Leucocyte β1,3 galactosyltransferase activity in IgA nephropathy

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Abstract

Background. Reduced galactosylation of the O-linked glycans of the IgA1 hinge region in IgAN has recently been described. To investigate the underlying defect resulting in this abnormality, we have measured the activity of β1,3 galactosyltransferase, the enzyme responsible for galactosylation of O-linked sugars.

Methods. A galactose-acceptor substrate was prepared from degalactosylated hinge region fragments of normal IgA1, and incubated with the T cell, B cell, and monocyte lysates from patients with IgAN and controls for acceptor regalactosylation. The extent of acceptor galactosylation was then measured with biotinylated Vicia villosa lectin (VV), which is specific for ungalactosylated moieties. Lectin binding of serum IgA from the same subjects was also measured.

Results. T cell and monocyte β1,3 galactosyltransferase activities did not differ between IgAN and control, but B cell lysates in IgAN showed significantly lower β1,3 galactosyltransferase activity than control (6.2 ± 0.71 vs. 9.5 ± 1.03 AU/mg, P = 0.018). Furthermore, B cell β1,3 galactosyltransferase activity showed a negative correlation (r = −0.87, P = 0.002) with VV lectin binding of serum IgA in IgAN, but not controls.

Conclusions. These data indicate that altered IgA1 O-galactosylation in IgAN results from a B cell-restricted reduction of β1,3 galactosyltransferase activity. This enzyme defect may be a fundamental pathogenic abnormality in IgAN.

Key words: β1,3 galactosyltransferase; IgA glycosylation; IgA nephropathy; Vicia villosa lectin

Introduction

IgA nephropathy (IgAN) is the commonest form of glomerulonephritis in developed countries, and a significant proportion of patients will progress to end stage renal failure [1]. The diagnostic hallmark of the condition is deposition of IgA in the glomerular mesangium; this is known to be of the IgA1 subclass and at least partly polymeric [2,3]. Immunoglobulins are glycoproteins: recently we, and others, have described abnormal carbohydrate composition within the IgA1 molecule in IgAN, which may be of pathogenic significance [4–7].

Protein glycosylation occurs in two major forms, N- and O-linked; these glycan types and biochemical pathways leading to their synthesis are distinct from one another. N-linked sugars are a common feature of serum proteins, but O-glycosylation is mainly restricted to membrane proteins, and is displayed by only a handful of serum proteins. IgA1 has a number of N-linked glycans, and is also one of the very few serum controls for acceptor regalactosylation. The extent of acceptor galactosylation was then measured with biotinylated Vicia villosa lectin (VV), which is specific for ungalactosylated moieties. Lectin binding of serum IgA from the same subjects was also measured.

This may be asialyl, or may be mono- or di-sialylated [12,13]. The O-linked sugars of IgA1 are apparently abnormal in IgAN. We, and others, have reported reduced binding of IgA1 to jacalin, a lectin which recognizes Gal β1,3GalNAc [4,5], increased binding to lectins which recognize terminal GalNAc [7], and reduced total Gal content of IgA1 [5]. The most consistent explanation of these findings is reduced galactosylation of the O-linked sugars of the IgA1 hinge region, leading to expression of truncated moieties consisting of terminal GalNAc only, though this has yet to be proven. This defect is probably synthetic...
rather than degradative, since Cl inhibitor, another of the few serum proteins with O-glycosylation, does not display the same pattern of lectin binding as IgA1 in IgAN [7].

Galactosylation of O-linked sugars is effected by the intracellular enzyme β1,3 galactosyltransferase (β1,3GT). This enzyme catalyses the transfer of galactose from the nucleotide donor, uridine 5’-diphospho-galactose (UDP-Gal), to the acceptor, GalNAc O-linked to serine residues in the IgA1 hinge region:

\[
\text{Acceptor} \quad \text{Donor} \quad \text{Product} \\
\text{GalNAc}-\beta1,3-Ser + \text{UDP-Gal} \rightarrow \text{Gal}-\beta1,3-\text{GalNAc}-\beta1,3-Ser
\]

β1,3GT has yet to be isolated or cloned, but its functional activity may be measured by the detection of increased galactosylation of a suitable acceptor substrate. Glycosyltransferases show exquisite specificity with respect to the monosaccharide donor, and can also be highly selective as to the acceptor; this latter selectivity may be influenced by the oligosaccharide chain and by the peptide backbone [14]. For this reason, the β1,3GT assay system developed for the experiments described in this study employed an acceptor derived from IgA1 hinge regions. Altered IgA1 hinge region O-glycosylation in IgAN can be detected by the differential binding of lectins with specificity for the various glycans. In previous studies [7] we have demonstrated increased terminal GalNAc in IgA1 in IgAN, using the lectin from Vicia villosa (VV), which displays a high degree of binding specificity for unsubstituted O-linked GalNAc moieties [15,16]. In the study now reported, increases in galactosylation of the acceptor after incubation with cell-lysate associated β1,3GT were detected by reduced binding of this lectin. The assay was carried out using an ELISA-type system, coating immunoplates with the IgA1-derived acceptor to tailor the assay to IgA1-galactosylating activity as much as possible. This is a novel approach, as is the use of a lectin to detect and quantify the glycosylation changes in the acceptor brought about by the action of the enzyme. This study demonstrates reduced activity of this enzyme in peripheral blood B cells from patients with IgAN.

Subjects and methods

Subjects

120 ml venous blood was obtained from nine patients with biopsy-proven IgAN (six male, mean age 33 years, range 23–51 years), and 12 normal individuals with no known renal or systemic disease (six male, mean age 38 years, range 20–64 years). These studies were approved by the Ethical Committee of Leicestershire Health Authority.

Serum samples and cell lysates

The blood samples were taken into preservative-free heparin (10 U/ml blood) and the plasma separated by centrifugation at 500 g. The plasma samples were clotted by the addition of 0.5 g/ml protamine sulphate and 0.5 U/ml bovine thrombin, serum separated after centrifugation at 10 000 g, and frozen in aliquots at −20 °C. The plasma volume removed from the blood cells was replaced with buffered Hank’s balanced salt solution (HBSS) (Life Technologies, Paisley, UK), and peripheral blood mononuclear cells (PBMCs) separated by density gradient centrifugation on Ficoll 400, density 1.077 (Histopaque; Sigma Chemical Co, Poole, UK ). The PBMCs were washed three times in HBSS and resuspended in 10 ml HBSS containing 1% foetal calf serum (FCS) (HBSS/FCS). The cells were fractionated into monoocyte, T, and B cell populations using anti-CD14, CD2, and CD19-conjugated magnetic beads (Dynabeads; Dynal Ltd, Wirral, UK) in a sequential fashion. The cell populations were washed in serum-free HBSS, pelleted by centrifugation and lysed by detergent treatment with 1 ml 0.5% Nonidet P40 in phosphate buffered saline (PBS), sonicated three times, and frozen in aliquots at −70 °C. The purity of the harvested cell populations was >90% by immunocytochemical staining of cytocentrifuge preparations.

A standard cell lysate was also prepared in the same way from unfractionated peripheral blood mononuclear cells pooled from four normal individuals. Protein content of the lysate samples was measured (Bio-Rad DC Protein Assay; Bio-Rad Ltd, Hemel Hempstead, UK).

Preparation of acceptor substrate for measurement of β1,3GT activity

Serum from six normal individuals was pooled and a crude immunoglobulin fraction prepared by precipitation with 20% polyethylene glycol 6000. The precipitate was redissolved in 0.2 M NH₄HCO₃ at a total protein concentration of ~50 mg/ml. This was digested overnight at 37 °C with TPCK treated trypsin (Sigma Chemical Co) at 1:50 w/w, freeze dried, redissolved in 5% formic acid and further digested overnight at 37 °C with pepsin at 1:50 w/w. The protein digest was passed through a Sephadex G10 column to exchange the buffer to 0.175M Tris–HCl buffer, pH 7.5, and the O-glycosylated peptide fragments isolated on jacalin-conjugated agarose (Vector Ltd, Peterborough, UK), removing unbound proteins with three washes in excess Tris–HCl. Bound fragments were eluted from the jacalin-agarose with 1 M galactose and passed through a Sephadex G10 column to remove free galactose and exchange the buffer to PBS. The preparation was then enzymatically degalactosylated by overnight incubation at 37 °C with acryl-bead conjugated β-galactosidase from E.coli (Sigma Chemical Co). The beads were removed by centrifugation and the acceptor preparation again passed through a Sephadex G10 column in PBS, adjusted to a total protein concentration of 100 μg/ml, and frozen in aliquots at −70 °C.

β1,3GT assay

96-well immunoplates (ICN Flow Ltd, Rickmansworth, UK) were coated with 50 μl/well of the acceptor preparation overnight at 4 °C; and washed four times in PBS. This washing step was repeated between each stage of the assay. The cell lysate samples were thawed rapidly, diluted in PBS to approximately 250 μg/ml, and duplicate 30 μl aliquots applied to the wells. 10 μl/well 4 × concentration reaction buffer (1 mM UDP-Gal/80mM CaCl₂ in PBS) was added. Blank (no lysate) wells containing 30 μl PBS and 10 μl 4 × reaction buffer alone, and a series of 11 doubling dilutions
of the standard cell lysate forming a standard curve (protein range 20 mg/ml to 5 μg/ml) were set up on each plate. The plates were sealed and incubated overnight (18 h) at 37°C. After further washing, the binding of the GalNAc-specific lectin Vicia villosa (VV) to the acceptor was measured. 40 μl/well biotinylated VV lectin (Vector Ltd) at 1 μg/ml in PBS was applied to the plates for 90 min at room temperature, followed by 40 μl/well horseradish peroxidase-conjugated avidin D (Vector Ltd) at 2.5 μg/ml for 90 min at room temperature. The colour was developed with 40 μl/well OPD/H₂O₂ substrate in 0.1 M citric acid/phosphate buffer, pH 5, stopping the reaction with 60 μl/well 1 M H₂SO₄ after 10 min at room temperature. The optical density (OD) was read at 492 nm, using an automated plate scanner (ICN Flow Ltd), and the means of duplicate wells calculated.

Calculation of β1,3GT activity in cell lysates

The degalactosylated acceptor coated on to the immunoplates expressed a high level of terminal GalNAc moieties, and consequently showed high binding to the GalNAc-specific VV lectin. Incubation with cell lysates resulted in re-galactosylation of some of the moieties by the action of β1,3GT, with a corresponding reduction in VV-binding proportional to the enzyme activity. Therefore, the OD value of the wells were subtracted from that of the blank (no lysate) for each plate. Standard curves were constructed from the subtracted ODs of a series of doubling dilutions of the standard lysate, which was assigned a β1,3GT activity of 10⁻⁵ AU at its neat concentration. β1,3GT activity values for the test lysates were then read from the standard curve. The actual measured protein values of the lysate dilutions were used to express the results as AU/μg total lysate protein.

Vicia villosa lectin binding of serum IgA

Galactosylation of the O-linked glycans of serum IgA1 from the same blood samples as the cells used in the above experiments was assessed by the binding of biotinylated VV lectin. 5 ml serum samples were diluted with an equal volume of PBS and a crude high molecular weight protein fraction prepared by precipitation with 2.8 g ammonium sulphate. The precipitates were redissolved in PBS and twice passed through Sephadex G25 columns. 96-well immunoplates were coated with 100 μl/well rabbit anti-human IgA (Dako Ltd, High Wycombe, UK) at 10 μg/ml in 0.05 M carbonate/bicarbonate buffer, pH 9.6 overnight at 4°C. washed four times in PBS and the excess protein binding sites blocked with 100 μl/well oxidized glutathione at 2 mg/ml in PBS. Ammonium sulphate precipitate samples adjusted to 3 μg/ml were applied at 50 μl/well in duplicate and incubated overnight at 4°C; this concentration had previously been shown to saturate the IgA binding capacity of the wells without appreciable non-specific binding of other proteins. VV lectin binding of the immobilized IgA was then measured as described for the β1,3GT assay above, expressing the results as OD at 492 nm.

Since the results of this assay system are expressed as raw OD values, results from assays run at different times cannot be compared directly to one another. For this reason, all the samples were run together in a single assay on one immunoplate. The intra-assay coefficient of variation was 3.7%.

Statistical analysis

β1,3GT activities of T cell, B cell and monocyte lysates in IgAN and controls were compared by unpaired Student t-tests, as were the lectin binding results of serum IgA. Linear regression analysis was carried out to investigate the relationships between β1,3GT activity of each cell type and lectin binding of serum IgA from the same subject in IgAN and controls. Results quoted in the text are mean values of IgAN or control groups, ± SEM.

Results

β1,3GT assay

The degalactosylated acceptor preparation displayed a high binding affinity for the GalNAc-specific lectin Vicia villosa (VV). Incubation with the standard cell lysate reduced the VV binding of the acceptor; the degree of this reduction was proportional to the protein concentration of this lysate over a range of 80–1200 μg/ml. The standard was assigned a β1,3GT activity of 10⁻⁵ AU and a series of doubling dilutions set up on each assay plate to allow the conversion of subtracted OD values of test lysate samples to AU/μg protein. The enzyme activity was shown to be time and temperature dependent, and the lectin binding of the acceptor was unaffected by incubation with irrelevant proteins or lysates of non-viable cells over the same concentration range as the standard lysate, or by β1,4 galactosyltransferase at a concentration range of 0.01–100 mU/ml. Bovine serum albumin, a non-glycosylated protein, and IgG, which carries similar N-linked carbohydrate moieties as IgA1, but which lacks O-glycans, did not bind VV lectin, and this was unaffected by incubation with the cell lysates if used as substrate in this assay system. Specificity of VV lectin for GalNAc was demonstrated by inhibition studies using various monosaccharides. 50% inhibition of VV binding was seen with GalNAc at 0.2 mM, while >20 mM concentrations of galactose, glucose, or N-acetylglucosamine all failed to produce 50% inhibition. 0.5 mM GalNAc gave 80% inhibition of VV binding to the acceptor, galactose gave 14% inhibition and N-acetyl glucosamine failed to inhibit at all at this concentration. These observations are entirely consistent with the reported specificity of VV lectin for GalNAc [15,16].

β1,3GT activity in IgAN and controls

PBMCs from nine patients with IgAN and 12 controls were fractionated into their constituent cell populations. Two monocyte lysates (1 IgAN, 1 control) contained insufficient protein for the assay (<100 μg/ml). β1,3GT activity of the remaining lysates was measured at 250 μg/ml (12 μg protein per well), and the results expressed as AU/μg. No difference was found between the enzyme activities of patient and control T cells (IgAN 3.3 ± 0.48 AU/μg, control 2.9 ± 0.35 AU/μg) or monocytes (IgAN 8.6 ± 1.14 AU/ μg, control 8.7 ± 1.14 AU/μg), as shown in Figure 1. However, the B cell lysates from patients with IgAN had significantly lower β1,3GT activity than those of controls (IgAN 6.2 ± 0.71 AU/μg, control 9.5 ± 1.03 AU/μg, P = 0.018) (Figure 2).
Vicia villosa lectin binding of serum IgA

VV lectin binds to terminal O-linked GalNAc, and increased binding of this lectin to IgA is seen in IgAN [7], probably indicating reduced galactosylation of the hinge region sugars. In agreement with our previous report involving a larger cohort of subjects [7], antibody-immobilized serum IgA displayed significantly higher VV lectin binding in the current group of patients with IgAN than in the controls (OD at 492nm: IgAN 0.2 ± 0.02; control 0.10 ± 0.02, $P = 0.007$) (Figure 3).

Relationship between cellular $\beta 1,3$GT activity and VV lectin binding of serum IgA

Linear regression analysis was carried out to look for relationships between the $\beta 1,3$GT activities of each cell type and the O-galactosylation status of IgA as assessed by VV lectin binding in IgAN and controls. IgA1 lectin binding was unrelated to T cell or monocyte enzyme activity in both the IgAN and the control groups (data not shown). In IgAN, B cell $\beta 1,3$GT activity had a strong inverse correlation with IgA1 lectin binding ($r = -0.872, P = 0.002$) (Figure 4); no such relationship was seen in the control subjects ($r = 0.200, P = \text{NS}$).

Discussion

The O-glycosylation defect of IgA1 in IgAN has not been fully characterized. Reports of increased binding to lectins with affinity for terminal GalNAc [7] and reduced binding to jacalin, a lectin with affinity for galactosylated Gal $\beta 1,3$GalNAc [4,5], indicate that it may take the form of a lack of terminal galactosylation of O-linked moieties, though other investigators report conflicting nature stresses the importance of further work to clarify the exact biochemical defect involved.

The experiments described in this study attempted to investigate the activity of the enzyme responsible for galactosylation of O-linked glycans in peripheral

Figure 1. $\beta 1,3$GT activity of T cell and monocyte lysates in patients with IgAN (●) and controls (○). There was no difference in the enzyme activity of either cell type between the two groups of subjects.

Figure 2. $\beta 1,3$GT activity of B cell lysates in patients with IgAN (●) and controls (○). In IgAN, B cell enzyme activity was significantly lower than controls ($P = 0.018$).

Figure 3. VV lectin binding of antibody-immobilized serum IgA from patients with IgAN (●) and controls (○). In IgAN, IgA shows significantly higher lectin binding than control ($P = 0.007$).

Figure 4. Correlation of B cell $\beta 1,3$GT activity with VV lectin binding of serum IgA in IgAN. VV lectin binds to terminal O-linked GalNAc, and is higher when galactosylation is reduced. The binding of this lectin to serum IgA shows an inverse relationship with B cell $\beta 1,3$GT activity in IgAN ($r = -0.87, P = 0.002$); no such relationship was found in controls.
blood cells from patients with IgAN. Since this enzyme has not been isolated or cloned, it can only be measured indirectly. The assay system used here employs degalactosed IgA1 as the acceptor substrate to tailor the system as closely as possible to the in vivo situation. Increases in terminal glycosylation were detected by reduction of VV lectin binding to exposed GalNAc units. This is assumed to be due to the enzymatic addition of \( \beta 1,3 \) galactosyltransferase, as this is the linkage usually found in O-glycans. It is possible that other glycosyltransferases may also be acting upon the substrate in this assay system, though in control experiments, \( \beta 1,4 \) galactosyltransferase did not affect the VV lectin binding of the acceptor. Structural analysis of the acceptor sugars would be necessary to demonstrate that the change in VV binding is entirely due to \( \beta 1,3 \)GT activity. However, the action detected in this assay system does mimic the abnormality of serum IgA1 described in IgAN, the precise nature of which has also yet to be confirmed. The results of this study show that B cell lysates from patients with IgAN have reduced ability to regalactosylate the IgA1-derived acceptor, and this is interpreted as reduced B cell \( \beta 1,3 \)GT activity. This finding supports the hypothesis that increased GalNAc expression by IgA1 is indeed Fc receptor and complement binding sites are located here [11]. The O-linked carbohydrates of IgA1 are adjacent to this region, and appear to be involved in the interaction with Fc receptors (FcRs) [10]. This may be because the glycans are actually part of the recognition motif, or because their presence is required to confer a molecular structure which renders the ligand accessible and recognizable to the receptor. Human mesangial cells have recently been shown to express an FcR [26,27], and though this is as yet uncharacterized, and its ligand undefined, it may be that the O-linked glycans of IgA1 could affect its interaction with this receptor. This may provide a pathway by which deposited IgA1 results in glomerular injury, since ligation of both leucocyte and mesangial Fc receptors leads to cell activation and initiation of inflammatory events.

The abnormality of IgA1 O-linked glycosylation in IgAN is inferred from altered lectin binding potential. The same mechanism could influence binding to other proteins in vivo, promoting non-immunological complex formation, and accounting for the increased levels of IgA complexes described in the serum of the patients [28,29]. It is also possible that binding to mesangial matrix components may be enhanced by lectin-like interactions. If altered O-glycosylation affects the structure of the IgA1 hinge region, possibly shortening it, antibody–antigen binding may be affected by steric hindrance resulting from the proximity of the rest of the IgA1 molecule: low IgA affinity has indeed been described in IgAN [30].

The reduced B cell \( \beta 1,3 \)GT activity reported in this study provides an explanation for the abnormality of O-linked glycosylation of serum IgA1 recently described in IgAN. This may prove to be a defect fundamental the pathogenesis of IgAN. The sequence of \( \beta 1,3 \)GT is still unknown. Once established, it will
facilitate future studies to define the molecular nature of this β1,3GT deficiency and its tissue distribution.

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