Aberrantly glycosylated serum IgA1 are closely associated with pathologic phenotypes of IgA nephropathy

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Aberrantly glycosylated serum IgA1 are closely associated with pathologic phenotypes of IgA nephropathy.

Background. IgA nephropathy (IgAN) is the most common glomerulonephritis with various histologic and clinical phenotypes. The mechanisms underlying the pathogenesis of IgAN remained unclear. But now altered O-glycosylation of serum IgA1 observed in these patients was considered to be a key contributory factor. The aim of the current study is to investigate whether aberrantly glycosylated IgA1 was associated with pathologic phenotypes of IgAN.

Methods. Sera from 107 patients with IgAN recently diagnosed were collected. Fifty patients were with mild mesangial proliferative IgAN, the others were with focal proliferative and sclerosing IgAN. Sera from 22 normal blood donors were used as normal controls. Biotinylated lectins were used in enzyme-linked immunosorbent assay (ELISA) to examine different glycans on IgA1 molecules. The α2,6 sialic acid was detected by elderberry bark lectin (SNA), the exposure of terminal galactose (Gal) and N-acetylgalactosamine (GalNAc) were detected by arachis hypogaea [peanut agglutinin (PNA)] and vilsa villosa lectin (VVL), respectively. The serum IgA1 glycans levels corrected by serum IgA1 concentrations were compared between patients and controls.

Results. Reduced terminal α2,6 sialic acid (1.16 ± 0.21 vs. 0.98 ± 0.31) (P = 0.008) and galactosylation (0.30 ± 0.29 vs. 0.16 ± 0.19) (P = 0.029) increased exposure of (GalNAc) (0.00 ± 0.08) and galactosylation (0.30 ± 0.017), but had no statistical difference in the pathogenesis of IgAN [9–13].

Conclusion. The desialylation and degalactosylation of IgA1 in sera of patients with IgAN were closely associated with pathologic phenotypes.

IgA nephropathy (IgAN) is the most common glomerulonephritis in the world. It is characterized by the deposition of polymeric IgA1 subclass in kidney mesangium [1]. But the mechanism of IgA1 deposition is unclear and it was suggested that IgA1 molecule was involved in the pathogenesis of IgAN [2–5]. Our previous study demonstrated that heat aggregated serum IgA1 from patients with IgAN had a higher binding capacity and stronger biologic effects to in vitro cultured human mesangial cells than that from healthy controls. It was suggested that serum IgA1 from patients with IgAN was different to healthy people [6, 7].

Different to IgG, IgM, and IgA2, human serum IgA1 is one of the most exceptional of the human serum immunoglobulins because it has five O-linked oligosaccharides in its hinge portion, in addition to the two N-linked carbohydrate chains in its structure [8]. The abnormal O-glycosylation of IgA1 has been well studied in recent years, and there is increasing evidence for its involvement in the pathogenesis of IgAN [9–13].

In general, it was agreed that IgA1 molecules from patients with IgAN displayed altered glycans moieties, usually with a reduced content of galactose (Gal) and sialic acid [14–16]. Altered glycosylation of serum IgA1 could favor the self-aggregation and/or the increased binding to circulating glycoproteins, including fibronectin or environmental alimentary, bacterial, or viral lectins as a result of carbohydrate interactions [17, 18]. Macromolecules could also be formed by the reaction between degalactosylated (deGal) IgA1 and specific IgG antibodies directed against IgA1 hinge O-glycans [19]. These IgA immune or nonimmune aggregates could escape the clearance by hepatic receptors for asialoglycoproteins [20]. Moreover, the targeting to mesangial area might be favored by carbohydrate interactions between aberrantly glycosylated IgA1 and mesangial matrix, leading to accumulation and/or prolonged persistence of IgA deposits.
within the mesangium [11]. Therefore, it was speculated that altered hinge-region O-glycosylation of IgA1 might play a pivotal role in the pathogenesis of IgAN.

It is well known that the histologic phenotypes of IgAN are highly variable. Light microscopy findings range from mild mesangial proliferation to a more diffuse pattern with focal segmental scarring and even to crescentic glomerulonephritis. But the association of different pathologic phenotypes of IgAN and aberrantly glycosylated serum IgA1 was not reported. Therefore, in this study, we attempted to determine the association between the altered O-glycans of serum IgA1 molecules and the pathologic phenotypes of IgAN.

METHODS
Patients and sera
A total of 107 patients with IgAN were enrolled in the current study. 53 were male and 54 were female, with a mean age of 32.2 ± 11.7 years. Of the 107 patients with IgAN, 50 were with mild mesangial proliferative glomerulonephritis in renal pathology which means the glomeruli showed no more than a minimal increase in mesangial cellularity, without segmental sclerosis or crescents and were defined as Haas-I, a pathologic scheme of IgAN proposed by Haas [21]. The proteinuria of these 50 patients was 2.79 ± 5.18 g/day and the mean serum creatinine was 63.6 ± 32.5 μmol/L, the mean duration of known disease at the time of biopsy was 15.4 ± 30.5 months. There were 57 with focal proliferative sclerosing glomerulonephritis in renal pathology, 42 of them were defined as Haas-III, which means that 50% or fewer of the glomeruli were hypercellular, less than 40% of the glomeruli were globally sclerotic. The other 15 were defined as Haas-V, which indicated that 40% or more of the glomeruli were globally sclerotic. The proteinuria of these 57 patients was 3.61 ± 5.42 g/day and the mean serum creatinine was 147.5 ± 136.1 μmol/L, the mean duration of known disease at the time of biopsy was 19.7 ± 28.8 months. Sera from patients were obtained at the time of renal biopsy and sera from 22 normal blood donors with comparable age and gender distribution were collected as normal controls.

Detection of glycans of serum IgA1 by sandwich enzyme-linked immunosorbent assay (ELISA)

The O-glycans in the hinge region of IgA1 were detected by specific lectin binding ELISA as previously reported [22]. Rabbit antihuman IgA (Dako, Glostrup, Denmark) diluted to 5.5 μg/mL in 0.05 mol/L bicarbonate buffer, pH 9.6, and were coated to the wells of one half of a polystyrene microtiter plates (Costar, Cambridge, MA, USA). The wells in the other half were coated with bicarbonate buffer alone to act as antigen-free wells. The volumes of each well for this step and for subsequent steps were 100 μL, all incubations were carried out at 37°C for 1 hour and the plate were washed by 0.01 mol/L phosphate-buffered saline (PBS) containing 0.1% Tween-20 (PBST) three times. Then the plate was blocked with PBST containing 1% bovine serum albumin (BSA) (PBST/BSA). The test sera diluted 1:200 in PBST/BSA were added in duplicate to both antigen-coated and antigen-free wells. Every plate contained blank control (PBST/BSA) and a known normal serum was used as a positive control. After incubation and washing, the biotinylated secondary antibodies or lectins in PBST/BSA were added, including 1:20,000 diluted monoclonal anti-human IgA1 (clone A1-18) (Sigma Chemical Co., St. Louis, MO, USA) to detect the IgA1 concentrations; 1:500 diluted elderberry bark lectin (Sambucus Nigra Agglutinin; SNA) (Vector Laboratory, Burlingame, CA, USA) to detect serum α,2,6 sialic acid; 1:200 diluted arachis hypogaea [peanut agglutinin (PNA)] (Sigma Chemical Co.) or viisa villosa lectin (VVL) (Vector Laboratory) to detect Gal and N-acetylgalactosamine (GalNAc), respectively. The wells were then incubated with 1:20,000 diluted avidin-horseradish peroxidase (HRP) (Sigma Chemical Co.). The results were revealed with 0.1 mol/L citrate phosphate buffer, pH 5.0, containing 0.04% O-phenylenediamine (OPD) and 0.1% H2O2 (vol/vol), then the reaction was stopped with 1 mol/L H2SO4. The absorbance at 490 nm (A) was recorded in an ELISA reader Bio-Rad 550 (Bio-Rad, Tokyo, Japan).

The relative concentration of serum IgA1 and the level of glycans were calculated as following: the A value of the blank control was regarded as 0, the A value of the known control was regarded as 100%, and the A value of each sample was calculated by log-transformed data. The relative lectin binding per unit IgA1 was calculated as the A value of lectin over the A value of IgA1 concentration.

Statistics
For statistical analysis, statistical software SPSS version 11.0 (SPSS, Chicago, IL, USA) was employed. Quantitative data were expressed as mean ± SD and median with range (minimum and maximum). For comparison between patients and controls, the Student t test, two-way analysis of variance (ANOVA), and Mann-Whitney U test were performed. Statistical significance was considered as P < 0.05.

RESULTS
Comparison of serum IgA1 glycosylation between patients with IgAN and normal controls
The levels of α,2,6 sialic acid and Gal of serum IgA1 were lower in patients with IgAN compared with that of normal controls (0.98 ± 0.31 vs. 1.16 ± 0.21) (P = 0.008) (0.16 ± 0.19 vs. 0.30 ± 0.29) (P = 0.029), respectively, and the exposure of GalNAc of serum IgA1 was higher...
Table 1. Comparison of levels (arbitrary units) of α2,6 sialic acid, galactose (Gal), and N-acetylgalactosamine (GalNAc) between patients with IgA nephropathy (IgAN) and controls

<table>
<thead>
<tr>
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<th>Normal controls (N = 22)</th>
<th>Patients with IgAN (N = 107)</th>
<th>P value</th>
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<tbody>
<tr>
<td>Sialic acid</td>
<td>1.16 ± 0.21</td>
<td>0.98 ± 0.31</td>
<td>0.008</td>
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<tr>
<td>Gal</td>
<td>0.30 ± 0.29</td>
<td>0.16 ± 0.19</td>
<td>0.029</td>
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<tr>
<td>GalNAc</td>
<td>0.00 (0.33)</td>
<td>0.03 (0.29)</td>
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in patients with IgAN than that in controls (0.03 vs. 0.00) (P = 0.024) (Table 1).

Comparison of serum IgA1 glycosylation between patients with different pathologic phenotypes and normal controls

When patients were further stratified, the levels of α2,6 sialic acid and Gal of serum IgA1 were much lower in patients with focal proliferative sclerosing IgAN compared with that in patients with mild mesangial proliferative IgAN (0.91 ± 0.34 vs. 1.05 ± 0.25) (P = 0.014) (0.108 ± 0.137 vs. 0.221 ± 0.219) (P = 0.018), respectively, and that in normal controls (0.91 ± 0.34 vs. 1.16 ± 0.21) (P = 0.001) (0.108 ± 0.137 vs. 0.297 ± 0.292) (P = 0.001), respectively (Figs. 1 and 2). The exposure of GalNAc of serum IgA1 in patients with focal proliferative sclerosing IgAN was higher than that in normal controls (0.02 vs. 0.00) (P = 0.017) but was comparable to that in patients with mild mesangial proliferative IgAN (P = 0.692) (Fig. 3). When the levels of α2,6 sialic acid, Gal, and GalNAc were compared between patients with mild mesangial proliferative IgAN and normal controls, no significant difference could be found (1.16 ± 0.21 vs. 1.05 ± 0.25) (P = 0.134) (0.297 ± 0.292 vs. 0.221 ± 0.219) (P = 0.147) (0.03 vs. 0.00) (P = 0.071), respectively (Table 2).

**DISCUSSION**

The IgA1 hinge region is a very unique mucin-like O-linked glycopeptide. The core peptide has a proline-, serine-, and threonine-rich amino acid sequence in which the five serines are able to carry O-linked oligosaccharides consisting of sialic acid, Gal, and GalNAc with microheterogeneity [23, 24]. Altered O-glycosylation of IgA1 in IgAN is a physicochemical abnormality of the IgA1 molecule that may contribute to pathogenic mechanisms in a number of ways. But the precise structural

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**Fig. 1.** The exposure of α2,6 sialic acid of serum IgA1 molecules measured by SNA between different pathologic phenotypes and normal controls. The serum IgA1 in normal controls (NC) and patients with mild mesangial proliferative IgA nephropathy (IgAN) (MIgAN) exposed more α2,6 sialic acid than in patients with focal proliferative sclerosing IgAN (FIgAN). SNA, sambucus nigra agglutinin.

**Fig. 2.** The exposure of galactose (Gal) of serum IgA1 molecules measured by peanut agglutinin (PNA) between different pathologic phenotypes and normal controls. The serum IgA1 in normal controls (NC) and patients with mild mesangial proliferative IgA nephropathy (IgAN) (MIgAN) exposed more Gal than in patients with focal proliferative sclerosing IgAN (FIgAN).

**Fig. 3.** The exposure of N-acetylgalactosamine (GalNAc) of serum IgA1 molecules measured by vilsa villosa lectin (VVL) between different pathologic phenotypes and normal controls. The serum IgA1 in patients with focal proliferative sclerosing IgAN (FIgAN) exposed more GalNAc than in normal controls. MIgAN is mild IgAN.
nature of defect remains to be fully characterized. Although the lectin-binding assays only afforded the indirect evidence of the glycans and could not give the precise structure of the sugar residues, it was suggested that the lectin-binding assay was a simple and effective method to detect different glycans of IgA1 molecules and it had a good correlation with fluorophore-assisted carbohydrate electrophoresis (FACE) and mass spectrometry in the detection of Gal and GalNAc [3, 22].

In the present study, a modified ELISA using anti-human IgA1 as the coating reagent and biotinylated-conjugated lectins as probes were performed to analyze the interaction of lectins with serum IgA1 of normal subjects and patients with IgAN. The results showed that the serum IgA1 from patients with IgAN had decreased exposure of α2,6 sialic acid and Gal but increased exposure of GalNAc, especially the IgA1 from patients with focal proliferative sclerosing IgAN (Haas-III or V). However, for patients with mild mesangial proliferative IgAN (Haas-I), they were similar to that in healthy controls. To our knowledge, this is the first study demonstrating that α2,6 sialic acid deficiency and Gal deficiency of serum IgA1 have close association with renal pathologic phenotypes of IgAN. It was well known that the advanced renal pathology usually indicated a worse prognosis in patients with IgAN. We speculated that the deficiency of sialylation and galactosylation of serum IgA1 might be associated with the prognosis of patients with IgAN. This is a strong support to the speculation of Coppo and Amore [25] that defects in IgA1 glycosylation might influence the presentation and natural history of patients with IgAN. However, no correlation between clinical parameter such as the duration of known disease at the time of biopsy, the serum creatinine, albumin, or the amount of the proteinuria and the lectin binding had been found.

Lectins are proteins derived from various plants and animals which display binding affinity for certain specific carbohydrate ligands. However, correlation between lectin-binding assays designed to identify alteration in sialic acid and precise sialic acid contents still need to be confirmed by sophisticated measurements.

Increasing evidences suggested that the serum IgA1 from patient with IgAN had deficiency of α2,6 sialic acid and Gal residues and increased exposure of GalNAc by different methods, such as lectin-binding assay, gas-phase hydrazinolysis analysis, mass spectrometry, or FACE [3, 4, 11, 15]. Iwase et al [17] studied the heat-aggregated serum IgA1 and heat-stable serum IgA1 and indicated that the heat-stable IgA1 contained a much higher amount of the sialylated Galβ1, 3 GalNAc. They also detected abundant asialo-Galβ1, 3 GalNAc (TF antigen) in the IgA1 molecule from patients with IgAN and indicated that the incompleteness of the sugar chain of IgA1 corresponded to a decrease in the sialic acid content of hinge O-glycan chain [26]. Milan et al [12, 13] found that serum IgA1 from patients with IgAN was deficient in Gal and that the serum IgA1 molecules with Gal deficiency could be bound with IgG to form high molar mass complexes, and they also demonstrated that circulating immune complexes in IgAN consisted of IgA1 with Gal deficiency in hinge region and antiglycan antibodies. When enzymatically deglycosylated IgA1 were injected into the renal artery of rats, distinct amounts of desialylated IgA1 and degalactosylated IgA1 could be observed in rat glomeruli. However, untreated IgA1 molecules (native IgA1) could not be found with obvious accumulation. These results suggested that underglycosylation of IgA1 might play an important role in the glomerular accumulation of IgA1 [9]. More important, Hiki et al [4] and Allen et al [5] had demonstrated that the sialic acid and Gal in the IgA1 eluted from renal biopsy tissue of patients with IgAN were significantly decreased and the deposited IgA1 had the same abnormal physicochemical features as serum IgA in IgAN.

Aberrantly glycosylated IgA could circulate in a monomeric form, and could participate in the formation of autoaggregates or true immune complexes. The sialic acid at the hinge region are large and bulky compared with the protein backbone of IgA1, and they carry negatively charge, any change in the carbohydrate moieties affects the tertiary structure as well as the electrostatic charges [27]. It was thought that electrostatic repulsion and steric hindrance due to the sialic acid residues might play an important role on stability of IgA1 molecule as Kokubo et al [11] described. The IgA1 molecular could bind to hepatic asialoglycoprotein receptors (ASGPR) by Gal, the deficiency of Gal might escape clearance by liver and make the serum IgA1 increased; furthermore, the

| Table 2. Comparison of the levels (arbitrary units) of α2,6 sialic acid, galactose (Gal), and N-acetylgalactosamine (GalNAc) in patients with different pathologic phenotypes |
|-------------------------------------------------|-----------------|-----------------|-----------------|-----------------|
| Normal controls (N = 22)                        | Mild mesangial IgA nephropathy (N = 50) | Focal proliferative sclerosing IgA nephropathy (N = 57) | Normal control vs. mild mesangial IgA nephropathy | Normal control vs. focal proliferative sclerosing IgA nephropathy | Mild mesangial IgA nephropathy vs. focal proliferative sclerosing IgA nephropathy |
| Sialic acid                                     | 1.16 ± 0.21     | 1.05 ± 0.25     | 0.91 ± 0.34     | P = 0.134      | P = 0.017      | P = 0.014 |
| Gal                                             | 0.297 ± 0.292   | 0.221 ± 0.219   | 0.108 ± 0.137   | P = 0.147      | P = 0.017      | P = 0.018 |
| GalNAc                                          | 0.000 (0, 0.33) | 0.031 (0.22)    | 0.018 (0.29)    | P = 0.071      | P = 0.017      | P = 0.692 |

- **Sialic acid**
  - Normal controls: 1.16 ± 0.21
  - Mild mesangial IgA nephropathy: 1.05 ± 0.25
  - Focal proliferative sclerosing IgA nephropathy: 0.91 ± 0.34
  - Normal control vs. mild mesangial IgA nephropathy: P = 0.134
  - Normal control vs. focal proliferative sclerosing IgA nephropathy: P = 0.017
  - Mild mesangial IgA nephropathy vs. focal proliferative sclerosing IgA nephropathy: P = 0.014

- **Gal**
  - Normal controls: 0.297 ± 0.292
  - Mild mesangial IgA nephropathy: 0.221 ± 0.219
  - Focal proliferative sclerosing IgA nephropathy: 0.108 ± 0.137
  - Normal control vs. mild mesangial IgA nephropathy: P = 0.147
  - Normal control vs. focal proliferative sclerosing IgA nephropathy: P = 0.017
  - Mild mesangial IgA nephropathy vs. focal proliferative sclerosing IgA nephropathy: P = 0.018

- **GalNAc**
  - Normal controls: 0.000 (0, 0.33)
  - Mild mesangial IgA nephropathy: 0.031 (0.22)
  - Focal proliferative sclerosing IgA nephropathy: 0.018 (0.29)
  - Normal control vs. mild mesangial IgA nephropathy: P = 0.071
  - Normal control vs. focal proliferative sclerosing IgA nephropathy: P = 0.017
  - Mild mesangial IgA nephropathy vs. focal proliferative sclerosing IgA nephropathy: P = 0.692
exposure of GalNAc could act as a neoautoantigen that might be recognized by naturally occurring antibodies (IgG or IgA1 specific for GalNAc) or in IgA1-IgA1 interactions [13, 28]. Macromolecular IgA1 complexes formed by either of these mechanisms might be particularly prone to mesangial trapping [13]. It was reported by Kokubo et al. [29] that the IgA1-IgA1 interaction could be mediated by the core structure, including the peptide and the sugars, except for sialic acid, and the removal of sialic acid and Gal residues from IgA1 molecules could result in a significant increase in the adhesion activities to the mesangial matrix glycoproteins and a significant increase in complement-binding properties [30, 31].

Our previous work clearly demonstrated that aggregated IgA1 from patients with IgAN had a higher binding capacity to human mesangial cells and could induce mesangial cells proliferation and enhance the production of profibrosing cytokine transforming growth factor-β (TGF-β) and extracellular matrix fibrinogen [6, 7]. The activation of mesangial cells by Gal-deficient IgA1-containing circulating immune complexes was considered to be the initiating event in the pathogenesis of IgAN. In vitro, mesangial cell activation was observed in many instances upon the binding of IgA or IgA1-immune complexes (depending on the amount and form of IgA) [32, 33]. In vivo, it was found that urinary interleukin (IL)-6, the tubular and interstitial expression of intercellular adhesion molecular type 1, and the intrarenal expression of proinflammatory cytokines and chemokines could correlate with renal injury and might have a prognostic value [34, 35].

The renal pathologic phenotypes of IgAN were highly variable, and not always correlated with clinical parameters. Based on our finding, we speculated that the aberrant glycosylations of IgA1 might affect the glomerular response to IgA1 deposition as well as to the mechanism of deposition. However, this hypothesis needs further investigation.

CONCLUSION

Undersialylation and undergalactosylation of serum IgA1 from patients with IgAN might be associated with the pathologic phenotypes.

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