Original Paper

Glucocorticoids Reduce Aberrant O-Glycosylation of IgA1 in IgA Nephropathy Patients

Petr Kosztu^a Martin Hill^b Jana Jemelkova^a Lydie Czernekova^a
Leona Raskova Kafkova^a Miroslav Hruby^c Karel Matousovic^d Karel Vondrak^e
Josef Zadrazil^c Ivan Sterzl^b Jiri Mestecky^{f,g,h} Milan Raska^{a,f,i}

^aDepartment of Immunology, Faculty of Medicine and Dentistry, Palacky University, Olomouc and University Hospital Olomouc, Olomouc, ^bDepartment of Steroids and Proteohormones and Department of Clinical Immunology, Institute of Endocrinology, Prague, ^cDepartment of Internal Medicine III Nephrology, Rheumatology and Endocrinology, Palacky University Olomouc, ^dDepartment of Medicine, Second School of Medicine, Charles University, Prague and University Hospital Motol, Prague, ^eDepartment of Pediatrics, Second School of Medicine, Charles University, Prague and University Hospital Motol, Prague, Czech Republic, ^fDepartment of Microbiology, University of Alabama at Birmingham, Birmingham, Alabama, USA, ^gDivision of Immunology and Gnotobiology Institute of Microbiology, Czech Academy of Sciences, Prague, ^hInstitute of Immunology and Microbiology, First School of Medicine, Charles University, Prague, ⁱInstitute of Molecular and Translational Medicine, Faculty of Medicine and Dentistry, Palacky University, Olomouc, Czech Republic

Key Words

IgA nephropathy • O-glycosylation • IgA1 hinge region • Circulating immune complexes • Galactose deficiency • Glycan-specific antibodies.

Abstract

Background/Aims: IgA nephropathy is associated with aberrant O-glycosylation of IgA1, which is recognized by autoantibodies leading to the formation of circulating immune complexes. Some of them, after deposition into kidney mesangium, trigger glomerular injury. In patients with active disease nonresponding to angiotensin-converting enzyme inhibitors or angiotensin II blockers, corticosteroids are recommended. **Methods:** The relationship between the corticosteroid therapy and serum levels of IgA, aberrantly O-glycosylated IgA1, IgA-containing immune complexes and their mesangioproliferative activity was analyzed in IgA nephropathy patients and disease and healthy controls. **Results:** Prednisone therapy significantly reduced proteinuria and levels of serum IgA, galactose-deficient IgA1, and IgA-IgG immune complexes in IgA nephropathy patients and thus reduced differences in all of the above parameters between IgAN patients and control groups. A moderate but not significant reduction of mesangioproliferative potential of IgA-IgG immune complexes and

Karel Matousovic
and Josef Zadrazil

Department of Medicine, Second School of Medicine, Charles University, University Hospital Motol, V Uvalu 84, Prague; Department of Internal Medicine III, Palacky University, University Hospital Olomouc, I.P. Pavlova 185/6, Olomouc (Czech Republic); E-Mail kmatusovic@hotmail.com, josef.zadrazil@upol.cz

IgA sialylation was detected. **Conclusion:** The prednisone therapy reduces overall aberrancy in IgA1 O-glycosylation in IgA nephropathy patients, but the measurement of IgA1 parameters does not allow us to predict the prednisone therapy outcome in individual patients.

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Introduction

IgA nephropathy (IgAN) is characterized by the deposition of IgA1 in the kidney mesangium, frequently accompanied by IgG, C3 complement component and/or IgM, and rarely also by C5b-9 [1, 2]. The mesangial IgA1 in IgAN is enriched for polymeric galactose-deficient (Gd-IgA1) glycoforms. The galactose (Gal) deficiency affects O-linked oligosaccharides attached to the amino acid backbone of the IgA1 hinge region. The formation of O-linked glycans takes place in the Golgi apparatus of lymphoblasts and plasma cells through the sequential addition of individual glycans to serine and threonine residues starting by the addition of N-acetylgalactosamine (GalNAc) followed by Gal and finally sialic acid (SA) which may be bound either to first or second glycan. This process is catalyzed by several glycosyltransferases including N-acetylgalactosaminyltransferase 2 (GalNAc-T2), Core1 β 1,3-galactosyltransferase 1 (C1GalT1), β 1,3-galactosyltransferase specific chaperone (Cosmc), α 2,3-sialyltransferase (ST3Gal) and a α 2,6-sialyltransferase (ST6GalNAc-II) [3-9].

Recent genome-wide association analyses confirmed C1GalT1 gene as one of critical genetic factors playing role in the Gd-IgA1 formation [10, 11]. Other recent studies suggested involvement of polymorphism in immune signalling molecules like IFN- γ and IL-10 [12, 13]. In patients with IgAN there is an increased proportion of O-linked oligosaccharides without Gal [14, 15], therefore with terminal GalNAc or GalNAc with SA. The sialylation of GalNAc prevents further addition of Gal and could be induced in IgA-producing cells from IgAN patient by inflammatory cytokine IL-6 [4]. Such mechanisms could contribute to the etiology of synpharyngitic hematuria. Recently, it was reported that modulation of IL-6-triggered intracellular signaling by STAT3 could alleviate Gal deficiency of IgA1 [16].

In IgAN patients, some Gal-deficient O-glycans attached to hinge region are recognized by autoantibodies of IgG or IgA1 isotype leading to the formation of immune complexes [17, 18]. There is compelling evidence that mesangial immunodeposits in IgAN are derived from IgA-containing circulating immune complexes (CIC) [19]. Thus, CIC likely play a key role in IgAN, and the kidneys are “innocent bystanders” [20, 21].

The hypertension and proteinuria are key prognostic factors for patients with a preserved estimated glomerular filtration rate (eGFR). Histological features of a less favorable prognosis include mesangial hypercellularity, endocapillary hypercellularity, segmental glomerulosclerosis, and tubular atrophy / interstitial fibrosis (MEST score), according to the Oxford classification [22].

If the disease exhibits persisting hematuria and proteinuria >1 g/24 h, decreased eGFR, and positive MEST, especially proliferative and necrotizing changes in glomeruli, glucocorticoid therapy is recommended [23]. It was reported recently that such therapy could alleviate Gal-deficiency of serum IgA after three months of therapy [24].

Here, we analyzed the levels of the total IgA, Gd-IgA1, IgA-IgG-containing CIC and their stimulatory activity on mesangial cells (MC), and IgA1 sialylation before, 7 and 30 days after the initiation of prednisone therapy in a group of IgAN patients, the disease, and healthy controls. Furthermore, we attempted to correlate each factor with the clinical status in individual patients to identify a potential biomarker applicable as a predictor of a clinical response.

Table 1. Study subjects characteristics. The dose of prednisone in treated subjects was (60 mg /day/m² of body surface) with a maximum 80 mg / 24 h). ND: not determined. * Rheumatoid arthritis, Systemic lupus erythematosus, Ankylosing spondylitis, Systemic sclerosis, Polymyalgia rheumatica, Gigantocellular arthritis. ** Idiopathic membranous nephropathy, Focal segmental glomerulosclerosis, Idiopathic nephrotic syndrome

Group	Number of subjects	Gender ratio (M/F)	Mean age (years)	Serum creatinine (μmol/l)	sUrea (mmol/L)	eGFR (mL/s per 1.73 m ²)	Proteinuria (g/L)	Blood pressure (mmHg)	ACE and/or ARB (treated/all)
IgAN patients D0	14	9/5	46	160	8.7	0.9	0.75	133/82	11/14
IgAN patients D7	14	9/5	46	150	11.2	0.8	1.00	141/89	11/14
IgAN patients D30	14	9/5	46	156	12.8	0.8	0.71	136/81	11/14
IgAN patients D90	14	9/5	46	113	7.6	0.8	0.67	137/82	11/14
IgAN patients D180	14	9/5	47	122	5.9	0.9	0.45	134/76	11/14
Prednisone-treated controls *	10	4/6	55	86.4	5.0	1.2	0.50	129/79	3/10
Disease controls untreated **	10	6/4	48	93.1	7.3	1.4	1.59	135/81	8/10
Healthy controls	10	1/9	43	ND	ND	ND	ND	120/80	0

Materials and Methods

Study subjects

14 IgAN patients with persisting proteinuria > 1 g / 24 h (mean value 1.8 g / 24 h) and mean eGFR 0.91 mL/s/1.73 m² were included in this study. Only 6 IgAN patients exhibited eGFR < 0.83 mL/s/1.73 m², and histological markers of disease activity (Table 1). The dose of prednisone in treated subjects was (60 mg / day/m² of body surface) with a maximum 80 mg / 24 h). The diagnosis of IgAN was based on biopsy-proven predominant mesangial deposition of IgA. Control subjects included 10 prednisone-treated control patients with connective tissue diseases, 10 disease controls w/o prednisone - non-IgAN glomerular nephritis patients (Table 1), and 10 healthy subjects. All study subjects signed an informed consent document approved by the Ethical Committee of the University Hospital in Olomouc, Czech Republic.

The ELISA measurement of serum level of total IgA and Gd-IgA1

The ELISA measurement of serum level of total IgA and Gd-IgA1 was determined according to previously described protocols [14] for Gd-IgA1 using the *Helix pomatia* lectin and sialidase treatment specifically recognizing GalNAc residues not occupied by Gal. Results were expressed as the ratio of individual tested sera absorbance to standard myeloma Gd-IgA1 absorbance [4].

IgG-IgA CIC were analyzed by ELISA using plates coated with anti-human IgG F(ab')₂ and detected by goat anti-human IgA F(ab')₂ [17]. Results were expressed as a relative to standard CIC-positive serum.

ELISA determination of IgA sialylation

Maxisorp ELISA plates were coated overnight with goat anti-human IgA F(ab')₂ at final concentration 2.5 μg/mL. Plates were then washed with PBS-T and blocked with blocking buffer for 3 h at room temperature. Tested sera were diluted in blocking buffer (in duplicates) and incubated overnight at 4°C. Plates were washed three times with PBS-T and biotinylated *Sambucus nigra* lectin (Vector Laboratories) diluted in blocking buffer 1:500 was added and incubated at 37°C for 3 h. Thereafter plates were washed three times with PBS-T and horseradish peroxidase-labeled avidin was added at dilution 1:20, 000 in blocking buffer. After 1 h incubation at 37°C, plates were washed, developed with OPD and the absorbance was read at 490 nm. Results were expressed as the ratio of absorbance measured for tested serum to standard serum.

MC proliferation

The MC proliferation was measured according to previously described protocol [25-27]. For analyses, 9 IgAN patients, 6 prednisone-treated controls, 6 untreated disease controls, and 6 healthy controls were

evaluated. The results were expressed as relative to negative control serum.

Statistical analysis

The statistical analysis was performed by repeated ANOVA model with the factors subject and within-subject factor stage of the treatment or by one-way ANOVA model with the factor group, followed by the least significant difference multiple comparison using. The original data were transformed by power transformations to attain Gaussian data distribution and constant variance prior statistical testing. Statgraphics Centurion, version XV statistical software from Manugistics (Herndon, MA, USA) was used for calculations.

Results

Prednisone therapy of IgAN patients was continuously administered for at least 6 months and was associated with an initial insignificant proteinuria increase 7 days after therapy initiation and followed by a slow decrease for 6 months, at which point the sampling of patients was terminated. The mean proteinuria decreased from 0.75 g/L to 0.45 g/L (repeated measures ANOVA; $P < 0.05$) (Fig. 1).

Prednisone therapy also led to a significant decrease in levels of the total serum IgA and Gd-IgA1 (Fig. 2A, B). The level of total IgA decreased significantly after 7 days, whereas Gd-IgA1 decreased significantly after 1 month of therapy (Fig. 2B). The comparison of Gd-IgA1 and total IgA levels between IgAN patients and control groups (see Concise methods) confirmed a significantly higher level of Gd-IgA1 and total IgA in IgAN patients before prednisone therapy (Fig. 2C, D). Just one week after the therapy initiation, the differences were abolished (Fig. 2C, D), indicating that prednisone therapy led to a prompt reduction of total IgA and Gd-IgA1 levels in IgAN patients. Nevertheless, the total IgA and Gd-IgA1 levels did not reach the values determined in any control groups even after one month of therapy (Fig. 2C, D).

Because the Gd-IgA1 production could be also caused by premature sialylation of GalNAc residues of forming O-glycans on IgA1[4], we analyzed the sialylation of IgA. Prednisone therapy of IgAN patients reduced the mean IgA sialylation only insignificantly, for about 10% of the pre-treatment mean value (Supplemental Fig. 1), and the sialylation remained significantly higher than in any control group ($P < 0.05$; Supplemental Fig. 1). For all supplemental material see www.karger.com/doi/10.1159/000487903.

Next we determined IgG-IgA CIC level. One month of prednisone therapy of IgAN patients reduced significantly the IgG-IgA CIC level (Fig. 3A) and reached the mean value similar to the control groups (Fig. 3B). Further, we determined nephritogenic activity of CIC *in vitro* by detecting their proliferative potential toward the MC (Supplemental Fig. 2). The sera from

Fig. 1. Proteinuria development in response to prednisone therapy. Changes in proteinuria were evaluated in IgAN patients before (N0) and 7 (N7), 30 (N30), 90 (N90), and 180 (N180) days after steroid therapy initiation. Statistical analysis was performed by repeated ANOVA model with factors subject and within-subject factor stage of the treatment followed by the least significant difference multiple comparison. The squares with error bars represent means with their 95% confidence intervals (least significant difference multiple comparisons). * = $P < 0.05$.

IgAN patients before prednisone therapy stimulated MC 57% more than those from healthy controls, whereas after one month of therapy the difference was reduced to 25%. Inherent variability in MC proliferation responses to IgAN sera, especially from those without macroscopic hematuria [26] preclude a positive significance confirmation (Supplemental Fig. 2).

Fig. 2. Prednisone reduces IgA and Gd-IgA1 levels in IgAN patient's sera. The development of levels of total serum IgA concentration and serum Gd-IgA1 in IgAN patients (A and B) before (N0), at 7 days (N7), and at 30 days (N30) after steroid therapy initiation. A repeated ANOVA model consisting of factors subject and within-subject factor stage of the treatment was employed. The differences between C) serum total IgA concentration and D) serum Gd-IgA1 levels between IgAN patients and untreated disease controls (CU), prednisone-treated controls (CT), or healthy control (CH) were assessed by the one-way ANOVA model with the factor group, followed by the least significant difference multiple comparisons. The squares with error bars represent means with their 95% confidence intervals (least significant difference multiple comparisons). * = $P < 0.05$.

Fig. 3. Prednisone therapy reduces serum IgG-IgA CIC level in IgAN patients. Changes in relative IgG-IgA CIC levels in IgAN patients (A) in before (N0), at 7 days (N7), and at 30 days (N30) after steroid therapy initiation were compared using a repeated measures ANOVA model with factors subject and within-subject factor stage of the treatment. B) Differences between relative IgG-IgA CIC levels in IgAN patients and healthy controls (CH), untreated disease controls (CU), or prednisolone treated controls (CT) were assessed with the use of a one-way ANOVA with the factor group, followed by the least significant difference multiple comparisons. The squares with error bars represent means with their 95% confidence intervals (least significant difference multiple comparisons). * = $P < 0.05$.

Fig. 4. Hypothesis about how corticosteroids could affect the course of IgAN. Glucocorticoids suppress immune response and are accepted as inhibitors of immunoglobulin production. In our study glucocorticoids reduced the serum concentration of the total IgA for about 26%. Because IgA1 subclass represents approximately 85% of the total IgA in serum, we can conclude that reduction of IgA could be attributed mainly to the reduction of IgA1. Further glucocorticoids also reduced serum Gd-IgA1 level. This was not

associated with changes in IgA sialylation, indicating that the basis of the glucocorticoids-induced lowering of serum Gd-IgA1 is not linked with the suppression of premature sialylation in Gd-IgA1-producing cells [4]. Based on this observation we hypothesize that glucocorticoids affect O-glycosylation of IgA1 at distinct steps of the O-glycosylation pathway [20, 21]. The effect of glucocorticoids on production of Gd-IgA1-specific IgG autoantibody was not tested in this study.

Discussion

In our group of IgAN patients, one month of prednisone therapy significantly reduced the levels of total IgA and Gd-IgA1, IgAN-specific serum biomarkers [20, 28]. Similar trend was reported recently by Kim et al. [24]. Nevertheless, in our study we compared both parameters with those in three control groups untreated disease controls, prednisone-treated controls, and healthy controls. Neither parameter reached the values detected in control groups. This suggests that the prednisone, at least at doses recommended by KDIGO (Kidney Disease: Improving Global Outcomes) [23], does not completely normalize the Gd-IgA1 production. Subsequent analysis of IgA sialylation confirmed moderate, non significant reduction of mean IgA sialylation, suggesting that the prednisone-caused reduction of serum Gd-IgA1 level is not substantially influenced through the reduction of IgA sialylation [4].

The initial level of total serum IgA was 3.7 mg/mL, which is similar to values reported by Ishiguro et al., and Moldoveanu et al., [14, 29] but higher than values reported by Tomino et al., and subsequently by Berthoux et al. who reported lowest mean serum IgA level 2.88 mg/mL in IgAN population [30, 31]. Nevertheless, when only IgAN patients with high blood pressure, high proteinuria, and severe light-microscopy pathologic lesions were included, the mean IgA level reached 3.2 - 3.3 mg/mL. Recently Kim et al. reported mean serum IgA level in the cohort of 36 IgAN patients to be 4.1 mg/mL [24]. Our group of patients treated with prednisolone was selected based on severity of clinical and laboratory findings - persisting hematuria and proteinuria >1 g/24 h, decreased eGFR, and positive MEST, especially proliferative and necrotizing changes in glomeruli. According to reports of others [31] such a group could exhibit higher serum IgA level than the mean values measured for cohort of all IgAN patients.

The levels of total serum IgG-IgA CIC decreased significantly after one month of prednisone therapy. The comparison with controls could not be statistically confirmed due to the high variability (Fig. 3B). Apart from CIC levels, the nephritogenic activity of CIC depends

also on molecular composition and molecular masses previously confirmed *in vitro* by MC proliferation response [25-27], which was not significantly reduced by prednisone therapy of our IgAN patients. Thus, we could not unambiguously conclude the corticosteroids' effect on overall nephritogenic activity of CIC.

After 180 days of continuous prednisone treatment, the significant reduction of proteinuria was observed in this study in agreement with a retrospective analysis of the European Validation Study of the Oxford Classification of IgA nephropathy (VALIGA) [32]. In our IgAN patients' group, the KDIGO guideline, defining the prednisone dosage (60 mg/m² every other day tapered to 30 mg/m² at 12 months) were followed [23], but significant changes were not detected in the eGFR at any time period (Table 1) although the mean values temporarily and moderately decreased. The KDIGO study reported reduction in proteinuria during prednisone therapy, but after 2 years of evaluation no difference in kidney function was observed [33]. It is questionable whether the increase in the prednisone dose could completely normalize the Gd-IgA1 level and whether such doses will be effective. Furthermore, it remains to be determined whether Gd-IgA1 level normalization could prevent the IgAN progression. Increasing the doses of corticosteroid may not be a realistic goal because Gd-IgA1 production is also affected by concurrent infections, which could alter the levels of cytokines [4] and thus the steady state of IgA production in general and Gd-IgA1 in particular [20, 21].

Finally, since we could detect the reduction of serum IgA and Gd-IgA1 levels early after prednisone therapy initiation (which contrasted with delayed reduction of proteinuria, requiring six months), we attempted to statistically compare the Gd-IgA1 or IgA reduction with the development of changes in proteinuria in individual IgAN patients at respective time points. No correlation between IgA or Gd-IgA1 and proteinuria levels development was identified.

All 14 IgAN patients enrolled in our study represent the majority of all prednisone-treated IgAN patients in the two of totally seven University hospitals in the Czech Republic. This number, although low, represents the population amenable to statistic evaluation to provide statistically significant results. Although several large studies characterizing involvement of IgA or Gd-IgA1 in the IgAN tested hundreds of subjects per group [14, 30], many others studies formulated their conclusions from only twice a higher number of subjects [24] or even smaller cohorts of IgAN patients and controls than in this report [34-37]. We are aware of limited potential for generalization of conclusions made from our study cohort but it needs to be highlighted that not only the size of the cohort but also the racial and regional factors could influence the clinical value of measured parameters. For example, tonsilectomy performed in cohort of 200 Japanese IgAN patients [38] showed beneficial effect on IgAN course but this was not proven in European cohort of 61 tonsillecctomized IgAN subjects selected from large cohort of 1, 147 VALIGA subjects [39].

Conclusion

We confirmed the reduction of proteinuria after the initiation of prednisone therapy, and we demonstrated that the levels of total as well as Gd-IgA1 were significantly reduced in IgAN patients. Thus, the prednisone therapy seems to be effective in the treatment of IgAN patients due to its potential involvement in several proposed "hits" (Fig. 4). that occur in the etiopathogenesis of IgAN [20, 21, 40]. The decrease of levels of serum Gd-IgA1 observed in this study is of a particular importance.

Disclosure Statement

All the authors declared no competing interests. The results presented in this paper have not been published previously in whole or part, except in abstract format.

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