ORIGINAL RESEARCH

The role of C1GALT1C1 in lipopolysaccharide-induced IgA1 aberrant O-glycosylation in IgA nephropathy

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Abstract

Purpose: IgA1 aberrant O-glycosylation is one of the main pathogenetic features of IgA nephropathy (IgAN). This study attempted to determine the role of C1GALT1C1 in aberrant IgA1 O-glycosylation induced by lipopolysaccharide (LPS) and identify potential therapeutic targets in IgAN.

Methods: Lymphocytes isolated from 22 patients with IgAN and 17 normal controls were cultured for 3 to 7 days with or without LPS and 5-azacytidine (5-AZA). Expression levels of C1GALT1C1 mRNA and protein were measured by real-time PCR and Western blot analysis, respectively. Concentration of IgA1 and level of O-glycosylation were determined by ELISA and Vicia villosa (VV) lectin-binding assay. Correlation analysis was performed between the expression of C1GALT1C1 protein and IgA1 O-glycosylation.

Results: Lymphocytes from patients with IgAN secreted more IgA1 than that from normal controls after LPS stimulation ($P=0.26$, 0.002 and 0.005 on the 3rd, 5th and 7th day, respectively) which could be inhibited by 5-AZA ($P=0.001$, 0.025 and 0.001 on the 3rd, 5th and 7th day, respectively). Moreover, LPS stimulation could obviously inhibit C1GALT1C1 expression in patients with IgAN (decreased by 71%, 82% and 92% on the 3rd, 5th and 7th day, respectively; $P<0.001$), and cause a significant decrease of IgA1 O-glycosylation compared with normal controls ($P=0.004$, 0.003 and 0.03 on the 3rd, 5th and 7th day, respectively). When 5-AZA was added, the level of C1GALT1C1

expression increased dramatically (1.98, 5.53 and 8.97 times on the 3rd, 5th and 7th day, respectively; $P<0.001$) along with an increase of IgA1 O-glycosylation ($P=0.295$, 0.09 and 0.003 on the 3rd, 5th and 7th day, respectively). However, normal controls showed no significant change in C1GALT1C1 expression and IgA1 O-glycosylation after LPS stimulation ($P>0.05$).

Conclusion: LPS induced IgA1 aberrant O-glycosylation and suppressed C1GALT1C1 expression in patients with IgAN. Upregulation of C1GALT1C1 expression by 5-AZA could reverse the IgA1 aberrant O-glycosylation. These results suggest that C1GALT1C1 may play a key role in the regulation of IgA1 O-glycosylation.

IgA nephropathy (IgAN) is the most common form of glomerulonephritis in the world.¹ Aberrant IgA1 O-glycosylation results from the abnormal function or expression of several specific glycosyltransferases, which may be one of the key pathogenetic mechanisms of IgAN.^{2,3} The activity of β 1,3-galactosyltransferase (β 1,3-Gal-T) is remarkably low in patients with IgAN. In our earlier study, no apparent decrease was observed in expression of β 1,3-Gal-T mRNA while the expression of C1GALT1C1 (core

TABLE 1. Baseline clinical characteristics of included subjects

	IgAN (n = 22)	Normal controls (n = 17)	P
Age (years)	24.17 ± 6.3	27.31 ± 7.15	NS
Males/females	14 / 8	9/8	NS
Systolic blood pressure (mmHg)	124 ± 21	102 ± 16	NS
Diastolic blood pressure (mmHg)	80 ± 16	70 ± 12	NS

β 1,3-Gal-T-specific molecular chaperone) mRNA is significantly decreased in patients with IgAN.^{4,5}

Clinically, the onset of IgAN is often related to upper respiratory tract infections caused by virus or bacteria. Therefore, external suppression derived from infectious agents has been considered to be an etiological factor in this disease. It was found that Epstein-Barr virus immortalized lymphocytes from patients with IgAN produced IgA1 molecules that were aberrantly glycosylated and had defective expression of C1GALT1C1.⁶ In addition, the level of O-glycosylation of serum IgA1 decreased in patients with IgAN who had received subcutaneous immunization with tetanus toxoid or were infected by mucosal *Helicobacter pylori* (HP).⁷ As the marker of gram negative bacterial infections, lipopolysaccharide (LPS) could trigger an acute aggravation of mesangioproliferative glomerulonephritis, induce experimental IgAN, and suppress C1GALT1C1 expression in patients with IgAN.⁸⁻¹⁰ Therefore, this study was designed to determine whether LPS could induce aberrant IgA1 O-glycosylation by suppressing C1GALT1C1 expression.

Previous studies indicated that no significant mutation was detected in the C1GALT1C1 gene in IgAN,¹⁰ and there was evidence that LPS could enhance the methylation of certain genes.¹¹ Moreover, treatment with 5-azacytidine (5-AZA, a potent inhibitor of DNA methylation) could reactivate the defective activity of β 1,3-Gal-T in Tn syndrome, which is also a disease characterized by aberrant glycosylation.¹² Thus, the secondary aim of this present study was to identify whether 5-AZA could reverse the aberrant IgA1 O-glycosylation by upregulating C1GALT1C1 expression in IgAN.

Materials and Methods

Subjects collection and clinical data

Twenty-two patients with biopsy-proven IgAN were included in this study after informed consent was obtained. The diagnostic criteria for IgAN were based on the manifestation of generalized glomerular mesangial proliferation with the presence of IgA as the sole or predominant deposition of immunoglobulin in the mesangial area of glomeruli. No patients included in this study had received corticosteroids or other immunosuppressive therapy. Patients with systemic diseases such as Henoch-Schönlein purpura, systemic lupus erythematosus, rheumatoid arthritis, diabetes mellitus or liver cirrhosis were excluded. Seventeen healthy volunteers were selected as normal controls after informed consent was obtained. As shown in Table 1, there were no differences in age, sex, baseline blood pressure between patients with IgAN and normal controls ($P > 0.05$). This study was approved by the clinical ethical committee of West China Hospital of Sichuan University.

Lymphocyte isolation, culture and treatment

Peripheral blood mononuclear cells (PBMCs) were separated from 20 ml venous blood of each participant by density gradient centrifugation on Lymphocyte-H isolation media (Cedarlane Laboratories Limited, Canada). Lymphocytes were isolated from lymphocytes after the depletion of monocytes via wall sticking method, where monocytes adhere to the plates while PBMCs remain suspended in the culture medium. Lymphocytes were cultured (10^6 cells/mL) with complete RPMI-1640 medium containing 15% fetal calf serum with 2 mM L-glutamine and 1 mM HE-

TABLE 2. Primers and fluorescence probes

	C1GALT1C1	GAPDH
Sense	5'-GTAACGGAGTGGTGCGCCAA-3'	5'-GGGTGTGAACCATGAGAAGT-3'
Antisense	5'-TTGCACTTCATCCGCGTCTAGA-3'	5'-CCAAAGTTGTCATGGATGACCT-3'
Probe	5'-FAM-CGTGCGCGGCTGCGCTTTCCTTAMRA-3'	5'-FAM-CTGCACCACCACTGCTTAGCTAMRA-3'

PES. Pokeweed mitogen (PWM, 5.0 µg/mL) was added to aid the proliferation and differentiation. Lymphocytes were divided into six groups including IgAN (A, B and C) and normal controls (a, b and c): Group A and a (Blank): RPMI + PWM; Group B and b (LPS): RPMI + PWM + LPS; and Group C and c (5-AZA): RPMI + PWM + LPS + 5-AZA. The concentrations of LPS and 5-AZA were 12.5 µg/mL and 1.0 µmol/L, respectively. Cells were cultured in 24-well plates at 37°C for 3 to 7 days. Evaluation of the proliferation and differentiation of lymphocytes was performed. Viability of PBMCs was about 85% as determined by trypan blue exclusion on Day 7, and no significant difference was observed among the three groups (Blank group, LPS group, 5-AZA group). Flow cytometric analysis with two-color staining was used to determine the differentiation of lymphocytes. B cells were defined as CD19⁺/CD38⁻, whereas plasma cell were defined as CD19⁻/CD38⁺. The results showed an increase of plasma cells accompanied by the enhanced concentration of IgA1 with increasing time (data not shown), implying that IgA1 was secreted by the plasma cells.

IgA1 ELISA analysis and Vicia villosa(VV) lectin-binding assay

As previously described, 96-well plates were coated with primary antibody (Southern Biotechnology Associates, USA) overnight at 4°C.⁵ After plates were blocked, samples were added in duplicate and incubated for 1 h at 37°C with the biotinylated secondary antibody (Southern Biotechnology Associates, USA). After plates were incubated for another 1 h at 37°C with peroxidase-avidin D (Vector Laboratories, UK), colour was developed using tetramethyl benzidine dilution (TMB) and detected at 450 nm. The concentra-

tion of IgA1 was calculated from the standard curve that was constructed with a serial dilution of IgA1 standard serum (Nordic Immunological Laboratories, Netherlands).

The levels of IgA1 O-glycosylation were determined by Vicia villosa (VV) lectin-binding assay as previously described.⁵ 96-well plates were coated with primary antibody and blocked as described above. Samples were added to the plates in duplicate and incubated for 1 h at 37°C. After washing, the plates were incubated for 1 h at 37°C with biotinylated VV lectin (Vector Laboratories, UK), washed again, and then incubated for a further 1 h at 37°C with peroxidase-avidin D (Vector Laboratories). Colour was developed and detected as above.

Real-time quantitative PCR

Total RNA was extracted from lymphocytes using the RNeasy Mini kit (QIAGEN, USA). Real-time quantitative PCR (RT-PCR) was performed with the Taqman probe technique after reverse transcription. The primers and probes of C1GALT1C1 and the internal calibrator, GAPDH (synthesized by Invitrogen, China), are listed in Table 2. The PCR reaction was amplified in a Roche Lightcycler (Roche Diagnostics, USA) as previously described.⁵ In order to examine the efficiency of RT-PCR, standard curves were established with serial dilutions of sample RNA (500 ng; 10×dilution). PCR products were purified and sequenced directly by Invitrogen (Shanghai, China). The sequencing result showed that the amplified fragment was in accordance with the GenBank record.

Western blotting

Protein samples of lymphocytes were separated on 10% sodium dodecyl sulfate polyacrylamide (SDS-

FIGURE 1. The concentration of IgA1 (ng/mL) secreted by cultured lymphocytes from patients with IgAN and normal controls. After LPS stimulation, lymphocytes from either patients with IgAN or normal controls secreted high concentration of IgA1 from 3 to 7 days, especially from those of the former. However, treatment with 5-AZA lead to a decrease in the secretion of IgA1.

FIGURE 2. IgA1 O-glycosylation assay (reciprocal of OD) in patients with IgAN and normal controls. The level of IgA1 O-glycosylation in each group of patients with IgAN was lower than that of the corresponding group in normal controls. LPS induced significant under-glycosylation in patients with IgAN, which could be reversed partly by 5-AZA. While no significant difference was observed in normal controls.

PAGE) gels and transferred to polyvinylidene fluoride (PVDF) membranes. After being blocked for 1 h, blots were incubated with primary antibodies (1:200; Santa Cruz Biotechnology, USA) overnight at 4°C. After washing, the blots were incubated with horseradish peroxidase-linked secondary antibodies (1:5000; Santa Cruz Biotechnology, USA) for 1 h. After further washing, the immunoreactivities of antibodies were detected via ECL reagents (GE Healthcare, USA). The measurement of β -actin was applied as an internal calibrator.

Statistical analysis

Data were expressed as mean $\pm$ standard error (SE). One-way ANOVA and Student's t-test were performed to analyze the concentration of IgA1, levels of O-glycosylation and C1GALT1C1 expression. $P < 0.05$ was taken as the level of statistical significance. Pearson correlation analysis was used to evaluate whether there was a correlation between the expression of C1GALT1C1 protein and the level of IgA1 O-

glycosylation. The above analyses were conducted with SPSS 13.0 statistical software.

Results

Effects of LPS and 5-AZA on IgA1 secretion and O-glycosylation

It was observed that *in vitro* culture led to an increase in the secretion of IgA1 from lymphocytes from both patients with IgAN ($P = 0.02$, 0.03 and 0.047 on the 3rd, 5th and 7th day, respectively) and normal controls ($P = 0.01$, 0.01 and 0.006 on the 3rd, 5th and 7th day, respectively), but especially from those of the former. Moreover, LPS stimulation resulted in a further increase of IgA1 secretion by lymphocytes from all participants (Patient with IgAN: $P = 0.032$, 0.006 and 0.004 on the 3rd, 5th and 7th day, respectively; Normal controls: $P = 0.018$, 0.035 and 0.03 on the 3rd, 5th and 7th day, respectively), particularly from patients with IgAN ($P = 0.26$, 0.002 and 0.005 on the 3rd, 5th and 7th day, respectively). However, treatment with 5-AZA inhibited the LPS-induced secretion of IgA1 at each time point ($P = 0.001$, 0.025 and 0.001 on

FIGURE 3. The expression levels of C1GALT1C1 mRNA represented by Delta Ct Ratio (C1GALT1C1/GAPDH) of patients with IgAN and normal controls. LPS stimulation significantly suppressed the C1GALT1C1 mRNA expression in patients with IgAN from 3 to 7 days, but the C1GALT1C1 mRNA expression was upregulated when 5-AZA was added. LPS showed no significant effect on the C1GALT1C1 expression in normal controls.

the 3rd, 5th and 7th day, respectively) (Figure 1). These data suggested that lymphocytes from patients with IgAN possess a large tendency to secrete IgA1 compared with those from normal controls under conditions of external stimulation such as with LPS. This activity could be inhibited significantly by treatment with 5-AZA.

As the increase of VV lectin binding denoted the decrease of IgA1 O-glycosylation, its reciprocal was used to reflect the IgA1O-glycosylation levels directly (Figure 2). It was noticed that the baseline levels of O-glycosylation of patients with IgAN were significantly lower than that of normal controls, which was similar to previous findings.^{4,5} In patients with IgAN, the VV lectin binding levels increased dramatically at each time point after LPS stimulation (Blank vs. LPS stimulation, $P=0.004$, 0.003 and 0.03 on the 3rd, 5th and 7th day, respectively), but this decreased after treatment with 5-AZA. On the 3rd day, the decrease was not statistically significant ($P=0.295$), on the 5th day the change approached significance ($P=0.09$), and on the 7th day a significant difference was found

($P=0.003$). However, no remarkable differences were observed in normal controls. These data suggest that LPS induced an increasing level of aberrant IgA1 O-glycosylation in patients with IgAN but not in normal controls; moreover, 5-AZA could reverse the aberrant IgA1 O-glycosylation in a time-dependent manner.

Effects of LPS and 5-AZA on C1GALT1C1 expression

The results of RT-PCR indicated that baseline C1GALT1C1 expression in lymphocytes from patients with IgAN was significantly lower than that from normal controls ($P<0.001$), which was similar to previous reports.⁵ C1GALT1C1 expression in patients with IgAN was markedly increased in the Blank group and LPS stimulation inhibited this upregulation. Interestingly, when 5-AZA was added, C1GALT1C1 expression increased dramatically. No significant change was observed in C1GALT1C1 expression in normal controls after the treatment mentioned above (Figure 3).

In order to compare the expression levels of C1GALT1C1 mRNA more directly, ratios of target gene expression between patients with IgAN and normal controls were calculated according to the mathematical model of relative quantification in RT-PCR developed by Pfaffl.¹³ It was found that baseline C1GALT1C1 expression in patients with IgAN was only 65% of the levels in normal controls. When the lymphocytes were cultured with RMPI-1640+PWM, this led to an increase in C1GALT1C1 expression in a time-dependent manner (Blank group vs Baseline, increased 259%, 398% and 510% on the 3rd, 5th and 7th day, respectively). However, LPS dramatically inhibited this increase. The analysis revealed that C1GALT1C1 expression dramatically decreased after LPS stimulation compared with the Blank group (decreased 71%, 82% and 92% on the 3rd, 5th and 7th day, respectively). Nevertheless, 5-AZA could reverse the suppression of C1GALT1C1 expression considerably (5-AZA group vs. LPS group, increased 1.98, 5.53

FIGURE 4. The expression levels of C1GALT1C1 protein of lymphocytes after cultured in presence or absence of LPS, 5-AZA in patients with IgAN. Western blotting assay supported the results that LPS could significantly suppress the C1GALT1C1 protein expression, but 5-AZA was able to reverse this repression in patients with IgAN.

FIGURE 5. Correlation between the levels of C1GALT1C1 expression and IgA1 O-glycosylation. Reciprocal of VV lectin binding (OD450) represented the levels of IgA1 O-glycosylation, and the expression of C1GALT1C1 protein was shown as the absorbance density of C1GALT1C1/β-actin. Each symbol represents the parameters of each group.

and 8.97 times on the 3rd, 5th and 7th day, respectively).

Western blotting was applied to measure the expression levels of C1GALT1C1 protein (Figure 4). These results were in accordance with that of RT-PCR: LPS suppressed C1GALT1C1 expression strongly from the 3rd day and the absorbance density was significantly lower than that of Blank group. However, 5-AZA could significantly reverse the LPS-repressed C1GALT1C1 expression, which persisted from the 3rd to 7th day.

Correlation between C1GALT1C1 expression and IgA1 O-glycosylation

As shown in Figure 5, the Pearson correlation analysis revealed that there was a significant positive correlation between expression levels of C1GALT1C1 protein and levels of IgA1 O-glycosylation ($r^2=0.706$, $P=0.005$).

Discussion

IgA nephropathy (IgAN) is characterized by the presence of IgA deposits in the glomerular mesangium,

accounting for more than 50% of biopsy-proven primary glomerulonephritis in Asia. Growing evidence suggests that the aberrance of IgA1 O-glycosylation is one of the key causes of IgAN.¹⁻³ This is likely because it promotes IgA1 self-aggregation thereby increasing its affinity towards mesangial cells and escaping clearance by liver, thus ultimately leading to deposition of IgA1 in glomeruli and initiation of renal injury via complement activation and proliferation of mesangial cells. IgA1 O-glycosylation depends on the activity of β1,3-Gal-T with assistance of its chaperone encoded by the C1GALT1C1 (core β1,3-Gal-T-specific molecular chaperone) gene. It was reported that β1,3-Gal-T activity was significantly decreased in peripheral B lymphocyte of patients with IgAN.⁴ A further study revealed that patients with IgAN had normal β1,3-Gal-T expression and decreased C1GALT1C1 expression, which implied that the variability of C1GALT1C1 influenced IgA1 O-glycosylation rather than the variability of β1,3-Gal-T.⁵

Bacterial infections are closely associated with the onset or relapse of IgAN. Lipopolysaccharide (LPS), which is one of the main pathogenic agents of Gram

negative bacteria, was found to suppress the expression of C1GALT1C1 mRNA in our previous study.¹⁰ Consequently, the current study was designed to determinate the direct relationship between LPS and IgA1 O-glycosylation, and evaluate the role of C1GALT1C1 in the regulation of IgA1 O-glycosylation. In this study, we noticed that the peripheral lymphocytes from patients with IgAN secreted significantly higher levels of IgA1 that had aberrant O-glycosylation compared with that from normal controls. Moreover, aberrant O-glycosylation dramatically increased after LPS stimulation at each time point in patients with IgAN, while this was not observed in normal controls. These data suggested that LPS could induce aberrant O-glycosylation in patients with IgAN, which supported the previous report that mucosal *Helicobacter pylori* (HP) infections caused decreased IgA1 O-glycosylation in IgAN.⁷ RT-PCR results showed that the expression levels of C1GALT1C1 mRNA in patients with IgAN were significantly lower than those of normal controls at baseline, and this level was upregulated after being cultured with RPMI-1640 + PWM. However, LPS could markedly inhibit the upregulation at each time point. The Western blot analysis for the levels of C1GALT1C1 protein showed similar results. Moreover, a highly significant correlation was observed between C1GALT1C1 expression and IgA1 O-glycosylation. These data suggest that the repressed C1GALT1C1 expression in IgAN may cause IgA1 aberrant O-glycosylation.

After LPS stimulation (mimicing bacterial infections), the lymphocytes from patients with IgAN possessed a high tendency to secrete IgA1 compared with that from normal controls; additionally, more underglycosylated IgA1 were secreted, while C1GALT1C1 expression was suppressed. The explanation may be that there was an imbalance between glycosylation demand and capacity, i.e., the biosynthesis of IgA1 O-glycosylation requires adequate β 1,3-Gal-T to catalyze, whereas C1GALT1C1 was not sufficient to sup-

port its function (because the activity of β 1,3-Gal-T depends on the presence of C1GALT1C1).

As suppressed C1GALT1C1 expression is considered to be the underlying mechanism of aberrant IgA1 O-glycosylation, the potential to reverse C1GALT1C1 expression in patients with IgAN became our next aim. Treatment with 5-azacytidine (5-AZA) could reactivate suppressed β 1,3-Gal-T in Tn syndrome, which indicates that epigenetic modifications may be involved in the regulation of O-glycosylation.¹⁴ Considering that IgAN is similar to Tn syndrome as a disorder of O-glycosylation and no common C1GALT1C1 gene mutation has been detected in IgAN,¹⁰ we proposed that epigenetic variability might also influence the development and progression of IgAN. DNA methylation is an epigenetic modification which usually takes place at CpG islands of promoter region and correlates with the inactivation or transcription of gene expression.¹⁵ Studies have shown that EBV and LPS could induce the hypermethylation of certain genes.^{11,16,17} Altered DNA methylation is involved in the abnormal post-transcriptional gene regulation in immune-mediated disease.¹⁸ Epigenetic agents (e.g. 5-AZA) have a reasonable safety profile and provide clinical benefits in the treatment of a number of diseases, such as leukemia, myelodysplastic syndrome (MDS), and non-small cell lung cancer (NSCLC).^{19,20} Calculations have indicated that there is a CpG island (GC% = 56.7%, ObsGpG/ExpCpG=0.724, length=540) in the DNA sequence of the C1GALT1C1 gene.²¹ Therefore, it is suspected that 5-AZA might enhance the levels of IgA1 O-glycosylation through regulating DNA methylation of C1GALT1C1.

The present study showed that treatment with 5-AZA could dramatically inhibit the secretion of IgA1 induced by LPS stimulation. Meanwhile, the expression levels of C1GALT1C1 mRNA increased on the 3rd, 5th and 7th day consecutively. Moreover, the levels of IgA1 O-glycosylation increased in IgAN groups in a time dependent manner. These results indicated that after treatment with 5-AZA, the high levels

of secreted IgA1 were reversed while the suppressed C1GALT1C1 expression was upregulated. This means that the equilibrium between the demand and capacity of the O-glycosylation was reestablished and resulted in normal IgA1 O-glycosylation. Although mucosal or systemic responses to *Helicobacter pylori* (HP) or Tetanus (TT) may induce increased IgA1 or abnormal O-glycosylation of IgA1 in IgAN, no studies have been performed addressing differential DNA methylation in these models of IgA nephropathy.^{22,23} At present, little is known about whether the abnormal epigenetic modification (e.g. DNA hypermethylation) is involved in IgA nephropathy. Knowledge of this may therefore enhance our understanding of IgA nephropathy.

In conclusion, this present study determined that C1GALT1C1 played a key role in the regulation of IgA1 O-glycosylation. Moreover, the balance between the secretion of IgA1 and C1GALT1C1 expression was crucial for IgA1 O-glycosylation. External influences on this gene expression, such as LPS, may induce aberrant IgA1 O-glycosylation in patients with IgAN by suppressing C1GALT1C1 expression. Treatment with 5-AZA could upregulate C1GALT1C1 expression and improve IgA1 O-glycosylation, which might help to identify new targets for future treatments of IgAN.

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