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Mesangial IgA1 in IgA nephropathy exhibits aberrant O-glycosylation: Observations in three patients

Alice C. Allen, Elaine M. Bailey, Paul E.C. Brenchley, Katharine S. Buck, Jonathan Barratt, and John Feehally

Department of Nephrology, Leicester General Hospital, Leicester, and Manchester Institute of Nephrology and Transplantation, Manchester Royal Infirmary, Manchester, England, United Kingdom

Mesangial IgA1 in IgA nephropathy exhibits aberrant O-glycosylation: Observations in three patients.

Background. In IgA nephropathy (IgAN), circulating IgA1 molecules display an abnormal pattern of O-glycosylation. This abnormality may potentially contribute to mesangial IgA1 deposition, but this is unproven because the O-glycosylation of mesangial IgA1 has not been analyzed.

Methods. IgA1 was eluted from glomeruli isolated from the kidneys of three IgAN patients obtained after nephrectomy or at postmortem. Serum from these patients, other patients with IgAN, and controls was subjected to the same treatment as the glomerular eluates. The O-glycosylation of eluted and serum IgA1 was measured by lectin binding using an enzyme-linked immunosorbent assay-based system.

Results. In all three cases, the lectin binding of IgA1 eluted from the glomeruli of IgAN patients was markedly higher than that of the serum IgA1 of the same individual, and also all but one of a series of serum IgA1 samples from other patients and controls.

Conclusions. The higher lectin binding of glomerular compared with serum IgA1 suggests that O-glycosylated IgA1 molecules abnormally and selectively deposit in the kidney. These results provide the first evidence that mesangial IgA1 is abnormally O-glycosylated, and support a direct role for abnormal IgA1 O-glycosylation in the mechanism of mesangial IgA deposition in IgAN.

The mechanism of mesangial deposition of IgA1 in IgA nephropathy (IgAN) is unknown, but a role has been suggested for altered O-glycosylation of IgA1 [1, 2].

Human IgA1 is unusual among serum proteins in its possession of a series of closely located O-glycans in the hinge region of the molecule [3.] These O-glycans are short chains, each based on a core N-acetyl galactosamine (GalNAc) unit linked to the amino acid backbone and usually extended with galactose to form the disaccharide Galβ1,3GalNAc, which may in turn be further extended with one or two sialic acid units [4]. Thus, each O-glycan may have one of four structures. Each IgA1 heavy chain has multiple potential O-glycosylation sites, and occupancy of these sites is variable. Therefore, an array of IgA1 "O-glycoforms" is possible. It is likely that serum contains of a mixture of IgA1 O-glycoforms, with varying degrees of galactosylation and sialylation.

We and others have shown that in IgAN, the O-glycosylation pattern of serum IgA1 is abnormal [1, 5–9]. This was initially demonstrated by the altered binding of lectins with specificity for oligosaccharides typically present within O-linked glycans to serum IgA1 [1, 5–7], the most consistent finding being the increased binding of lectins with an affinity for ungalactosylated terminal GalNAc [5, 6]. This abnormality of lectin binding is probably due to the reduced occurrence of galactosylated and sialylated chains, leading to increased frequency of O-glycans consisting of single GalNAc units alone. This abnormal O-glycosylation of serum IgA1 has now been confirmed directly by more precise analytical methods [7–9].

There has been considerable speculation about the possible pathogenic consequences of this serum IgA1 abnormality [1, 2], but the contribution of aberrant O-glycosylation to the mechanism of mesangial IgA1 deposition in human IgAN has yet to be fully elucidated. To date, no study has addressed the O-glycosylation pattern of mesangial IgA1. Elution from renal biopsy specimens can yield nanogram quantities of IgA1 [10], but available methods of carbohydrate analysis require more than this to obtain reliable results.

In this study, we had the unique opportunity to analyze the O-glycosylation of IgA1 eluted from nephrectomy or postmortem kidneys of three patients with IgAN. In all three cases, the O-glycosylation of the mesangial IgA1 was highly abnormal in comparison to the serum IgA1 of the same patient, other patients with IgAN, and controls.

Key words: glomerulonephritis, *Vicia villosa* lectin, *Helix aspersa* lectin, lectin binding, sialylation.

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METHODS

Subjects

Serum was obtained with informed consent from 20 patients with biopsy-proven IgAN (14 male, mean age 43.7, range 32 to 63 years) and 16 healthy control subjects (9 male, mean age 40.3, range 21 to 70 years) with no evidence of renal or systemic disease.

Renal cortex was obtained at nephrectomy or autopsy from three patients, A, B and C, all with biopsy-proven IgAN.

Patient A

A 64-year-old man presented in 1991 with proteinuria (1.5 g/24 h), microscopic hematuria, hypertension with preserved renal function, and serum creatinine 116 μmol/L (1.3 mg/dL). Renal biopsy showed characteristic features of IgAN: diffuse mesangial hypercellularity with mesangial IgA and C3 deposits. One year later, he had an episode of macroscopic hematuria, and a solid mass lesion was identified in the upper pole of the left kidney on computed tomography scanning. He underwent left nephrectomy: The mass proved to be a renal cell carcinoma, and the features of IgA in the remainder of the kidney were unchanged from the previous biopsy. The tumor-free lower pole of the kidney was obtained for this study.

Patient B

A 41-year-old man presented in 1978 with loin pain in association with hematuria and proteinuria (1.3 g/24 h). A renal biopsy was performed in 1982 and showed characteristic features of progressive IgAN: Glomeruli showed changes ranging from normal to diffuse globular or segmental mesangial sclerosis with diffuse granular deposits of mesangial IgA and C3. In 1989, the patient (aged 55) was admitted to Manchester Royal Infirmary and died of cardiorespiratory failure. At postmortem, kidney tissue was obtained for glomerular elution studies.

Patient C

A 48-year-old man developed end-stage renal disease caused by IgAN in 1991. He received a cadaveric renal transplant in 1992, which failed in 1996 because of chronic rejection, although there was immunohistological evidence of recurrent mesangial IgA deposition at the time of graft failure. He received a second cadaveric transplant in 1996. Function was satisfactory for 18 months, but then there was a relentless decline into renal failure with nephrotic range proteinuria and no evidence of rejection. Biopsy showed recurrent IgAN: mesangial IgA with diffuse glomerular hypercellularity and progressive glomerulosclerosis. The graft failed in 1999. He returned to maintenance dialysis, and immunosuppression was gradually withdrawn. One month later, the graft became increasingly swollen and tender with macroscopic hematuria and transplant nephrectomy was required. Histology showed extensive glomerulosclerosis with interstitial fibrosis and hemorrhage. Mesangial IgA deposits persisted. One half of the kidney was available for this study.

Preparation of glomeruli from nephrectomy specimens

In each case, the outer cortex was dissected from perfused kidney tissue, minced, and washed to remove all traces of blood, and glomeruli were isolated by conventional sieving techniques. Briefly, the tissue was forced through a series of sieves of decreasing mesh size (500, 250, and 75 μm), washing through with buffer. Glomeruli from the top of the 75 μm mesh were washed thoroughly and examined by microscopy to check for purity. The glomeruli were stored at −80°C in a buffer containing 0.5 UL^{-1} aprotinin as the protease inhibitor prior to the elution of IgA1.

Elution of IgA1 from glomeruli

Experiments A and C. The glomeruli were thawed and washed four times in phosphate-buffered saline (PBS), with repeated passes through a 21-gauge needle to disrupt the glomerular structure. The wash supernatants were pooled and dialyzed against water, and these eluates were concentrated by centrifugal evaporation at ambient temperature.

Experiment B. Kidney B was obtained during a study investigating the antigen specificity of mesangially deposited antibodies, and the elution protocol was designed to dissociate the eluted IgA from other possible complexed proteins. The glomeruli were eluted in 0.1 mol/L citrate buffer at pH 3.0 for 20 minutes at 4°C. The eluate was neutralized to pH 7.5 with Tris and concentrated by positive pressure ultracentrifugation.

To ensure that these protocols were effective and were not selective with respect to the IgA1 glycoforms eluted, glomerular tissue from kidney A was first subjected to PBS elution and then to a second citrate elution as described for kidney B. The concentration and O-glycosylation of the IgA1 contained in the two kidney-A eluates was compared by enzyme-linked immunosorbent assay (ELISA) and lectin binding assay as described later in this article. Finally, after further washing with PBS, the postelution glomerular tissue was stained with peroxidase-conjugated anti-IgA and examined microscopically to check for residual IgA.

Preparation of serum IgA

The lectin binding of eluted glomerular IgA1 from each experiment was compared with that of serum IgA1 from the nephrectomized subject and from other individuals. In each case, the serum samples were subjected to the same "elution" treatment and stored under the same conditions as the glomerular eluate.

Experiments A and C. Duplicate aliquots of serum from patients A and C, 25 other IgAN patients and 21 controls were diluted in an equal volume of PBS/aprotinin buffer and stored at −80°C.

Experiment B. Serum IgA from patient B, five other IgAN patients and six controls was exposed to 0.1 mol/L citrate elution buffer for 30 minutes at 4°C and then restored to PBS immediately prior to the lectin binding assays.

Dissociation of possible IgA1 complexes

The elution protocol used for kidney B (0.1 mol/L sodium citrate, pH 3) was designed to dissociate IgA1 from complexes with other proteins. The lectin binding of this eluate was compared with serum IgA1 treated in the same manner. We chose to elute kidneys A and C with PBS and mechanical disruption alone, since this proved sufficient to eluate the IgA from the glomeruli and was a gentler treatment less likely to affect the IgA1 O-glycans. However, to demonstrate that the lectin binding of the IgA1 eluted from these glomeruli was indeed to IgA1 and not to other complexed proteins, these eluates also were treated with sodium citrate and the lectin binding results compared with those of IgA1 eluted with PBS alone.

Kidney A. After PBS elution, glomeruli from kidney A were subjected to a second elution with sodium citrate, as described previously for kidney B. The lectin binding of the PBS and citrate eluates were then compared with each other.

Kidney C. The glomerular eluate obtained from kidney C was incubated with 0.1 mol/L sodium citrate, pH 3, for 30 minutes at 4°C and then restored to PBS immediately before measuring its lectin binding in parallel with the same eluate, which had been exposed to PBS only.

Lectin binding assays

The O-glycosylation profile of IgA1 samples was measured by the binding of lectins from *Vicia villosa* (VV) and *Helix aspersa* (HA), which are specific for terminal GalNAc and have previously been used to demonstrate abnormal IgA1 O-glycosylation in IgAN [5, 6, 11]. Briefly, 96-well immunoplates were coated with rabbit anti-human IgA antibody, washed, and blocked with 2% bovine serum albumin in PBS. Glomerular eluates and serum samples, which had been treated in the same manner, were diluted to 1 μg/mL IgA1 in PBS; 20 μL aliquots of each sample were applied to three sets of duplicate wells to capture IgA. Biotinylated VV and HA lectins and monoclonal anti-IgA1 were applied to the wells. Lectin binding was detected with horseradish peroxidase-conjugated avidin and anti-IgA1 binding with horseradish peroxidase-conjugated anti-mouse immunoglobulin antibody. The reaction was developed with OPD/H_2O_2 substrate and stopped with 1 mol/L H_2SO_4, and the results were read as absorbance at 492 nm. For each sample, the lectin binding results were expressed per unit IgA1 (A_{492} lectin/A_{492} IgA1). We have found that these lectin binding assays give constant values for lectin binding/IgA1 when samples are used at IgA1 concentrations above 0.1 μg/mL (data not shown). Dose response curves of anti-IgA1 and lectins binding to both serum and to the glomerular eluate in these assays were parallel and reached saturation and background binding at the same dilution points. This indicated that the lectin binding is to IgA1 rather than the unlikely possibility of any other proteins nonspecifically bound to the anti-IgA–coated wells.

Statistical analysis

The lectin binding of serum IgA1 from patient and control groups was compared by unpaired *t* tests.

RESULTS

Between 5 and 20 μg IgA1 was eluted from the glomeruli of each of the three kidney cortex specimens. The IgA concentration and lectin binding of the first (PBS) and the second (citrate) eluates from kidney A were compared. The PBS eluate contained 78% of the IgA obtained from this kidney, and the eluates obtained by the two methods did not differ in their lectin binding profiles. The postelution kidney tissue was negative for IgA by immunoperoxidase staining.

The lectin binding of the glomerular IgA1 samples was compared with that of serum samples treated in the same manner (Fig. 1). In all the experiments, the VV and HA lectin binding of serum IgA1 from IgAN was significantly higher than controls. All the glomerular IgA1 samples had higher VV and HA lectin binding than the serum IgA1 of the same subject treated in the same fashion. Indeed, the glomerular eluates showed the highest VV lectin binding of all the samples, and the HA lectin binding of the glomerular eluates was higher than all but one serum IgA1.

For kidneys A and C, which were initially eluted with PBS alone, the lectin binding of eluates after complex dissociation with 0.1 mol/L sodium citrate, pH 3, was also measured. No change in the lectin binding of these samples was observed after citrate complex dissociation, indicating that lectin binding was indeed to IgA1 and not to other proteins that may be complexed to it (Table 1).

DISCUSSION

Abnormal O-glycosylation of serum IgA1 in IgAN is now well established. The available evidence points to a lack of terminal galactosylation and sialylation leading to increased frequency of truncated O-glycan chains consisting of GalNAc alone [5–9].

There are plausible reasons why this O-glycosylation

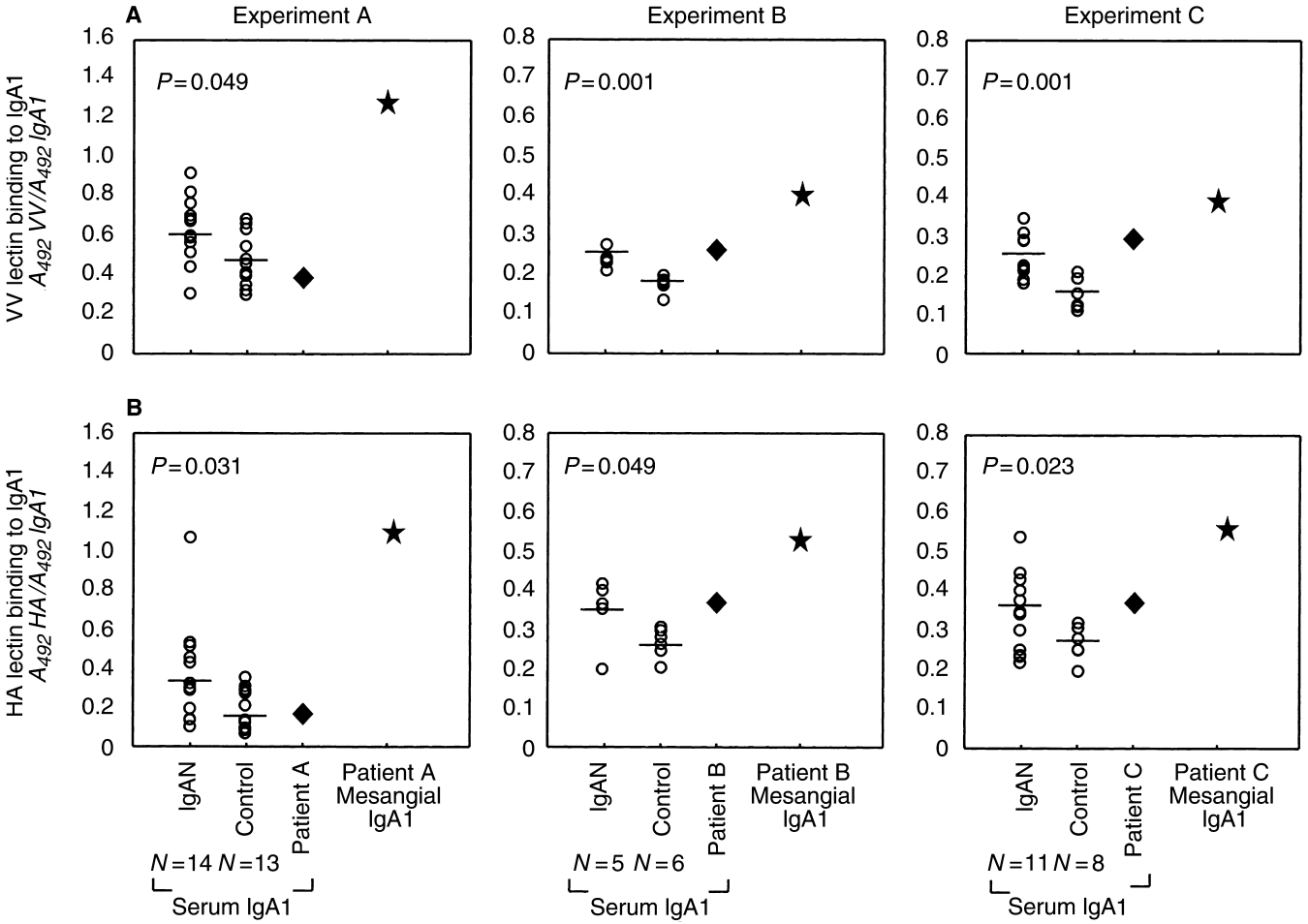

Fig. 1. Binding of *Vicia villosa* (VV) and *Helix aspersa* (HA) lectins to serum and mesangial IgA1. Binding of VV (*A*) and HA (*B*) lectins to serum IgA1 from IgAN and controls (○); serum IgA1 from patients A, B, and C (◆); and IgA1 eluted from the glomeruli of patients A, B, and C (★). In all of the experiments, both the VV and HA lectin binding of serum IgA1 from patients with IgAN was significantly higher than that of controls (as indicated by the *P* values on each graph), and the lectin binding to mesangial IgA1 was higher than to serum IgA1 of the same subject. Note the ratios of A_{492}lectin:A_{492}IgA1 were derived from raw data and are arbitrary values that vary between assay runs. Therefore, the ordinate scales differ between the experiments and direct comparisons between the different experiments should not be made.

Table 1. Effect of complex dissociation on lectin binding of IgA1 eluted from glomeruli

	VV binding (A_{492}VV/A_{492}IgA1)		HA binding (A_{492}HA/A_{492}IgA1)	
	PBS	Citrate	PBS	Citrate
Eluate A	1.23	1.19	0.89	0.74
Eluate B	—	0.41	—	0.53
Eluate C	0.35	0.34	0.54	0.55

Eluates from kidneys A and C were eluted by washing glomeruli in PBS with mechanical disruption. To ensure that lectins were binding to IgA1 rather than other proteins complexed to it in the subsequent lectin binding assays, any complexes were dissociated by incubation in 0.1 mol/L sodium citrate, pH 3 for 30 minutes at 4°C and the lectin binding of this dissociated IgA1 compared to that exposed to PBS only. Lectin binding of eluted IgA1 was not altered by citrate treatment, and remained higher than that of serum IgA1. Kidney B was eluted directly with sodium citrate, and no values are available for PBS-eluted IgA1.

abnormality may be directly pathogenic in IgAN [1, 2]. Altered O-glycosylation might favor self-aggregation of IgA1 [12] or act as an autoantigen in immune complexes with IgG [13]. Macromolecular IgA1 complexes formed by either of these mechanisms might be particularly prone to mesangial trapping. There is also evidence for increased binding of abnormally O-glycosylated IgA1 to extracellular matrix components [14, 15], again promoting mesangial deposition. Furthermore, abnormally O-glycosylated IgA1 from human IgAN patients has been shown to accumulate preferentially in the glomeruli of perfused rat kidneys [16], but the pattern of deposition in this model is somewhat different to that seen in human IgAN, and the cross-species nature of the experiment makes it difficult to interpret its true significance.

Currently, there is no direct evidence that aberrant

O-glycosylation really is involved in the mechanism of mesangial IgA1 deposition in human IgAN. Hitherto, it has not been known whether the IgA1 molecules deposited in the mesangium are abnormally O-glycosylated, because obtaining such evidence is extremely difficult. Lectin staining of renal biopsy sections is uninformative, as many cell surface proteins are O-glycosylated, and extensive background staining masks the signal from the deposited IgA1. Elution from renal biopsy sections yields insufficient IgA1 for reliable O-glycosylation analysis by currently available methods. More substantial amounts of renal tissue are needed, and the study reported here required the collection of three nephrectomy specimens from two UK centers over a period of 10 years.

Despite the different circumstances that led to kidney tissue becoming available, the findings were remarkably consistent. In each case, the O-glycosylation pattern of mesangial IgA1 appeared to be more abnormal than that of IgA1 in synchronous serum samples. The lectin binding patterns indicated that O-glycoforms of IgA1 deficient in galactose and/or sialic acid were preferentially deposited in the mesangium. Lectin binding studies require relatively small amounts of eluted IgA1, but even from the substantial amounts of kidney tissue available from the nephrectomy specimens, it was not possible to obtain amounts of IgA1 sufficient for the current requirements of analytical techniques such as mass spectroscopy, carbohydrate electrophoresis, or chromatography. The nephrectomy specimens were kept cold and processed as quickly as possible to avoid O-glycan degradation, although some degree of loss, particularly of sialic acid, cannot be excluded. In addition, serum samples for comparison were subjected to as similar treatment to the glomerular eluates as possible.

The conclusions that can be drawn from this study of three kidneys inevitably must be cautious. Nevertheless, the evidence is consistent that abnormally O-glycosylated IgA1 appears to selectively deposit in the mesangium, and strongly suggests that aberrant IgA1 O-glycosylation is not an epiphenomenon but is pathogenic in IgAN. As such, it warrants further study.

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Reprint requests to Alice C. Allen, M.D., Department of Nephrology, Leicester General Hospital, Leicester LE5 4PW, England, United Kingdom.
E-mail: aa50@le.ac.uk

REFERENCES

1. Mestecky J, Tomana M, Crowley-Nowick PA, *et al*: Defective galactosylation and clearance of IgA1 molecules as a possible etiopathogenic factor in IgA nephropathy. *Contrib Nephrol* 104:172–182, 1993
2. Feehally J, Allen AC: Structural features of IgA molecules which contribute to IgA nephropathy. *J Nephrol* 12:59–65, 1999
3. Kerr MA: The structure and function of human IgA. *Biochem J* 271:285–296, 1990
4. Field MC, Dwek RA, Edge CJ, Rademacher TW: O-linked oligosaccharide from human serum immunoglobulin A1. *Biochem Soc Trans* 17:1034–1035, 1989
5. 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
6. 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
7. Hiki Y, Iwase H, Kokubo T, *et al*: Association of asialo-galactosylβ1-3 N-acetyl galactosamine on the hinge with a conformational instability of jacalin reactive immunoglobulin A1 in immunoglobulin A nephropathy. *J Am Soc Nephrol* 7:955–960, 1996
8. 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
9. 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
10. Monteiro RC, Halbwachs-Mecarelli L, Roque-Barreira MC, *et al*: Charge and size of mesangial IgA in IgA nephropathy. *Kidney Int* 28:666–671, 1985
11. Allen AC, Willis FR, Beattie TJ, Feehally J: Abnormal IgA glycosylation in Henoch-Schönlein purpura restricted to patients with clinical nephritis. *Nephrol Dial Transplant* 13:930–934, 1998
12. Kokubo T, Hiki Y, Iwase H, *et al*: Evidence for involvement of IgA1 hinge glycopeptide in the IgA1-IgA1 interaction in IgA nephropathy. *J Am Soc Nephrol* 8:915–919, 1997
13. 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
14. 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
15. Coppo R, Amore A, Gianoglio B, *et al*: Serum IgA and macromolecular IgA reacting with mesangial matrix components. *Contrib Nephrol* 104:162–171, 1993
16. 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