precise structure of *O*-glycans in the deposited IgA1 in the glomeruli of IgAN by MALDI-TOFMS analyses. In our previous studies using an early MALDI-TOFMS model [11, 13, 20], it was impossible to analyze the native IgA1 hinge region. Only the molecular weights of the IgA1 fragments treated by the exo-glycosidases (neuraminidase, β-galactosidase, and N-acetyl galactosaminidase) could be estimated. In the present study, we could directly analyze the native IgA1 hinge using the newer MALDI-TOFMS apparatus, which had a enough capacity for estimating the molecular weights of the native IgA1 hinge glycopeptides.

We could differentiate the structure of the IgA1 hinge glycopeptides in the patients with IgAN from those in the non-IgAN controls. The results supported the series of previous studies [6–11], indicating that the IgA1 hinge of IgAN patients was underglycosylated. The information obtained in these previous studies had been rather indirect and limited. The majority of the data were obtained by the binding capacity of IgA1 to the lectins recognizing each carbohydrate part of *O*-glycans in the IgA1 hinge. In the biochemical analyses, we succeeded in the specific release of the Galβ1,3GalNAc side chains from the IgA1 hinge by gas-phase hydrazinolysis [21], and found an increase in the asialo-Galβ1,3GalNAc residue in the patients with IgAN [16]. However, these methods failed to find any free GalNAc residue. Therefore, we could not obtain any information about the content of Gal, which was bound to the outer side of GalNAc.

Recently, a few reports have appeared in which this problem was overcome using physicochemical methods. Allen et al precisely analyzed the *O*-glycan structure of serum IgA1 using FACE and demonstrated that the IgA1 *O*-glycans chains were truncated with increased terminal GalNAc in IgAN [10]. In our most recent study, Odani et al developed a method for the direct analysis of the IgA1 hinge using electrospray ionization/liquid chromatography/mass spectrometry (ESI/LC/MS) and observed a decreased sialylation and galactosylation of the hinge glycopeptides of serum IgA1 in IgAN [22].

However, the physicochemical techniques such as hydrazinolysis, FACE, or ESI/LC/MS were unable to analyze the *O*-glycan structure of the deposited IgA1 in the glomeruli because of its small quantity. The MALDI-TOFMS apparatus used in our current study, however, had enough sensitivity to obtain the information from such a small IgA1 sample. According to the manufacturer's instructions, the apparatus is capable of estimating molecular weights from 50 fmol/L of a calibration sample

(Neurotensin) within a 0.1% error. It was observed that the underglycosylated, especially the asialo-type, *O*-glycans were predominantly present in the deposited IgA1. Recently, Allen et al also observed an increased reactivity of the glomerular IgA1 to *Helix asperosa*, which has an affinity to GalNAc, suggesting a decrease in the Gal content in the mesangial IgA1 (abstract; Allen et al, *J Am Soc Nephrol* 10:506A, 1999).

There have been a number of reports analyzing the role of the *O*-glycan structure of IgA1 for the pathogenic role of IgAN. The removal of sialic acid and galactose from the *O*-glycan side chains in the IgA1 hinge served to accelerate the self-aggregation and adhesion to extracellular matrix proteins in the glomeruli [17]. Furthermore, aggregated IgA1 induced by neuraminidase treatment had a lower number of *O*-linked sugar side chains in the hinge region [18]. Recently, we isolated and characterized the IgA1 molecules that could accumulate in rat glomeruli [19] and called them the "glomerulophilic" IgA1. Structural analyses clarified that the hinge region in the "glomerulophilic" IgA1 was highly deglycosylated. Conversely, it was clarified that enzymatically deglycosylated IgA1 accumulated and induced interleukin-6 production in rat glomeruli (abstract; Sano et al, *J Am Soc Nephrol* 10:115A, 1999). These results indicated that glomerular IgA deposition could occur because of the nonimmunologic mechanisms induced by the aberrant *O*-glycosylation in the IgA1 molecules. It has also been suggested that the under-*O*-glycosylated IgA1 had the following other characteristics: a conformational instability [16], stickiness [23], and binding to IgG [8, 24]. Recently, Tomana et al suggested that the deficiency of Gal in the IgA1 hinge resulted in the generation of antigenic determinants containing GalNAc residues that were recognized by naturally occurring IgG and IgA antibodies [25].

In conclusion, we could present the first information, directly analyzing the detailed structure of the hinge region of the glomerular IgA1 and determining the definite under-*O*-glycosylation of its hinge glycopeptides. Especially, the decrease in the sialic acid (NANA) residue was noted in the glomerular IgA1. Along with the series from previous investigations, it was confirmed that the under-*O*-glycosylation of IgA1 played an essential role in its glomerular deposition, resulting in the occurrence of IgAN. It will be necessary to investigate the actual cause of the under-*O*-glycosylation of IgA1 molecules in the future.

ACKNOWLEDGMENTS

The study was supported by grants from the Ministry of Education (No. 11671047) and the Aichi Kidney Foundation. The authors are grateful to Mr. R. Yamakawa of the Education and Research Apparatus Center of Nagoya University for his valuable suggestions about mass spectrometry.

Reprint requests to Yoshiyuki Hiki, M.D., Ph.D., Department of Medicine, Daiko Medical Center, Nagoya University, 1-1-20, Daiko-Minami, Higashi-ku, Nagoya, Aichi 461-0047, Japan.
E-mail: yhiki@med.nagoya-u.ac.jp

REFERENCES

1. Conley ME, Cooper MD, Michael AF: Selective deposition of immunoglobulin A1 in immunoglobulin A nephropathy, anaphylactoid purpura nephritis, and systemic lupus erythematosus. *J Clin Invest* 66:1432–1436, 1980
2. Baenziger J, Kornfeld S: Structure of the carbohydrate units of IgA1 immunoglobulin. II. Structure of the *O*-glycosidically linked oligosaccharide units. *J Biol Chem* 249:7270–7281, 1974
3. Mattu TS, Pleass RJ, Willis AC, *et al:* The glycosylation and structure of human serum IgA1, Fab, and Fc regions and the role of *N*-glycosylation on Fcα receptor interactions. *J Biol Chem* 273:2260–2272, 1998
4. Cunningham LW: Microheterogeneity and function of glycoproteins, in *Glycoprotein of Blood Cells and Plasma*, edited by Jamieson GA, Greenwalt TJ, Philadelphia, J.B. Lippincott Company, 1971, pp 16–34
5. Frangione B, Wolfenstein-Todel C: Partial duplication in the "hinge" region of IgA1 myeloma proteins. *Proc Nat Acad Sci USA* 69:3673–3676, 1972
6. Mestecky J, Tomana M, Crowley-Nowick P-A, *et al:* Defective galactosylation and clearance of IgA1 molecules as a possible etiopathogenic factor in IgA nephropathy. *Contrib Nephrol* 104:172–183, 1993
7. Allen AC, Harper SJ, Feehally J: Galactosylation of N- and O-linked carbohydrate moieties of IgA1 and IgG in IgA nephropathy. *Clin Exp Immunol* 100:470–474, 1995
8. Tomana M, Matousovic K, Julian BA, *et al:* Galactose-deficient IgA1 in sera of IgA nephropathy patients is present in complexes with IgG. *Kidney Int* 52:509–516, 1997
9. Hiki Y, Iwase H, Saitoh M, *et al:* Reactivity of glomerular and serum IgA1 to Jacalin in IgA nephropathy. *Nephron* 72:429–435, 1996
10. Allen AC, Bailey EM, Barratt J, *et al:* Analysis of IgA1 *O*-glycans in IgA nephropathy by fluorophore-assisted carbohydrate electrophoresis. *J Am Soc Nephrol* 10:1763–1771, 1999
11. Hiki Y, Tanaka A, Kokubo T, *et al:* Analyses of IgA1 hinge glycopeptides in IgA nephropathy by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *J Am Soc Nephrol* 9:577–582, 1998
12. Westberg NG, Michael AF: Human glomerular basement membrane preparation and composition. *Biochemistry* 9:3837–3846, 1970
13. Iwase H, Tanaka A, Hiki Y, *et al:* Estimation of the number of *O*-linked oligosaccharides per heavy chain of human serum IgA1 by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOFMS) analysis of the hinge peptide. *J Biochem* 120:393–397, 1996
14. Berger J, Hinglais N: Lés depôts intercapillaires d'IgA-IgG. *J Urol Nephrol* 74:694–695, 1968
15. Woodroffe AJ, Gormly AA, McKenzie PE, *et al:* Immunologic studies in IgA nephropathy. *Kidney Int* 18:366–374, 1980
16. Hiki Y, Iwase H, Kokubo T, *et al:* Association of asialo-galactosylβ1-3N-acetylgalactosamine on the hinge with a conformational instability of Jacalin-reactive immunoglobulin A1 in immunoglobulin A nephropathy. *J Am Soc Nephrol* 7:955–960, 1996
17. Kokubo T, Hiki Y, Iwase H, *et al:* Protective role of IgA1 glycans against IgA1 self-aggregation and adhesion to extracellular matrix proteins. *J Am Soc Nephrol* 9:2048–2054, 1998
18. Iwase H, Tanaka A, Hiki Y, *et al:* Aggregated human serum immunoglobulin A1 induced by neuraminidase treatment had a lower number of *O*-linked sugar chains on the hinge portion. *J Chromatogr B* 724:1–7, 1999
19. Hiki Y, Kokubo T, Iwase H, *et al:* Underglycosylation of IgA1 hinge plays a certain role for its glomerular deposition in IgA nephropathy. *J Am Soc Nephrol* 10:760–769, 1999
20. Hiki Y, Horii A, Iwase H, *et al: O*-linked oligosaccharide on IgA1 hinge region in IgA nephropathy. *Contrib Nephrol* 111:73–92, 1995

21. Iwase H, Ishii-Karakasa I, Fujii E, *et al:* Analysis of glycoform of *O*-glycan from human myeloma immunoglobulin A1 by gas-phase hydrazinolysis following pyridylamination of oligosaccharides. *Anal Biochem* 206:202–205, 1992
22. Odani H, Hiki Y, Nishimoto A, *et al:* Direct evidence for decreased sialylation and galactosylation of human serum Fc O-glycosylated hinge peptides in IgA nephropathy by mass spectrometry. *Biochem Biophys Res Commun* 271:268–274, 2000
23. Iwase H, Ohkawa S, Ishii-Karakasa I, *et al:* Study of the relationship between sticky human serum IgA1 and its *O*-glycan glycoform. *Biochem Biophys Res Commun* 261:472–477, 1999
24. Iwase H, Yokozeki Y, Hiki Y, *et al:* Human serum immunoglobulin G3 subclass bound preferentially to asialo-, agalactoimmunoglobulin A1/sepharose. *Biochem Biophys Res Commun* 264:424–429, 1999
25. Tomana M, Novak J, Julian BA, *et al:* Circulating immune complexes in IgA nephropathy consist of IgA1 with galactose-deficient hinge region and antiglycan antibodies. *J Clin Invest* 104:73–81, 1999