Oxidative Stress and Galactose-Deficient IgA1 as Markers of Progression in IgA Nephropathy

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Summary

Background and objectives We assessed the activation of the oxidative stress pathway in patients with IgA nephropathy (IgAN), while evaluating the classic marker of the disease (galactose-deficient serum IgA1).

Design, setting, participants, & measurements Sera from 292 patients and 69 healthy controls from Italy and the United States were assayed for advanced oxidation protein products (AOPPs), free sulfhydryl groups on albumin (SH-Alb), and IgA1 with galactose-deficient hinge-region O-glycans (Gd-IgA1). Gd-IgA1 was detected by binding to Helix aspersa agglutinin (HAA) and expressed as total Gd-IgA1 or as degree of galactose deficiency relative to a standard Gd-IgA1 myeloma protein (%HAA).

Results Sera from IgAN patients showed higher levels of Gd-IgA1, %HAA, and AOPPs, but lower levels of SH-Alb in comparison to that from healthy controls. Serum levels of AOPPs significantly correlated with serum Gd-IgA1 and %HAA. The relationship between these biomarkers and clinical features at sampling and during follow-up was assessed in 62 patients with long-term follow-up. AOPPs and %HAA correlated with proteinuria at sampling and independently associated with subsequent proteinuria. Levels of AOPPs correlated with rate of decline in renal function after sampling. The combination of a high level of AOPPs and a high level of %HAA associated with decline in estimated GFR.

Conclusions Serum levels of aberrantly glycosylated IgA1 are elevated and oxidative stress pathways are activated in patients with IgAN; the intensity of the stress correlated with expression and progression of the disease. We speculate that oxidative stress may modulate the nephrotoxicity of aberrantly glycosylated IgA1 in IgAN.

Introduction

IgA nephropathy (IgAN) is characterized by a dysregulation of the immune system, with aberrantly glycosylated polymeric IgA1 in the circulation and mesangial deposits. This aberrant IgA1 has galactose-deficient O-glycans (1,2). Normally, a N-acetylgalactosamine (GalNAc) residue is attached to a serine or threonine in the IgA1 hinge region of the heavy chain and the structure is extended by the addition of galactose (Gal) to form a Galβ1,3GalNAc disaccharide. In patients with IgAN, activities of key enzymes involved in this process are altered, leading to production of an increased number of carbohydrate side chains that lack Gal.

IgA1 isolated from sera of patients with IgAN contains elevated levels of Gal-deficient IgA1 (Gd-IgA1) within immune complexes. These complexes stimulate cultured human mesangial cells (3–5), resulting in activation of oxidative stress pathways (6–8). Evidence of increased oxidative stress has been noted in renal tissue of patients with IgAN (9) as well as in their sera and/or erythrocytes (i.e., increased levels of lipoperoxide or malondialdehyde and reduced activity of superoxide dismutase, catalase, and glutathione peroxidase) (10,11). Moreover, increased serum levels of another marker of oxidative stress, advanced oxidation protein products (AOPPs), have been recently associated with proteinuria and disease progression in patients with IgAN (12). Although the serum level of Gd-IgA1 is considered a disease marker for IgAN, its unique role in inducing renal pathology or disease progression has been questioned. Notably, levels of Gd-IgA1 are commonly elevated in healthy first-degree relatives without clinical evidence of renal injury (13). In patients with IgAN, the specific degree of Gal deficiency of O-glycans on serum IgA1 remains constant over long intervals (2). Serum levels of Gd-IgA1 are increased in patients with severe IgAN who progressed to end-stage renal failure and renal transplantation. Nonetheless, because the levels overlap between patients grouped by recurrence of IgAN in the grafted kidney, altered IgA1 O-glycosylation alone is
insufficient to trigger disease development and progression (14). Indeed, 4% to 16% of apparently healthy individuals in the general population have IgA deposits in their glomeruli (15,16), although it is unknown whether these deposits consist of Gd-IgA1. Therefore, we have postulated that the pathogenesis of IgAN requires multiple hits in the setting of an increased serum level of Gd-IgA1 to become fully manifest (17). The aim of this study was to investigate the potential of circulating Gd-IgA1 and measures of oxidative stress as risk markers for development and progression of IgAN.

We measured serum Gd-IgA1 and AOPPs and free sulfhydryl groups on serum albumin (SH-Alb) in large cohorts of patients with IgAN from Italy and the United States. In a representative subgroup, we assessed the correlations of these parameters with proteinuria and renal clearance function at sampling as well as with renal function decline and proteinuria during the subsequent follow-up. We found that although both an increased proportion of Gd-IgA1 in the total serum IgA1 and markers of oxidative stress were associated with clinical activity of IgAN, the stronger risk marker of progression was the serum level of AOPPs.

**Materials and Methods**

**Patients**

We assayed serum samples from 292 patients with IgAN followed by centers in Turin, Italy, and New York. Patients with Henoch–Schönlein nephritis, systemic lupus erythematosus, celiac disease, chronic liver disease, or diabetes were excluded. Seventy-nine patients were Italian, all of Caucasian ethnicity, and 213 patients were from the United States (83% Caucasians, 7% African Americans, and 10% Asians). Healthy controls were selected from healthy blood donors of the same areas from which patients were recruited (northern Italy and Atlantic coast of the United States); sera from 69 healthy individuals from Italy and from the United States, matched for ethnicity and sex ratios of the patients, were used. All serum samples were stored at −20°C before analyses.

For 230 patients, clinical data were incomplete; therefore, we did not include them in further statistical analyses.

For 62 patients, complete clinical data were available, including demographics, past medical history, serum creatinine, proteinuria at the time of study, and serial clinical assessments (serum creatinine and proteinuria at least three times over an interval longer than 3 years; Table 1) over a mean follow-up of 4.46 (range 3 to 10) years. Estimated GFR (eGFR) was calculated using the four-variable Modification of Diet in Renal Disease formula for adults (18) and the Schwartz formula for children younger than 18 years of age (19). Rate of decline in renal clearance function was determined by fitting a straight line through available data for eGFR using the principle of least squares for each patient (Table 1).

Proteinuria was measured with a 24-hour collection; in children, results were corrected for body surface area, to homogenize units of measurement for patients of various ages (20). An average proteinuria (21) was determined for each 1-year block during follow-up; the average of all 1-year measurements was defined as time-average proteinuria (TA-proteinuria).

**Determination of Serum IgA and Gd-IgA1 by ELISA**

Serum total IgA and Gd-IgA1 were measured as described previously (22). Briefly, ELISA plates were coated...
with an F(ab′)2 fragment of goat IgG anti-human IgA (Jackson ImmunoResearch, Inc., West Grove, PA), 1 μg/ml. Bound IgA was detected with a biotin-labeled F(ab′)2 fragment of goat IgG anti-human IgA (Biosource, Camarillo, CA). Binding was measured after the addition of avidin-horseradish peroxidase conjugate (ExtrAvidin; Sigma-Aldrich, St. Louis, MO); the reaction was developed with the peroxidase chromogenic substrate o-phenylenediamine (OPD)-H2O2 (Sigma-Aldrich), and absorbance at 490 nm was measured (EL312 Bio-Kinetics microplate reader, BioTek Instruments Inc., Winooski, VT). The standard for IgA consisted of a pool of normal human sera (Binding Site, Birmingham, UK) previously calibrated for Ig isotype concentrations, including that of IgA.

To measure serum total Gd-IgA1, 96-well plates (MaxiSorp; Nunc, Thermo Fisher Scientific, Rochester, NY) were coated with the F(ab′)2 fragment of goat anti-human IgA (Jackson ImmunoResearch), 3 μg/ml. Plates were blocked with 2% BSA (Sigma-Aldrich) in PBS with 0.05% Tween 20. Diluted samples were added and incubated overnight at 4°C. Captured IgA was subsequently desialylated by treatment for 3 hours at 37°C with 10 mU/ml neuraminidase from *Vibrio cholerae* (Roche, Indianapolis, IN) in 10 mM sodium acetate buffer, pH 5. Samples were incubated for 3

Figure 1. | Serum levels of galactose-deficient IgA1 and markers of oxidative stress. Serum levels of Gal-deficient IgA1, as total amount ([A] Gd-IgA1) or as a relative degree of galactose deficiency (percentage of standard Gal-deficient IgA1 myeloma protein) ([B] %HAA); serum levels of markers of oxidative stress, i.e., advanced oxidation protein products ([C] AOPPs) and albumin containing free SH groups ([D] SH-Alb) in 69 healthy controls and in 292 patients with IgAN. P = significance of the difference between patients and healthy controls. AOPPs, advanced oxidation protein products; Gd-IgA1, galactose-deficient IgA1; %HAA, % Helix aspersa agglutinin; SH-Alb, free sulfhydryl groups on albumin.
hours at 37°C with GalNAc-specific biotinylated HAA lectin (Sigma-Aldrich) diluted 1:500. Bound lectin was detected with avidin-horseradish peroxidase conjugate. Naturally Gal-deficient IgA1 myeloma protein purified from plasma of a patient with IgA1 (Ale) multiple myeloma was used as the standard for Gd-IgA1. (This standard differs from the previously published standard; see references 13,23.) Results were expressed as relative Gal deficiency of IgA1 (percentage of standard Gd-IgA1 myeloma protein; %HAA) or as total amount of Gd-IgA1 (U/ml).

Measurement of AOPPs

Serum AOPPs levels were measured (24) by spectrophotometry on a microplate reader (MR 5000; Dynatech, Paris) calibrated with chloramine-T solutions (Sigma-Aldrich) which, in the presence of potassium iodide, absorb light at 340 nm. In test wells, 200 μl of serum diluted 1:5 in PBS was placed on a microtiter plate (Becton Dickinson Labware, Lincoln Park, NJ) and 20 μl of acetic acid was added. In standard wells, 20 μl of acetic acid was added to 200 μl of chloramine-T solution (0 to 500 μmol/L). Ten microliters of 1.16 mol/L potassium iodide (Sigma-Aldrich) followed by 20 μl of acetic acid were added immediately before reading. Absorbance of the reaction mixture was read at 340 nm on the microplate reader against a blank containing 200 μl of PBS, 10 μl of potassium iodide, and 20 μl of acetic acid. Because chloramine-T absorbance at 340 nm was linear within the range of 0 to 100 μmol/L, AOPP concentrations were expressed as μmol/L of chloramine-T equivalents.

Measurement of Albumin-Free SH Groups

Any decrease in titratable free SH for a given protein is an indirect marker of oxidative stress (25). Synthesis of SH-specific cyanines, their validation, and methods for their application have been detailed (26). Samples from patients and controls were normalized for protein content (CBB silver colloidal) and solubilized in 8 M urea, 4% wt/vol CHAPS, 10 mM Tris-HCl, pH 8.8, 1 mM EDTA, and 5 mM TBP. Labeling with iodoacetamide cyanines C3NIASO3 and C5NIASO3 dyes was done in the same solution, at concentrations of each cyanine varying from 10 to 400 pmol of dye per mg of proteins, for 1 hour, and the Cy-SH reaction was quenched with 65 mM DTE for 1 hour at room temperature in a dark box and gentle shaker. Specificity of the reaction for SH residues was assessed by competition with 150 mM unlabeled iodoacetamide. Determination of SH-labeled proteins was done with SDS-

Table 2. Proteinuria at sampling and time-average proteinuria calculated over follow-up subsequent to sampling in patients with abnormal circulating levels of AOPPs, SH-Alb, %HAA, or Gd-IgA1

<table>
<thead>
<tr>
<th>Proteinuria at sampling (g/d)</th>
<th>P</th>
<th>Time-average proteinuria (g/d)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>AOPP abnormal (30 patients)</td>
<td>1.2 (0.8 to 1.8)</td>
<td>0.01</td>
<td>1.1 (0.7 to 1.8)</td>
</tr>
<tr>
<td>normal (32 patients)</td>
<td>0.4 (0.1 to 1.4)</td>
<td>0.02</td>
<td>0.3 (0.1 to 0.8)</td>
</tr>
<tr>
<td>SH-Alb abnormal (25 patients)</td>
<td>1.2 (0.5 to 2.7)</td>
<td>0.02</td>
<td>1.1 (0.3 to 1.9)</td>
</tr>
<tr>
<td>normal (37 patients)</td>
<td>0.8 (0.3 to 1.4)</td>
<td>0.02</td>
<td>0.6 (0.2 to 1.3)</td>
</tr>
<tr>
<td>%HAA abnormal (25 patients)</td>
<td>1.3 (0.5 to 2.4)</td>
<td>0.02</td>
<td>1.0 (0.3 to 2.4)</td>
</tr>
<tr>
<td>normal (37 patients)</td>
<td>0.7 (0.3 to 1.2)</td>
<td>0.86</td>
<td>0.6 (0.2 to 1.4)</td>
</tr>
<tr>
<td>Gd-IgA1 abnormal (42 patients)</td>
<td>1.1 (0.3 to 2.0)</td>
<td>0.6 (0.3 to 1.3)</td>
<td>0.6 (0.3 to 1.2)</td>
</tr>
</tbody>
</table>

Abnormal values were those exceeding the 90th percentile for healthy controls for AOPPs, %HAA, and Gd-IgA1, and those lower than 10th percentile for healthy controls for SH-Alb. AOPPs, advanced oxidation protein products; Gd-IgA1, galactose-deficient IgA1; %HAA, % Helix aspersa agglutinin; SH-Alb, free sulphhydryl groups on albumin.
PAGE in 8 to 16 T% polyacrylamide gels (27). Results were expressed as arbitrary units (AU) corresponding to the fluorescence intensity at Ex532/Em555 corrected for the concentration of albumin.

Statistical Analyses

For each test, values within the 90th percentile for healthy controls were considered normal.

The t test and Mann–Whitney test were used to compare normally and not normally distributed variables, respectively; similarly, Pearson and Spearman tests were used to correlate variables.

Rate of decline in renal function for each patient was determined by fitting a straight line through the eGFR data points according to least squares and used in linear regression models. To analyze data using receiver operating characteristic (ROC) curves, eGFR slope was dichotomized in two groups: positive when ≥0 ml/min per 1.73 m²/yr or negative when <0 ml/min per 1.73 m²/yr.

The relationships between biomarkers and TA-proteinuria (dichotomized in 2 groups using 1 g/d as the cutoff point) were tested using ROC curves. Multiple linear regressions were used to determine whether biomarkers were independent of each other.

On the basis of AOPPs and %HAA, we divided patients into three categories, those with (1) normal AOPPs and normal %HAA, (2) abnormal AOPPs or %HAA, or (3) abnormal AOPPS and %HAA. Relationships between these categories and eGFR slope were assessed using the trend test. The same method was applied for AOPPs and GdIgA1 or Sh-Alb.

All P values were two-tailed and those <0.05 were considered significant. Confidence intervals (CI) were calculated at the 95% level. Analyses were performed using SPSS software (version 16, SPSS Inc., Chicago, IL).

Results

Circulating Levels of Gd-IgA1 and Markers of Oxidative Stress

Sera from the 292 patients with IgAN contained, in comparison with those from healthy controls, higher levels of total IgA (3.62 ± 1.86 versus 1.79 ± 0.93 mg/ml, P < 0.0001) and Gd-IgA1, both as total levels (Gd-IgA1: 183.4 ± 156.4 versus 56.1 ± 33.8 U/ml, P < 0.0001) and as a relative degree of IgA1 Gal deficiency (%HAA: 49.6% ± 15.8% versus 32.5% ± 11.8%, P < 0.0001) (Figure 1, A and B).

IgAN patients had higher serum AOPP levels (103.9 ± 49.10 versus 47.8 ± 37.5 μmol/L, P < 0.0001) and lower amounts of SH-Alb (3.9 ± 2.6 versus 7.7 ± 3.5 AU, P < 0.0001) when compared with healthy controls (Figure 1, C and D).

Figure 3. | Correlation between loss of eGFR and AOPPs. Correlation between eGFR slope (ml/min per 1.73 m²/yr) calculated over the follow-up after sampling and serum levels of AOPPs. Sera positive for %HAA (A) or Gd-IgA1 (B) are indicated with closed circles; sera negative for %HAA (A) or Gd-IgA1 (B) are indicated with open circles. AOPPs, advanced oxidation protein products; eGFR, estimated GFR; Gd-IgA1, galactose-deficient IgA1; %HAA, % Helix aspersa agglutinin.
Values for Gd-IgA1 and %HAA correlated ($r^2 = 0.32$, $P < 0.0001$). Levels of total IgA correlated with levels of Gd-IgA1 ($r^2 = 0.67$, $P < 0.001$). Levels of AOPPs correlated with those of %HAA ($r = 0.24$, $P = 0.01$), which are independent of total IgA. Levels of AOPPs and SH-Alb did not correlate.

Correlation with Clinical Data

The subgroup of 62 patients with complete clinical data had, as did the whole cohort of 292 cases, levels of Gd-IgA1, %HAA, and AOPPs higher than those of the healthy controls (44.8 ± 79.3 versus 56.1 ± 33.8 U/ml, $P < 0.0001$; 49.4% ± 14.4% versus 32.5% ± 11.8%, $P < 0.0001$; and 111.1 ± 45.2 versus 47.8 ± 37.5 μmol/L, $P < 0.0001$, respectively) and lower levels of SH-Alb (4.7 ± 2.3 versus 7.7 ± 3.5 AU, $P < 0.0001$). No correlation was detected between markers of oxidative stress or serum Gd-IgA1 level and eGFR, gender, or age at time of sampling. AOPPs and %HAA correlated with proteinuria at sampling ($r = 0.58$, $P = 0.003$ and $r = 0.25$, $P = 0.03$, respectively; Figure 2, A and B), whereas an inverse relationship with SH-Alb did not reach statistical significance.

Patients with abnormal levels of AOPPs, %HAA, and SH-Alb had greater proteinuria at sampling and TA-proteinuria over follow-up than did patients with normal levels of these biomarkers (Table 2).

Levels of AOPPs correlated with rate of decline in renal function ($r = -0.33$, $P = 0.008$; Figure 3), whereas no such correlation was observed with levels of SH-Alb, total Gd-IgA1, or %HAA. In the ROC analysis (Figure 4), the best cutoff value for AOPPs was 100.7 μmol/L, yielding a sensitivity of 76% and specificity of 73% in predicting eGFR slope.

The association of increased levels of AOPPs and high %HAA was a risk marker of loss of eGFR (trend test $P = 0.01$; Figure 5); also the association of high values of AOPPs and decreased levels of SH-Alb was a risk marker of loss of eGFR (trend test $P = 0.002$).

TA-proteinuria correlated with eGFR slope ($r = -0.348$, $P < 0.05$) and also with AOPPs ($r = 0.49$, $P < 0.0001$; Figure 6A) and %HAA ($r = 0.29$, $P = 0.007$; Figure 6B), but not with SH-Alb. At multivariate analysis, serum levels of AOPPs and %HAA were associated with TA-proteinuria over the follow-up interval after sampling, independently of each other ($B = 0.09$, $P < 0.0001$ and $B = 0.016$, $P = 0.01$, respectively). Because TA-proteinuria >1 g/d is a significant risk factor for progression (21), we used this value to dichotomize TA-proteinuria for ROC analysis (Figure 7); the best cutoff value was 121.5 μmol/L for AOPPs (sensitivity 70% and specificity 79%) and 48% for %HAA (sensitivity 67% and specificity 66%). Patients with AOPPs >121.5 μmol/L had higher TA-proteinuria compared with those with AOPPs <121.5 μmol/L (1.41 ± 0.74 versus 0.69 ± 0.82, $P = 0.001$).

Discussion

This study investigated, for the first time, the relative contribution of circulating Gd-IgA1 and a pro-oxidant milieu on the expression of disease activity and progression in patients with IgAN. The major conclusion is that although both biomarkers are associated with IgAN and with clinical activity of disease, systemic oxidative stress, as denoted by circulating AOPPs, was the stronger risk marker of disease progression. Notably, progression was more likely when the %HAA was also high.

AOPPs are a family of oxidized, di-tyrosine-containing protein compounds that are generated by monocyte acti-
vation, myeloperoxidase release, and contact with reactive oxygen species (28). In addition to being markers of oxidative protein damage, AOPPs play an important role as effector molecules, by activating monocytes and triggering respiratory burst and inflammation (29), inducing vascular endothelial dysfunction and accelerating atherosclerosis by activating endothelial cells (30). AOPPs also act on resident renal cells to increase synthesis of TGF-β1 and matrix components that may lead to renal fibrosis (31). AOPPs induce podocyte depletion and proteinuria when injected into experimental animals and when added in very low concentrations to podocyte cultures (32). The cascade of intracellular signaling events elicited by AOPPs leads to nuclear translocation of nuclear factor kappa B (NF-κB) and synthesis of pro-inflammatory and prosclerotic mediators in podocytes. The series of events finally induces p53-dependent apoptosis that likely plays a critical role in promoting proteinuria and accelerating glomerulosclerosis (32,33). It is difficult to state whether the association we found between signs of oxidative stress and proteinuria in patients with IgAN is causative (due to an effect of AOPPs on podocytes leading to development of proteinuria) or simply indicates a phase of clinical activity and potential progression of renal disease. The observational nature of this study precludes determination of a causal relationship between higher serum levels of AOPPs and progressive renal injury.

In spite of the important pathologic implication of AOPPs, we still lack a clear structural characterization of these molecules. Albumin is likely a major target of oxidative stress (25,28,34,35), as its thiol groups are almost always targeted in oxidation processes. Hence, SH-Alb gives a quantification of available SH-buffering groups. In patients with IgAN, we detected high levels of AOPPs and low amounts of SH-Alb; each finding correlated with severity of proteinuria. These associations were observed also in patients with preserved renal function, thereby suggesting oxidative stress early in the clinical course of IgAN. The presence of enhanced oxidative stress may not be unique to patients with IgAN, and additional studies including such patients are necessary to examine this point. However, the aim of our study was to look for interplay between aberrantly glycosylated IgA and pro-oxidative milieu, rather than demonstrating specificity of these findings with respect to patients with other glomerular diseases.

We confirmed the presence of an increased level of Gd-IgA1 in the circulation of the patients with IgAN and...
examined this biomarker as a risk marker of progression. Some weak correlations were indeed found with proteinuria, albeit at levels of significance lower than those for the markers of oxidative stress. When we considered the prognostic value of both series of biomarkers, only the serum level of AOPPs strongly correlated with decline in renal function. However, the combination of a high AOPP level with a high %HAA was a strong risk marker of progression. Aberrantly glycosylated IgA1 can activate transcription factor NF-kB in mononuclear cells (7). We recently reported increased NF-kB nuclear translocation in peripheral blood mononuclear cells of patients with IgAN, particularly during phases of clinical activity (36). AOPPs can activate NF-kB also in mesangial cells (31). NF-kB activation triggers transcription of inducible nitric oxide synthase (iNOS), a powerful mediator of oxidative stress (37). Circulating mononuclear cells as well as resident renal cells are also targets of angiotensin II and TGF-β1, both capable of triggering generation of intracellular superoxide and initiating the oxidative stress pathway (38). Increased serum levels of markers of oxidative stress may derive from circulating cells as well as resident renal cells, sharing receptors and activating common pathways of inflammatory response (39).

In summary, levels of Gd-IgA1 and signs of oxidative stress are increased in the circulation of patients with IgAN in comparison with healthy controls. Progression to reduced renal function was predicted by sustained proteinuria during follow-up, but not by proteinuria at sampling, likely because half the cases had stable or even improved renal function during the follow-up interval in spite of initially high proteinuria. An increased serum level of AOPPs was the only biomarker that strongly associated with renal function decline and proteinuria during follow-up. Combining a high serum level of aberrantly glycosylated IgA1 with a high serum level of AOPPs strengthened the association. These data suggest that the nephrotoxicity of Gd-IgA1 in patients with IgAN may be enhanced in the presence of systemic oxidative stress and support the hypothesis that the intensity of the oxidative stress alters expression and progression of this common renal disease.

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Disclosures
None.

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