The nucleotide sequence of the IgA1 hinge region in IgA nephropathy

Morag R. Greer1, Jonathan Barratt1, Steven J. Harper2, Alice C. Allen1 and John Feehally1

1Department of Nephrology, Leicester General Hospital, Leicester, 2Richard Bright Renal Unit, Southmead Hospital, Bristol, UK

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Abstract

Background. Mesangial IgA1 deposition is characteristic of IgA nephropathy (IgAN). Structural abnormalities of the IgA1 glycoprotein may play a key role in its mesangial deposition, particularly the recently described abnormalities of O-glycosylation of the IgA1 hinge region. The mechanism of abnormal O-glycosylation has not yet been elucidated; it is not clear whether there is an alteration in the amino acid sequence of the hinge region, modifying the number of O-glycosylation sites available, or whether there is a post-translational defect in the glycosylation process.

Methods. The O-glycosylation of serum IgA1 from a series of patients with IgAN and matched controls was assessed by lectin binding assay. We then used dideoxysequencing of the PCR-amplified hinge region of the α1 heavy chain gene to compare the hinge region nucleotide sequence in IgAN and controls. We also compared cDNA transcripts of α1 hinge region mRNA to look for evidence for a transcriptional abnormality in IgAN.

Results. Lectin binding assays confirmed that the IgAN subjects used in this study did indeed display the previously reported abnormality of IgA1 O-glycosylation. However, the hinge region nucleotide sequence of the α1 gene was identical in IgAN and controls. There was also no difference in the sizes of cDNA transcripts of hinge region mRNA from patients with IgAN and controls.

Conclusions. We found no evidence for any nucleotide sequence alteration or transcriptional abnormality of the α1 hinge region in IgAN, and we conclude that the O-glycosylation defect is post-translational.

Introduction

In IgA nephropathy (IgAN) IgA molecules of the IgA1 subclass are deposited in the renal mesangium.

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In IgA nephropathy, lectin binding assays [2–4] and matrix-assisted laser desorption mass spectroscopy [11] have indicated significant differences in the hinge region O-glycans which may have structural and functional implications relevant to the pathogenesis of the disease [6].

This closely located cluster of O-glycans is large in comparison to the protein backbone, and carries a high negative charge, due to sialylation of the sugars. Series of O-glycans tend to confer an extended structure upon the protein domain in which they occur [12]. Alterations in IgA1 O-glycosylation may affect interactions with other proteins, such as other immunoglobulin molecules, antigen, complement and extracellular matrix components. Furthermore, IgA1 binding to key cell surface receptors may be affected. The major route of IgA1 catabolism is via the hepatic asialoglycoprotein receptor, which has high affinity for the O-glycans [13]; impairment of this process may lead to failure of IgA1 clearance from the circulation. Lastly, enhanced or compromised ligation of leucocyte and mesangial cell IgA receptors could be involved in the development of glomerular inflammation.

Defective O-glycosylation may be post-translational, but it is important to exclude an altered amino acid structure of the hinge region changing the number of serine and threonine residues available for O-glycosylation. To investigate the possibility of point mutations or deletions in the nucleotide sequence which could modify the amino acid sequence, we compared the α1 hinge region DNA nucleotide sequence in IgAN and controls. We also synthesised hinge region cDNA by reverse transcription from mRNA and compared the sizes of these transcripts in IgAN and controls, to look for evidence for abnormally transcribed mRNA species. The O-glycosylation of serum IgA1 from the same subjects was assessed by lectin binding.

**Subjects and methods**

**Materials**

All chemicals were purchased from Sigma Chemical Co, Poole, UK, except where otherwise specified.

**Subjects**

Venous blood was obtained with informed consent from 10 patients (9 male) with renal-biopsy-proven IgAN and 10 healthy age, gender- and race-matched controls recruited from hospital staff. The median age of the patients was 47.5 years (range 27–62 years) and of the controls was 41.5 years (range 17–62 years). This study was approved by the Ethical Committee of Leicestershire Health Authority.

**Preparation of peripheral blood mononuclear cells (PBMCs) and serum IgA1**

PBMCs were isolated from the blood by density gradient centrifugation. IgA1 was purified from the serum of the same subjects by affinity chromatography on jacalin-agarose as previously described [2].

**Lectin binding assay**

To confirm that the IgAN subjects used in this study did indeed display abnormal IgA1 O-glycosylation, we used *Vicia villosa* lectin (VV), which recognizes O-linked N-acetyl galactosamine, and which shows increased affinity for IgA1 in IgAN [2]. The binding of biotinylated VV lectin to serum IgA1 was measured in our published ELISA-type assay [2]. Briefly, duplicate wells of immunoplates were saturated with IgA1 samples, and after washing with PBS containing 0.3 M NaCl and 0.1% Tween 20, excess binding sites were blocked with 5% (w/v) oxised glutathione in PBS. The plates were washed again and 1 μg/ml biotinylated VV lectin applied. After further washing, bound VV lectin was detected with 2.5 μg/ml peroxidase-conjugated avidin, finally developing the colour with OPD substrate solution and stopping the reaction with 1M H₂SO₄. The absorbances of the wells were read at 492 nm and the VV binding of each IgA1 sample expressed in arbitrary units (AU).

**DNA extraction, PCR, and sequencing**

Total cellular DNA was extracted from the PBMCs of six patients and seven controls using TRIzol-LS reagent (Life Technologies, Paisley, UK). A portion of the α1 gene containing the hinge region (illustrated in Figure 2) was amplified by PCR using the primers 5'- AAT CCC AGC CAG GAT GTG AC - 3' (sense) and 5'-AGG CAG CAG TGC TGA AG - 3' (anti-sense). After a hot start at 98°C for 10 min and cooling to 72°C, 1U *Thermus aquaticus* polymerase was added and the PCR carried out with four cycles of denaturation at 95°C for 1 min 30 s, annealing at 63.5°C for 1 min, and extension at 72°C for 1 min, followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 63.5°C for 1 min and extension at 72°C for 1 min.

The PCR-amplified products were separated by electrophoresis through 1.5% agarose, and each band excised and purified using the silica matrix-based Sephaglas™ Bandprep kit (Pharmacia, St Albans, UK). For sequencing, DNA was synthesized using the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer, Beaconsfield, UK). The extension products were precipitated with ethanol, separated by 6% denaturing polyacrylamide gel electrophoresis through 1.5% agarose, and each band excised and purified using the silica matrix-based Sephaglas™ Bandprep kit (Pharmacia, St Albans, UK). For sequencing, DNA was synthesized using the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer, Beaconsfield, UK). The extension products were precipitated with ethanol, separated by 6% denaturing polyacrylamide gel
electrophoresis, and analysed using an automated sequencer (Perkin Elmer, Beaconsfield, UK).

**mRNA extraction, cDNA synthesis, and size analysis**

For 10 subjects (5 IgAN and 5 control), mRNA was extracted from PBMCs using TRizol-LS reagent as above, and cDNA synthesized with oligo (dT) primer using Avian Myeloblastosis Virus reverse transcriptase, dNTPs, and RNase inhibitor (all from Promega, Southampton, UK), following the manufacturer’s instructions. The PCR products were separated by electrophoresis through 1.5% agarose gels, and the bands examined under UV light to compare the size of the transcripts from the 10 individuals investigated.

**Statistics**

The binding of VV lectin to IgA1 from IgAN and control groups was compared by Student’s *t* test.

**Results**

**O-glycosylation of serum IgA1**

Serum IgA1 from patients with IgAN showed significantly higher binding to VV lectin than control IgA1 (*P* = 0.04) (Figure 3), confirming that these patients had abnormal IgA1 O-glycosylation.

**ζ1 hinge region nucleotide sequence in IgAN**

PCR amplification of the IgA1 hinge region from total cellular DNA yielded a single band consistent with the expected 563-bp fragment in all six patient and seven control samples. The nucleotides from positions 693 to 774 of the published sequence [8] were sequenced; this region contains the hinge and is illustrated in Figure 2. The genomic DNA for this region, spanning 81 nucleotides, was identical in all 13 subjects and was 100% homologous to the published sequence:

CCC TCA ACT CCA CCT ACC CCA TCT CCC TCA ACT CCA CCT ACC CCA TCT CCC TCA TGC TGC CAC CCC CGA TCA CTG CTC CAC.

**Size separation of cDNA transcripts from mRNA**

RT-PCR amplification of IgA1 hinge region mRNA yielded a band of the expected 349-bp size in all 10 subjects (5 IgAN and 5 controls). The bands obtained from all individuals were of identical size, as shown in Figure 4; with no differences between IgAN and controls.

**Discussion**

In this study we sought an abnormality of the nucleotide sequence or mRNA transcripts of the ζ1 hinge region. It is important to establish whether such an abnormality exists, since an altered IgA1 amino acid sequence, presenting an altered template for post-translational O-glycosylation, could account for the potentially pathogenic abnormality of IgA1 O-glycosylation described in IgAN.

The group of patients we studied displayed characteristic abnormal IgA1 O-glycosylation, as demonstrated by the increased binding of VV lectin to their serum IgA1 in comparison to matched controls. However, there was no difference in the nucleotide sequence of the ζ1 hinge region from the subjects with IgAN and race and sex-matched controls, and nor was there evidence for any difference in the mRNA.

![Fig. 3. Binding of VV lectin to IgA1 in IgAN (■) and controls (□); mean ± SEM. VV shows significantly raised binding to serum IgA1 from patients with IgAN (*P* = 0.04), confirming that these patients display abnormal IgA1 O-glycosylation.](image-url)

![Fig. 4. Agarose gel electrophoresis of RT-PCR products derived from ζ1 hinge region mRNA. Lanes 1 and 10: 4X174 Hae III DNA size markers. Lanes 2, 7, 8, and 9: cDNA from control subjects. Lanes 3–6: cDNA from IgAN subjects. Transcripts from all subjects are of identical size (349 bp, arrowed) and there is no difference between IgAN and controls.](image-url)
transcripts derived from the genomic DNA sequence in IgAN.

It is likely that the abnormal IgA1 O-glycosylation in IgAN is due to a post-translational defect, and this is supported by our recent report of reduced activity of the O-galactosylating enzyme β1,3 galactosyltransferase in peripheral blood B cells in IgAN [14]. Little else is known about the nature of this enzyme defect, but the observation reported here indicates that future studies should be directed towards the further elucidation of IgA1 O-glycosylation mechanisms in IgAN, rather than a defect of the amino acid content of the IgA1 protein per se.

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References


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