Analyses of IgA1 Hinge Glycopeptides in IgA Nephropathy by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry

YOSHIYUKI HIKI,* ATSUSHI TANAKA,† TOHRU KOKUBO,* HITOO IWASE,† JOJI NISHIKIDO,‡ KYOKO HOTT A,† and YUTAKA KOBAYASHI*
Departments of *Medicine and †Biochemistry, School of Nursing and Medicine, Kitasato University, Sagamihara, Kanagawa, Japan; and ‡Analytical Research Center, Asahi Chemical Industry, Fuji, Shizuoka, Japan.

Abstract. This study was performed to analyze the structural variety of O-glycans on the IgA1 hinge in IgA nephropathy (IgAN). The IgA1 fragments containing the hinge glycopeptide (33-mer hinge peptide core (HP) + O-glycans) were separated from 13 IgAN patients, eight healthy control subjects, and 11 patients with other primary glomerulonephritides by pyridyl-ethylation, trypsin treatment, and Jacalin affinity chromatography. Because of the use of Jacalin, only the Galβ1-3GalNAc residue containing IgA was analyzed. The molecular weights (MW) of the IgA1 fragments treated by the following sequential treatment by exoglycosidases were estimated using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry: (1) Sialidase treatment: the MW of the two observed peaks A and B were compatible with (A) HP + 4GalNAc + 4Gal and (B) HP + 5GalNAc + 4Gal. (2) Sialidase and galactosidase: the MW of the two identified peaks a and b were consistent with (a) HP + 4GalNAc and (b) HP + 5GalNAc. (3) Sialidase, galactosidase, and α-N-acetylgalactosaminidase. All subjects revealed one peak, indicating the 33-mer IgA1 hinge peptide core. The intensity rate of peak B/A was significantly decreased in the IgAN group (mean ± SD, 1.01 ± 0.08) compared with the negative control subjects (healthy group, 1.15 ± 0.06, P = 0.0048; other glomerulonephritis group, 1.13 ± 0.10, P = 0.0049; Scheffe’s F test). These results suggested the presence of a defect in the Gal and/or GalNAc residues in the IgA1 hinge glycopeptides in IgAN. (J Am Soc Nephrol 9: 577-582, 1998)

The IgA1 molecule, which is the predominant subtype to deposit in the glomeruli in IgA nephropathy (IgAN), has a unique glycosylated hinge possessing O-glycan side chains (1). Previously, we succeeded in the specific release of galactosyl β1-3 N-acetylgalactosamine (Galβ1-3GalNAc) side chains from the IgA1 hinge by gas-phase hydrazinolysis (2) and found an increase in the asialo-Galβ1-3GalNAc residue in the patients with IgAN (3). However, the actual glycoform (the distribution of a number of O-glycan side chains on the IgA1 molecule) remained unclear. Furthermore, the methods of gas-phase hydrazinolysis failed to find the GalNAc residue, which had been found by the alkaline degradation method of Baenziger and Kornfeld (1). On the other hand, Mestecky et al. (4) and Allen et al. (5) suggested a decrease in the Gal content in the IgA1 molecule of IgAN patients.

We have attempted to investigate the structural variety of the O-glycan side chains in the IgA1 hinge region, using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS) and faced difficulty because of the presence of sialic acid residue (6). Most recently, the problem has been overcome by the serial treatments of sialidase, galactosidase, and α-N-acetylgalactosaminidase, and the microheterogeneity of the O-glycan glycoform on the IgA1 hinge has been elucidated (7).

The study presented here, therefore, was performed to analyze the structural varieties of O-glycans on the IgA1 hinge region in IgAN, determining the number of enzyme-treated O-glycan side chains using MALDI-TOFMS.

Materials and Methods
Materials
Thirteen patients with biopsy-proven IgAN, eight healthy individuals, and 11 patients with other primary glomerulonephritides (GN) (membranous nephropathy, lipid nephrosis, focal glomerulosclerosis, and mesangial proliferative GN without IgA deposits, 2 respectively; acute glomerulonephritis, membranoproliferative glomerulonephritis, and diabetic nephropathy, 1 respectively) were the subjects of this study.

The following materials were purchased from the sources indicated: PD-10 from Pharmacia Biotech AB (Uppsala, Sweden); 4-vinyl pyridine from Aldrich Chemical Co. (Milwaukee, WI); Jacalin-agarose from Vector Laboratories (Burlingame, CA); neuraminidase from Arthrobacter ureafaciens from Boehringer Mannheim (Germany); β-galactosidase from bovine testis and trypsin from Sigma Chemical
Co. (St. Louis, MO); and α-N-acetylgalactosaminidase from Acremonium sp from Seikagaku Co. (Tokyo, Japan).

**Preparation of IgA1 by Jacalin-Agarose Affinity Chromatography**

Three-to-five milliliters of sera from each subject were treated with 50% ammonium sulfate precipitation and applied to the Jacalin column (3 ml). After the column was thoroughly washed with 0.01 M phosphate buffer, 0.15 M NaCl, pH 7.5, containing 0.01% sodium azide (PBS), it was first eluted with 0.8 M glucose and then with 0.1 M melibiose as described previously (7). All procedures were carried out at room temperature (RT). 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 precipitated with 50% ammonium sulfate, and the precipitated IgA1 was dialyzed against distilled water and lyophilized.

**Preparation of the S-Pyridylethylated α1 Chain from the Separated IgA1**

Each IgA1 sample (1 to 3 mg) was dissolved in 500 μl of 0.4 M Tris-HCl buffer, pH 8.6, containing 6 M guanidine-HCl and 0.2 M ethylenediamine tetra-acetic acid. To dissociate the disulfide linkage, 5.0 μl IgA1 mg of dithiothreitol solution (200 mg/ml) was added and stirred. After heating at 50°C for 4 h, 1.6 μl/μl IgA1 mg of 4-vinyl pyridine was added, and the reaction mixture was allowed to stand for 90 min at RT. The reaction was terminated by the addition of 50 μl of 2 M formic acid, and the mixture was then dialyzed against distilled water overnight at 4°C.

**Preparation of the Hinge Glycopeptide from a Trypsin Digest of the Heavy Chain**

Approximately 0.1 mg of the S-pyrudylethylated α1 chain was dissolved in 160 μl of 50 mM Tris-HCl buffer, pH 8.0, containing 2 M urea. Twenty microliters of trypsin solution (10 μg of trypsin/20 μl of the above buffer) and 20 μl of 0.1 M CaCl2 were added, and the reaction mixture was incubated overnight at 37°C. The trypsin digest was made up to 2 ml by adding 0.175 M Tris-HCl buffer, pH 7.6, and the reaction mixture was incubated at 4°C for 18 h. Purification of the glycopeptide by HPLC was carried out using a linear gradient for 60 mm from 0 to 90% acetonitrile in 0.1% trifluoroacetic acid. Detection was performed by monitoring the ultraviolet absorption at 220 nm. The material eluted at RT was collected and concentrated.

**Treatment of the Glycopeptide with Exoglycosidases**

The purified glycopeptide was dissolved in 95 μl of 0.2 M sodium acetate buffer, pH 5.0. Five microliters of neuraminidase from Arthrobacter ureafaciens (1 U/100 μl) were added to the solution followed by incubation at 37°C overnight. The desialylated glycopeptide was purified by HPLC under the gradient conditions from 0 to 90% acetonitrile in 0.1% trifluoroacetic acid. A part of the purified desialylated glycopeptide (IgAN patients, n = 13; healthy control subjects, n = 8; patients with other GN, n = 11) was analyzed by MALDI-TOFMS.

The rest of the solution of asialoglycopeptide was then treated with β-galactosidase as follows (IgAN patients, n = 10; healthy control subjects, n = 7; patients with other GN, n = 10): The samples were dissolved in 30 μl of 0.2 M acetate buffer, pH 5.0, and then 20 μl of β-galactosidase from bovine testis (1.0 U/ml) was added to each sample. The reaction mixture was incubated overnight at RT. HPLC purification of the asialo-β-galacto-glycopeptide was carried out as described previously. A part of the product of asialo- and agalacto-glycopeptide was then measured by MALDI-TOFMS.

For the final treatment of the glycopeptide with α-N-acetylgalactosaminidase, the asialo-, agalacto-glycopeptide (IgAN patients, n = 5; healthy control subjects, n = 7; patients with other GN, n = 4) was dissolved in 83 μl of 50 mM sodium citrate buffer, pH 4.5, and then 17 μl of α-N-acetylgalactosaminidase from Acremonium sp. (13 U/ml) was added to the solution. The incubation of the reaction mixture was performed overnight at RT. Purification of the deglycosylated peptide was carried out by HPLC on a Cosmosil 5C18-300 column (4.6 × 150 mm) under the same conditions as described previously. The molecular weight (MW) of the purified deglycosylated peptide was estimated by MALDI-TOFMS.

**MALDI-TOFMS Analyses**

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, United Kingdom). The matrix used was α-cyano-4-hydroxy cinnamic acid (10 mg/ml) in a 70% acetonitrile solution. The mass spectra were obtained with almost the same laser power, and 10 shots were summed. The accuracy of the mass spectra was less than 0.6% error. MALDI-TOFMS analysis of the glycopeptide sequentially treated with neuraminidase, β-galactosidase, and α-N-acetylgalactosaminidase was carried out under the above conditions.

**Analyses of Amino Acid Sequence of the Hinge Peptide Core**

The sequence of 16 amino acids from the N-terminal and that of five amino acids from the C-terminal of the hinge peptide core were analyzed by the conventional procedures of the Edman degradation method.

**Statistical Analyses**

The relative content of each peak was calculated according to its respective height. The intensity rate of the peaks observed from the serial treatment of the enzymes (peak B/A rate was calculated in each sample from peaks A and B observed by the treatment of sialidase and peak b/a rate by sialidase and β-galactosidase) was then compared among healthy, IgAN, and other GN groups. Scheffe’s F test was used for estimating the statistical significance. A P value less than 0.05 was considered significant.

**Results**

**Estimation of the MW of Each Peak by MALDI-TOFMS**

The mean MW of each peak taken by MALDI-TOFMS in the IgAN and control groups is summarized in Table 1. As in our previous studies (6,7), the peak of the nontreated IgA1 hinge glycopeptide ranged so broadly (Mr, 4500 to 7000)
Table 1. Molecular weights of IgA1 hinge glycopeptides

<table>
<thead>
<tr>
<th>Group</th>
<th>Theoretical MW</th>
<th>Healthy (n = 8)</th>
<th>IgA-N (n = 13)</th>
<th>Other GN (n = 11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SA removed HGP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>peak A: HP + 4GalNAc + 4Gal</td>
<td>4936</td>
<td>4952.5 ± 8.8</td>
<td>4966.4 ± 14.9</td>
<td>4962.0 ± 11.7</td>
</tr>
<tr>
<td>peak B: HP + 5GalNAc + 4Gal</td>
<td>5139</td>
<td>5152.1 ± 3.6</td>
<td>5155.5 ± 4.5</td>
<td>5157.9 ± 5.8</td>
</tr>
<tr>
<td>SA and Gal removed HGP</td>
<td></td>
<td>(n = 7)</td>
<td>(n = 10)</td>
<td>(n = 10)</td>
</tr>
<tr>
<td>peak a: HP + 4GalNAc</td>
<td>4288</td>
<td>4296.7 ± 2.2</td>
<td>4296.2 ± 3.1</td>
<td>4300.7 ± 4.0</td>
</tr>
<tr>
<td>peak b: HP + 5GalNAc</td>
<td>4491</td>
<td>4502.3 ± 4.7</td>
<td>4503.1 ± 4.8</td>
<td>4505.7 ± 3.9</td>
</tr>
<tr>
<td>SA, Gal, and GalNAc removed HGP</td>
<td></td>
<td>(n = 7)</td>
<td>(n = 5)</td>
<td>(n = 4)</td>
</tr>
<tr>
<td>(33-mer hinge peptide core)</td>
<td>3476</td>
<td>3480.1 ± 6.5</td>
<td>3481.6 ± 2.6</td>
<td>3490.5 ± 8.2</td>
</tr>
</tbody>
</table>

MW, molecular weight; SA, sialic acid; HGP, 33-mer glycopeptide including hinge; HP, 33-mer hinge peptide core.

(Figure 1) that no estimation could be made based on the results. The measured MW (m/z) of the observed peaks of IgA1 hinge glycopeptides serially treated by exoglycosidases were quite consistent with those of the theoretically calculated MW (Table 2) as follows.

**Sialidase Treatment.** Two similar peaks were observed in each subject of the three groups. The mean ± SD MW of peak A were 4952.5 ± 8.8 in healthy control subjects (n = 8), 4966.4 ± 14.9 in IgA1 patients (n = 13), and 4962.0 ± 11.7 in the patients with other GN (n = 11), respectively, and those of peak B were 5152.1 ± 3.6, 5155.5 ± 4.5, and 5157.9 ± 5.8. The observed MW of the two peaks were compatible with the calculated MW of (A) hinge peptide core (HP) + 4GalNAc + 4Gal (4936) and (B) HP + 5GalNAc + 4Gal (5139), respectively.

Small peaks having a compatible MW (4784.1 ± 2.7) with that of HP + 4GalNAc + 3Gal (4774) and having the approximate MW of 5320 (compatible with HP + 5GalNAc + 5Gal (5301)) were also identified in some cases.

**Sialidase and β-Galactosidase.** Two definite peaks (peaks a and b) were also differentiated. The MW of peak a (healthy control subjects [n = 7], 4296.7 ± 2.2; IgA1 [n = 10], 4296.2 ± 3.1; and other GN [n = 10], 4300.7 ± 4.0) and (b) (healthy control subjects, 4502.3 ± 4.7; IgA1, 4503.1 ± 4.8; and other GN, 4505.7 ± 3.9) were consistent with those of (a) HP + 4GalNAc (4288) and (b) HP + 5GalNAc (4491), respectively. Tiny peaks (mean MW 4094.3 ± 2.7 and approximate MW 4730) were also identified in some cases and were regarded to be HP + 3GalNAc (theoretical MW, 4085) and HP + 6GalNAc (4694), respectively.

**Sialidase, β-Galactosidase, and α-N-Acetylgalactosaminidase.** All subjects revealed one peak (healthy control subjects [n = 7], 3480.1 ± 6.5; IgA1 [n = 5], 3481.6 ± 2.6; and other GN [n = 4], 3490.5 ± 8.2), which indicated the
Table 2. Theoretical molecular weights of asialo-hinge glycopeptides

<table>
<thead>
<tr>
<th>No. of Gal</th>
<th>No. of GalNAc</th>
</tr>
</thead>
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<tr>
<td>0</td>
<td>3476</td>
</tr>
<tr>
<td>1</td>
<td>3841</td>
</tr>
<tr>
<td>2</td>
<td>4206</td>
</tr>
<tr>
<td>3</td>
<td>4571</td>
</tr>
<tr>
<td>4</td>
<td>4936</td>
</tr>
<tr>
<td>5</td>
<td>5301</td>
</tr>
</tbody>
</table>

* Underlined numbers are discussed in this study.
* Theoretically these structures are not present in IgA1 hinge glycopeptide.

33-mer IgA1 fragment containing the hinge peptide core (HP; MW, 3476). Two samples (IgAN and healthy control) of the mass spectra are shown in Figure 1.

Distribution of Sialidase-Treated IgA1 Hinge Glycopeptides

As shown in Figure 2, the intensity rate of peak B/A was significantly decreased in the IgAN group (mean ± SD, 1.01 ± 0.08) compared with those of the negative control groups (Scheffe's F test, versus healthy control subjects, 1.15 ± 0.06, \( P = 0.0048 \); versus other GN patients, 1.13 ± 0.10, \( P = 0.0049 \)). The peaks compatible with HP + 3GalNAc + 3Gal and HP + 5GalNAc + 5Gal were so small that comparative study of the intensity of these peaks could not be performed.

Figure 2. Sialidase-treated IgA1 hinge. The intensity rate of peak B/A is significantly decreased in the IgA nephropathy (IgAN) group (mean ± SD, 1.01 ± 0.08) compared with that of healthy control subjects (1.15 ± 0.06, \( P = 0.0048 \)) and that of other GN patients (1.13 ± 0.10, \( P = 0.0049 \)).

Sialidase- and β-Galactosidase-Treated Hinge

No significant differences were found among the three groups in this study (peak b/a: healthy control subjects, 1.34 ± 0.26; IgAN, 1.31 ± 0.11; other GN, 1.37 ± 0.20) (Figure 3). Comparative study of the peaks regarded as HP + 3GalNAc and HP + 6GalNAc were impossible because of the limited sensitivity of MALDI-TOFMS.

Analyses of Amino Acid Sequence of the Hinge Peptide Core

The sequence of 16 amino acids from the N-terminal and that of five amino acids from the C-terminal were clarified to be HYTNPSQDVTVPCPVP and TPSPS, respectively. Therefore, it was clarified that the amino acid sequence of the hinge peptide core was determined to be a 33-mer peptide (MW,
Discussion

MALDI-TOFMS analysis provided some information of the structural varieties of O-glycan side chains in the IgA1 hinge glycopeptides differentiating the tiny differences in their MW. Peaks A and B observed in the sialidase-treated IgA1 hinge and peaks a and b in the sialidase- and β-galactosidase-treated hinge were found to have identical MW with the IgA1 hinge containing 4 GalNAc + 4Gal (peak A) and 5GalNAc + 4 Gal (peak B), and 4 (peak a) and 5 GalNAc (peak b), respectively. Furthermore, it was clarified that the peak B/A rate in the IgAN group was significantly lower than those of the control groups. On the other hand, no significant differences of peak b/a were found among the three groups in this study.

The tiny peak of HP + 4GalNAc + 3Gal in the sialidase-treated hinge and that of HP + 3GalNAc in the sialidase- and galactosidase-treated hinge were also observed in some cases. Small peaks of higher MW than peak B or b in each enzyme-treated hinge peptides (peak C and c, respectively) were also observed. The approximate MW were 5320 in peak C that was 165 bigger than peak B, and 4730 in peak c, 225 higher than peak b. Theoretically, MW increase 162 and 203 in every Gal and GalNAc residue, respectively. Therefore, the MW of these peaks were compatible with HP + 4GalNAc + 5Gal in peak C and HP + 6GalNAc in peak c, respectively. Because the IgA1 hinge peptide has five serines, it may be speculated that one GalNAc bound not to serine but to threonine in peak c. However, the intensities of these peaks were so small or invisible that the comparative studies could not be performed. Therefore, in this study, the restricted peaks definitely identified (peaks A, B and a, b) were analyzed.

Theoretically, the absolute content of the hinge fragment containing 4 GalNAc residues (4 GalNAc hinge), which was observed as peak a after the treatment with sialidase and β-galactosidase, would equal the sum of the 4 GalNAc hinge containing 0, 1, 2, 3, and 4 Gal (peak A) residues under the condition of the sialidase treatment. Similarly, that of the sialidase- and β-galactosidase-treated 5 GalNAc hinge (peak b) would be composed of the total contents of the sialidase-treated 5 GalNAc hinge possessing 0, 1, 2, 3, 4 (peak B), and 5 Gal residues. Therefore, to explain the lack of difference in peak b/a among the three groups and the significant decrease in peak B/A in IgAN, it should be reasonable to speculate the presence of a shift from the 4 GalNAc hinge having 0, 1, 2, and 3 Gal residues to that having the 4 Gal residue (peak A), and/or the deviation from the 5 GalNAc hinge possessing the 4 Gal residue (peak B) to those containing 0, 1, 2, 3, and 5 Gal residues in IgAN. However, probably because of the limited sensitivity of MALDI-TOFMS, we could not clearly identify the peaks in the sialidase-treated hinge fragments except for peaks A and B. Therefore, in this study, the actual abnormality(ies) of O-glycosylation could not be clarified. Furthermore, the possibility of the abnormal GalNAc residue (a selective defect of the GalNAc residues) resulting in the decrease of peak B/A should also be considered.
IgAN, using a chemical method such as MALDI-TOFMS. Mestecky et al. (4) and Allen et al. (5) found a decreased terminal galactose content by examination with various lectins and reduced β1-3 galactosyl transferase activity in the B cells (10). However, we could not obtain sufficient information to support their data in this study.

As far as the role of the aberrant O-glycosylation on the IgA1 hinge region for the pathogenesis of IgAN is concerned, we have recently reported the role of the asialo Galβ1-3GalNAc residue for the formation of macromolecular IgA1 due to the conformational instability of the IgA1 molecule (3,11). Several years ago, we found a significant increase in serum IgA1 possessing an affinity for human IgA in patients with IgAN (12). In the most recent study, we observed that the IgA1-IgA1 interaction was inhibited by Galβ1-3GalNAc, as well as the synthesized IgA1 hinge peptide core (13). From these observations, we suspect that the aberrant O-glycosylation on the IgA1 hinge plays a role in the formation of macromolecule IgA, resulting in its glomerular deposition in IgAN. Mestecky et al. (4) proposed that the decreased galactosylation of IgA1 hinge altered the physiologic clearance of IgA1 molecules by hepatocytes. We found data suggesting a reduced Gal and/or GalNAc content in this study. Although the hypothesis of the role of the O-glycans is different in these two studies, their data and ours indicate that the underglycosylation of the IgA1 hinge region is involved in the pathogenesis of IgAN.

This study provided the first information on the difference in the glycoform of the O-glycan residue on the IgA1 hinge in IgAN, using the chemical approach of MALDI-TOFMS. Although we could not directly observe the O-glycan residues specific to IgAN in this study, such a chemical approach would help clarify the actual abnormalities of the glycosylation of IgA1 in IgAN in the near future.

Acknowledgments

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References