Glycosylation of Circulating IgA in Patients with IgA Nephropathy Modulates Proliferation and Apoptosis of Mesangial Cells

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Abstract. Abnormalities in circulating IgA1 have been demonstrated in patients with IgA nephropathy (IgAN). This study addresses the question of the functional significance of this alteration in creating mesangial injury. Biologic effects of selected IgA glycoforms isolated from serum of IgAN patients and controls and *in vitro* deglycosylated normal IgA were tested on cultured human mesangial cells (MC). IgA glycoforms, ranging from 250 to 500 kD molecular weight, were isolated by lectin affinity chromatography followed by HPLC. IgA and IgG content was measured by enzyme-linked immunosorbent assay. HPLC fractions were incubated with MC to evaluate proliferation and apoptosis rates and nitric oxide synthesis. Moreover, MC were conditioned with *in vitro* desialylated and degalactosylated normal IgA. Patients with IgAN displayed increased levels of IgA glycoforms exposing sialic acid in α2,6 linkage with N-acetylgalactosamine (Neu5Acα2,6GalNAc) ($P < 0.02$) and GalNAc ($P < 0.05$), indicating truncation of O-linked glycans of IgA1. Moreover, IgA glycoforms with increased exposure of mannose were observed ($P < 0.03$), suggesting a defective N-linked glycosylation. No modification in IgG glycosylation was detected. When incubated with MC, the IgA glycoforms isolated from patients with increased exposure of GalNAc, Neu5Acα2,6GalNAc, or mannose, significantly depressed the proliferation and increased the apoptotic rate and nitric oxide synthesis activity of cultured MC, in comparison with fractions isolated from controls. Similarly, *in vitro* desialylated and degalactosylated IgAs significantly depressed the proliferation and enhanced the apoptosis rates of MC. In conclusion, a significant modulation of several human MC functions exerted by serum IgA with increased exposure of GalNAc, Neu5Acα2,6GalNAc, and mannose residues isolated from IgAN patients is reported for the first time.

IgA nephropathy (IgAN) is characterized by mesangial deposits of IgA (1). The pathogenetic factors that lead to IgA deposition, glomerular inflammation, and progression toward sclerosis remain to be elucidated fully (2,3).

Recent attention has been focused on the character of IgA produced by patients with IgAN. Human IgA is highly glycosylated (4). IgA1 subclass bears 5 O-linked glycans that are linked to serine or threonine residues between the CH1 and CH2 domains in the hinge region of each heavy chain (Figure 1) (5). N-acetylgalactosamine (GalNAc) is linked with the amino acid residues, and galactose (Gal) is linked in β1,3 to GalNAc. Sialic acid (Neu5Ac) has an α2,6 linkage with GalNAc and an α2,3 linkage with Gal residues. Bi-branched glycan residues of variable structure, mostly rich in mannose (Man) and N-acetylglucosamine (GlcNac) and N-linked to asparagine residues, are represented in both IgA1 and IgA2 subclasses in the CH3 domain. IgA1 has Cα2 N-glycosylation sites that contain biantennary carbohydrates. The tailpiece site contains mostly triantennary glycans.

Several aberrant patterns of glycans have been detected in circulating IgA1 from patients with IgAN, including increased exposure of GalNAc residues and decreased content of Gal (6–10), all consistent with a truncation of O-glycan chains (3,11). We previously demonstrated that IgA from patients with IgAN has increased binding to environmental lectins (pokeweed, soybean, and gliadin) (12,13) and to mesangial matrix glycoproteins (fibronectin, laminin, type IV collagen) (14) as a result of carbohydrate interactions. Moreover, these properties were most evident in sera of transplanted patients with recurrent IgAN (15). We demonstrated also that a carbohydrate interaction regulates the reactivity of IgA and lysosomal enzymes (antineutrophil cytoplasmic antibody antigens) in IgAN related to Henoch-Schoenlein syndrome (16). Incomplete glycation of the hinge region can result from a deficiency of β1,3 galactosyltransferase due to genetic factors (17). An altered balance between Th1 and Th2 lymphocytes has been proposed in mouse models (18), but the relevance to human IgAN should be proved, because mouse IgA has N-glycosylated residues but lacks O-linked ones.

Interest has been focused recently on the potential pathogenetic role of aberrantly glycosylated IgA molecules in the
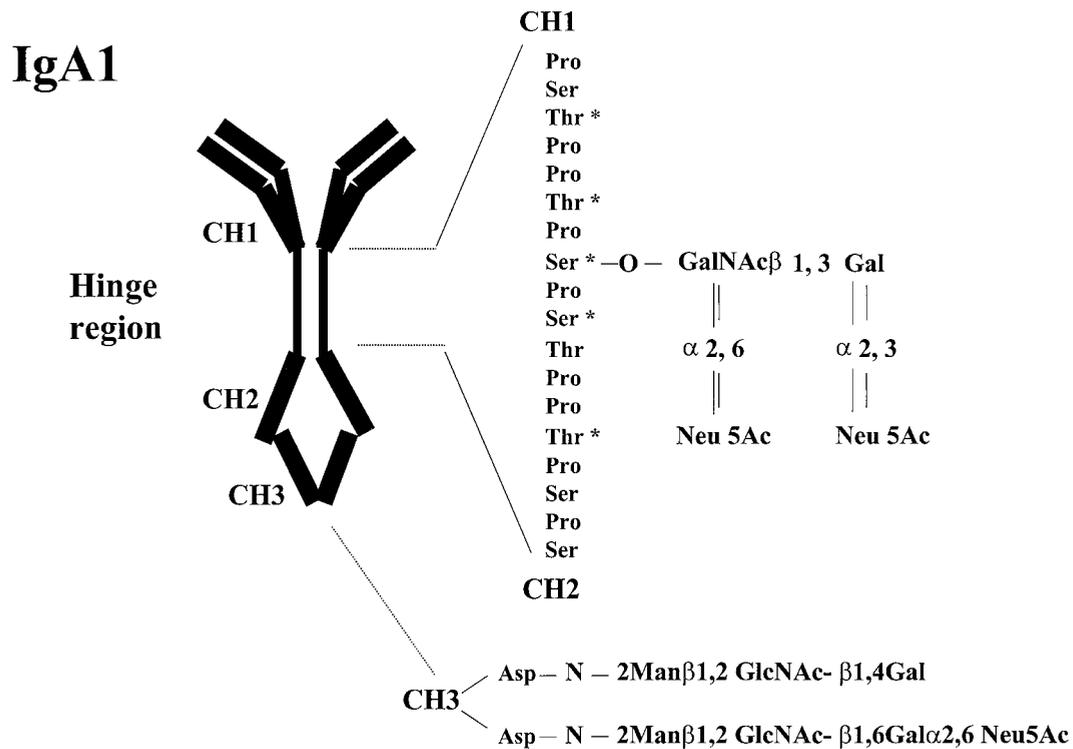
Figure 1. IgA1 glycosylation sites. IgA1 subclass bears five O-linked glycans linked to serine or threonine residues between the CH1 and CH2 domains in the hinge region of each heavy chain. N-acetylgalactosamine (GalNAc) is linked with the amino acid residues, and galactose (Gal) is linked in β1,3 to GalNAc. Sialic acid (Neu5Ac) has an α2,6 linkage with GalNAc and an α2,3 linkage with Gal residues. N-linked to asparagine residues bi-branched glycans, of variable structure, rich in mannose (Man), and N-acetylglucosamine (GlcNAc) are represented in both IgA1 and IgA2 subclasses in the CH3 domain. IgA1 has Cα2 N-glycosylation sites containing biantennary carbohydrates (5).

development of IgAN. An altered glycosylation of serum IgA could favor the self-aggregation (19) and/or the increased binding to circulating glycoproteins, including fibronectin (20) or environmental alimentary, bacterial, or viral lectins (21) as a result of carbohydrate interactions. Macromolecules also can form by the reaction between degalactosylated (deGal) IgA1 and specific IgG antibodies directed against the IgA1 hinge O-glycans (10). These IgA immune or nonimmune aggregates escape the clearance by hepatic receptors for asialoglycoproteins (22). Moreover, the targeting to mesangial area may be favored by carbohydrate interactions between aberrantly glycosylated IgA and mesangial matrix (14,19), leading to accumulation and/or prolonged persistence of IgA deposits within the mesangium.

The contact between MC and deposited IgA, mediated by a specific IgA receptor (23), leads to activation of intracellular pathways only partially known (24). Desialylated (deSia) IgA binds to rat MC with release of proinflammatory cytokines and superoxide generation (25). We recently demonstrated that *in vitro* prepared aberrantly glycosylated IgA can modulate human mesangial cell integrin expression (26) and vascular endothelial growth factors synthesis (27). Altered glycosylation of IgA also may promote complement activation (28), which plays a pivotal role in glomerular inflammation.

No investigation has attempted the analysis of the reactivity of circulating aberrantly glycosylated IgA molecules isolated

from patients with IgAN. Therefore, we aimed in this study to fractionate serum IgA in these patients on the basis of IgA glycosylation patterns then investigate the reactivity of cultured human MC to selected IgA glycoforms in respect to the proliferation and apoptosis rate and the nitric oxide (NO) synthesis. The results were compared with those obtained by testing glycoforms prepared *in vitro* by enzymatic treatments of normal IgA (deSia and deGal IgA).

We found increased exposure of GalNAc, Neu5Acα2,6 GalNAc, and Man residues in circulating macromolecular IgA of patients with IgAN, confirming the defective glycation of O-linked carbohydrate chains of IgA1 but suggesting a coexistence of IgA N-linked carbohydrate truncation. The aberrantly glycosylated IgA isolated from patients with IgAN modulate several important MC functions, which could play a role in development and progression to sclerosis of this nephropathy.

Materials and Methods
Study Design
Human MC were cultured in the presence of serum fractions isolated from patients with IgAN or healthy control subjects that contained IgA with different glycosylation patterns and IgA from healthy control subjects that were treated *in vitro* with glycosidases. Results on MC proliferation and apoptosis rates and NO synthesis modulation in cells that were cultured with selected stimuli were compared with those of cells that were maintained in standard culture

medium. Proliferation rate was done with different concentrations of deSia and deSia/deGal IgA at concentrations ranging from 0 to 100 μg/ml in presence or absence of N-nitro-L-arginine ω methyl ester 0.01 M.

IgA Glycoforms from Sera of Patients with IgAN and Healthy Control Subjects

Fractions from sera of 10 patients with IgAN and 10 healthy control subjects that contained IgA with various glycosylation patterns were isolated by affinity chromatography using columns of lectins immobilized on Sepharose. Proteins eluted by the corresponding sugars were fractionated in HPLC to isolate the peaks of 250- to 500-kD glycoproteins. IgA and IgG content was measured by enzyme-linked immunosorbent assay (ELISA).

Preparations of Columns. Sepharose 4B cyanogen bromide (CNBr) was activated (Pharmacia, Uppsala, Sweden) was prepared as recommended by the manufacturer. Selected lectins were cross-linked to activated Sepharose by incubation in 0.1 M NaHCO$_3$, 0.5 M NaCl buffer at 4°C for 12 h. Each lectin used (all from Sigma, St Louis, MO) belonged to separate lectin classes following Damjanov (29): *Canavalia ensiformis* (Con A) specific for αmannose (Man) group; *Triticum vulgare* (WGA) specific for N-acetylglucosamine (GlcNAc) group; *Glycine maximus* (SBA) specific for N-acetylgalactosamine (GalNAc) group; *Ulex europaeus* (UEA-I) specific for αL-fucose (Fuc); *Limulus polyphemus* specific for sialic acid (Neu5Ac)α2,6GalNAc group. The gel beds were packed in 5-ml columns (Economopack; BioRad Laboratories, Hercules, CA) and washed with 0.1 M phosphate-buffer saline (PBS; pH 7.4).

Affinity Chromatography. Each of the 5 columns was loaded with 1 ml of serum from each patient and control subject. The efflux was clamped, and the column was incubated for 1 h at room temperature. The effluent was passed through the column twice. The columns were washed for approximately 3 h with flowing PBS, until the optical density (OD) at 280 nm was negligible. Finally, the bound glycoproteins were eluted from the columns by washing with solutions of the appropriate monosaccharide specific for each lectin: 0.1 M Man, 0.1 M GlcNAc, 0.1 M GalNAc, 0.1 M Fuc, and 0.05 M Neu5Ac. The eluates were collected in 0.5-ml fractions, and the absorbance values at 280 nm were considered to isolate the main peak of eluted glycoproteins. Each serum had this procedure done three times, and the main peak of each serum passed on a single column were pooled together for further analysis.

HPLC. The fractions isolated from each column eluate were fractionated by BioRad 5000T HPLC System on a size exclusion Bio-Sil SEC 250 to 5 column (300 $\times$ 78 mm; BioRad), which separates biologic molecules in the 30- to 500-kD molecular mass range. The elution buffer was 0.5 M PBS, 0.15 M NaCl, 0.1% sodium azide (pH 7.4). The calibration profile was the following: ferritin molecular weight (MW) 440.000 kD = 17 min, catalase 232.000 kD = 30 min, bovine IgG 180.000 kD = 35 min, bovine serum albumin (BSA) 66.000 kD = 45 min, ovalbumin 44.000 kD = 50 min. The material eluted after 5 to 25 min, corresponding to molecules of molecular weight ranging between 250 and 500 kD, was isolated and assayed for IgA content by sandwich ELISA (values were expressed in μg/ml). The same peak of human polymeric IgA (from colostrum, Sigma) also was resolved by HPLC as a control. The area of the peak or peaks eluted between 5 and 25 min and corresponding to 250 to 500 kD was measured and expressed in mm^2. Concentration of total IgA and IgG in the HPLC 250 to 500 kD effluents was expressed in arbitrary units (μg/peak), calculated as follows: (μg/peak) = IgA or IgG content (μg/ml) $\times$ peak area (mm^2).

Enzymatic Modification of IgA Carbohydrate Side Chain Residues

DeSia IgA. Human IgA was incubated for 6 h at 37°C with neuraminidase (from *Clostridium perfringens*; Sigma), which acts on α2,3 or α2,6 linked to Neu5Ac using 0.1 U of enzyme per milligram of IgA in 25 mM potassium acetate buffer containing 0.25 μM CaCl$_2$, 0.1% BSA (pH 4.5). The effluent from columns of Sepharose cross-linked to *Sambucus nigra* lectin (Vector Laboratories, Inc., Burlingame, CA) was deficient in terminal Neu5A 2,3 linked to Gal residues (30).

DeSia and DeGal IgA. A portion of the neuraminidase-treated IgA, nonadherent to *Sambucus nigra* columns, was incubated subsequently for 12 h at 37°C with 32 β-galactosidase (from bovine testis; Sigma) at a ratio of 0.1 U of enzyme per 200 μg of IgA in 0.2 M sodium phosphate, 0.1 M citrate buffer containing 0.1% BSA (pH 4.5). The IgA bearing residual terminal Gal was removed by affinity chromatography using Sepharose cross-linked with *Ricinus communis* (Vector Laboratories), which recognizes terminal Gal (31).

Verification of IgA-Bound Oligosaccharide Termination. ELISA plates were coated with 10 μg/ml unmodified or enzyme-treated IgA in 0.015 M carbonate buffer (pH 9.6), and unsaturated sites were blocked with 1% BSA-PBS. After overnight incubation at 4°C, plates were washed with 0.1% BSA and 0.05% Tween 20-PBS, and peroxidase-labeled *Arachis hypogaea* (PNA) specific for Galβ1,3GalNAc or *Vicia villosa* (VV) specific for ungalactosylated GalNAc (32) (5 μg/ml; both from Sigma) were added for 1 h at room temperature. After three washes, the enzyme substrate was added (phenylenediamine dihydrochloride 0.4 mg/ml in 0.03% vol/vol hydrogen peroxide, 0.1 M disodium hydrogen phosphate, and 0.05 M citric acid monohydrate; Sigma). The results of this colorimetric reaction were read at 490 nm at the end of the linear phase.

MC Culture

Human MC were obtained as described previously (33). Briefly, cells were isolated from cortical fragments obtained, with the patient's consent, from the normal part of kidneys surgically removed for renal carcinoma. The fragments were minced and passed through sieves of different pore size. The cell suspension, after treatment with collagenase type IV, 750 U/ml (Sigma) at 37°C for 30 min, was washed and seeded on culture plates (Becton Dickinson, Lincoln Park, NJ) containing RPMI 1640 supplemented with 20% FCS, ITS (5 μg/ml insulin, 5 ng/ml transferrin, 5 ng/ml selenium) antibiotics and antimycotics (media and other reagents were purchased from Sigma). Cells were cultured in a humidified atmosphere with 5% CO$_2$ at 37°C. MC were subcultured three times before use, and purity was assessed by negative staining for coagulation factor VIII and cytokeratin and positive staining for desmin.

Cell Conditioning

MC were incubated for 12 h at 37°C with deSia IgA, deSia/deGal IgA, native or heat aggregated polymeric IgA (Sigma), each at the concentration of 50 μg/ml. HPLC fractions that contained the different IgA glycoforms isolated from IgAN patients and control subjects were incubated similarly with MC.

In all cases, stimuli—tested with the LAL test (LAL and substrate supplied by Kabi, Stockholm, Sweden) to ensure endotoxin-free conditions— were added to the same supplemented culture medium used to maintain the cells in culture, in a humidified atmosphere containing 5% CO$_2$ and incubated for 12 h. Each experiment included cells similarly cultured and incubated in the same medium and atmosphere, without any treatment (basal conditions).

Cell Proliferation Rate

[^{3}H]-thymidine incorporation assay was performed in 96-well microtiter plates in 200 μl total volume. At the end of the 24-h incubation, cells were pulsed overnight with [^{3}H]-thymidine (1 μCi/well; Amersham, Arlington Heights, IL). Cells were collected on glass microfiber filters (934-AH; Whatman, Clifton, NJ) using a cell harvester, and radioactivity was assessed, after placing the filters in liquid scintillation fluid, by a β-counter.

Terminal Uridine Nick 3' End Labeling (TUNEL) Method

After a 5-min incubation with 1% paraformaldehyde, cells were fixed in 2:1 vol/vol ethanol:acetic acid for 10 min at room temperature. After three washings with PBS, cells were incubated with 100 U/ml terminal deoxyuridine transferase (TdT), 0.5 μg/ml biotinylated uridine in 1 M potassium cacodylate, 125 mM Tris-HC1, and 2.5 mM cobalt chloride (pH 6.6; Boehringer, Mannheim, Germany) for 1 h at 37°C in a humidified chamber. After washes, a 1:40 solution of fluoresceinated streptavidin (Boehringer) was incubated for 30 min at room temperature. Slides were counterstained with 0.3 μg/ml propidium iodide (Sigma) in PBS for 1 min at room temperature. An epifluorescence microscope (Ernst Leitz, Inc., Rockleigh, NJ) was used to detect apoptotic cells, which were quantified by counting the number of fluorescein-positive cells relative to the total number of cells in at least 10 microscopic fields.

Tumor Suppressor Protein p53 Flow Cytometry Analysis (FACS)

For the detection of p53, a molecule involved in the apoptotic mechanisms (34), 5×10^4 cells were allowed to adhere to 75 cm^3 flasks for 8 h at 37°C; then, the medium was substituted with complete medium containing one of the various stimuli noted above. Flasks were incubated for 6 h at 37°C, then cells were detached by trypsinization, washed in PBS, and incubated with the primary antibody at 4°C for 30 min. After two washes with PBS, MC were incubated at 4°C for 30 min with a FITC-rabbit anti-mouse antibody (Biosoft, Varilhes, France), rinsed in PBS, and analyzed, after fixation with 1% paraformaldehyde, in an EPICS XL counter (Coulter, Hialeah, FL) set to analyze 5000 cells per sample. Data were elaborated by the Immuno-4 program (Coulter).

Quantification of p53 Molecules

For quantification of the average number of p53 per cell, the Quantum 27 R-phycoerythrin–conjugated microbead kit (Flow Cytometry Standard Corp., San Juan, Puerto Rico) was used (32). This contains a set of calibrated standards, with four populations of phycoerythrin-conjugated microbeads displaying graded and predetermined fluorescence intensity (expressed in terms of the number of molecules of equivalent soluble fluorochromes [MESF]) and one reference blank population. With the use of this method, the relative channel number obtained by flow cytometry analysis of a cell population is transformed directly into the number of MESF. The linear regression equation, correlating the channel number with the specific MESF value, was calculated using a specific software (Quickcal V2.0; Flow Cytometry Standard Corp.). Results express the mean MESF $\pm$ SEM of four separate experiments, each done in triplicate.

NO Synthase Activity Measurement

After washing three times in PBS, MC were frozen in 1 ml of reaction buffer (20 mM Hepes, 0.5 M ethylenediaminetetraacetate, 1 mM dithiothreitol [pH 7.2]), and homogenized on ice with three 20-s bursts in a Polytron homogenizer. Each reaction used 100 μl of homogenate in a mixture to contain 2 mM NAD, 1.5 mM CaCl$_2$, 1 to 100 μM L-arginine, 2.5 μCi (0.4 μM) L-[2,3,4,5-^{3}H] arginine monohydrochloride (62 Ci/mmol; Amersham International, Bucks, UK). After a 30-min incubation at 37°C, the reaction was stopped by addition of 2 ml of 20 mM Hepes, 2 mM disodium ethylenediaminetetraacetate (pH 6). The whole reaction mixture was applied to 2-ml columns of Dowex AG50WX-8 (Na+ form; Aldrich, Milano, Italy) and eluted with 4 ml of water. At pH 6, arginine is negatively charged, whereas citrulline is neutral; the Dowex resin is a cationic exchanger that binds arginine but not citrulline under these conditions. The radioactivity corresponding to [^{3}H]citrulline content in 6 ml of eluate was measured by liquid scintillation counting. The protein content of cells was assessed with the modified micro-Lowry method (Sigma). NO synthase (NOS) activity was expressed as picomoles of citrulline generated per minute of incubation per milligram of cell protein. NOS activity was expressed as folds increase of values obtained with the same cell line under basal condition. To inhibit NOS activity, we used the competitive NOS inhibitor ω-nitro-L-arginine methyl ester (L-NAME) 0.01 M in selected experiments.

Statistical Analyses

For the statistical elaboration, a statistical software (Statistica; StaSoft, Padova, Italy) was used. Values are expressed as mean $\pm$ SEM. Comparison between group means were performed using t test. $P < 0.05$ was considered statistically significant. Pearson's correlation and linear regression analysis were used to calibrate receptor count by flow cytometry, as detailed above.

Results

Analysis of Serum Glycoproteins Eluted by Lectin Columns from Patients with IgAN and Healthy Control Subjects

In the HPLC system used, purified human IgG (MW 180 kD) gave a narrow peak at 35 min, whereas human polymeric IgA showed a broad peak after 22 to 25 min of elution, corresponding—according to the producer's information and to molecular markers—to molecules ranging from 250 to 350 kD.

Serum glycoforms isolated by affinity chromatography on Sepharose cross-linked lectins from patients and control subjects showed in HPLC one major peak and sometimes one or two additional small peaks in the material eluted between 5 and 25 min, averaging 250 to 500 kD (Figure 2). The 250- to 500-kD peaks, pooled in a single sample for each serum, were analyzed for IgA and IgG content and used as selected stimuli for cultured MC. The sodium dodecyl sulfate-polyacrylamide gel electrophoresis (4 to 15% gradient gel) of HPLC fractions from three patients with IgAN who were highly positive for IgA glycoforms detected one single band (data not shown). The HPLC pattern obtained fractionating samples from the same three patients in acidic conditions were strictly superimposable to that obtained by acidic treatment of polymeric IgA (data not shown).

Patients with IgAN showed, in comparison with control subjects, increased levels of IgA glycoforms with affinity for SBA ($P < 0.05$), suggesting increased exposure of GalNAc, and affinity for *Limulus polyphemus* ($P < 0.02$), indicating increased exposure of sialic acid bound to Gal-
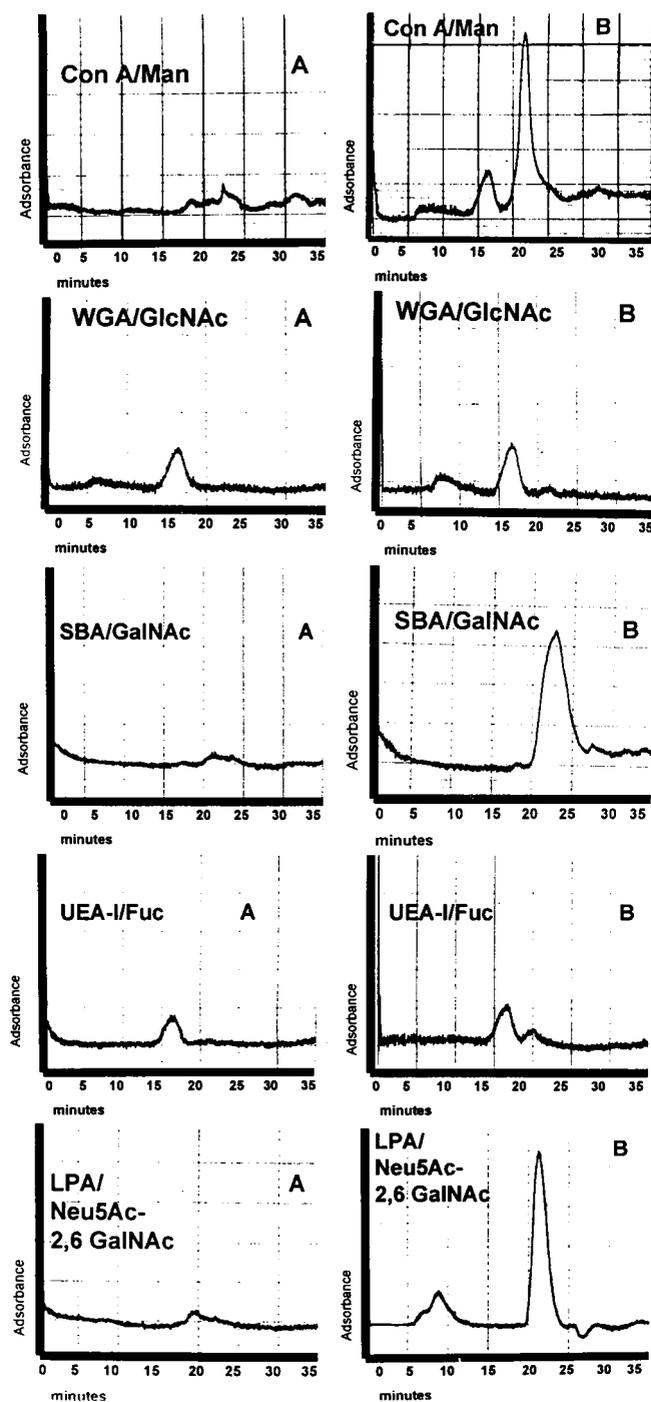
Figure 2. Representative HPLC profile of serum glycoforms isolated by affinity chromatography on insolubilized lectins form healthy control subjects (A) and patients with IgAN (B). Lectins and corresponding sugars used in affinity chromatography: *Canavalia ensiformis* (Con A), αmannose (Man), *Triticum vulgare* (WGA), N-acetylglucosamine (GlcNAc), *Glycine maximus* (SBA), N-acetylgalactosamine (GalNAc), *Ulex europaeus* (UEA-I), αL-fucose (Fuc), *Limulus polyphemus* (LPA), and sialic acid (Neu5Ac)α2,6GalNAc.

NAc (Neu5Acα2,6GalNAc; Figure 3). Moreover, patients with IgAN showed, in comparison with control subjects, increased levels of IgA glycoforms with affinity for Con A

($P < 0.03$), indicating an increased exposure of Man. The peaks isolated by HPLC from glycoproteins purified on WGA and on UEA-I, exposing GlcNAc and Fuc, respectively, had an IgA content similar in patients and healthy subjects. No modification in IgG glycosylation pattern between patients and control subjects was detected (Figure 3).

Effects of In Vitro IgA Desialylation and Degalactosylation of IgA from Healthy Control Subjects

The effects of enzymatic hydrolysis of sialic acid and galactose to generate deSia IgA and deSia/deGal IgA were confirmed by reciprocal changes in the binding of the treated IgA to PNA and to VV lectins. Neuraminidase-treated IgA bound 2.3-fold more PNA and 2.6-fold less VV than IgA treated sequentially with neuraminidase and β-galactosidase.

Reactivity of In Vitro DeSia IgA and DeSia/DeGal IgA with Cultured MC

Both deSia IgA and deSia/deGal IgA significantly depressed the MC proliferation rate ($P < 0.01$ in comparison to basal conditions and to native polymeric IgA; Figure 4), and the effect was dose dependent (Figure 5). *In vitro* prepared aberrantly glycosylated IgA significantly enhanced apoptosis as detected by the increased percentage of positive cells in the TUNEL test (deSia IgA, $P < 0.01$; deSia/deGal IgA, $P < 0.01$ *versus* basal conditions) and higher expression of the tumor suppressor protein p53 (deSia/deGal, $P < 0.02$ *versus* basal conditions). DeSia IgA and even more deSia/deGal IgA significantly enhanced NOS activity of MC in comparison with basal conditions and native IgA ($P < 0.01$; Figure 4).

Of interest, the coincubation with the NOS specific inhibitor L-NAME, 0.01 M, blunted each effect of aberrantly glycosylated IgA. By coincubating L-NAME, aberrantly glycosylated IgA not only failed to enhance NOS activity, but also failed to have an antiproliferative and pro-apoptotic effect ($P < 0.001$ *versus* each effect of deSia/deGal IgA, Figure 4)

Reactivity of Serum Fractions that Contained Aberrantly Glycosylated IgA from Patients with IgAN and Control Subjects with Cultured MC

Glycoproteins of 250 to 500 kD from patients with IgAN and control subjects expressing terminal GalNAc, Neu5Ac2,6GalNAc, or Man were incubated with MC in culture for investigation of the possible modulation of some cell functions. The IgA glycoforms from patients with IgAN who highly expressed GalNAc or Neu5Ac-2,6GalNAc significantly depressed the MC proliferation rate (both $P < 0.01$), whereas those that were rich in Man were ineffective (Figure 6).

Apoptosis was significantly enhanced by IgA glycoforms isolated in patients with IgAN in comparison with healthy control subjects, as detected by the increased percentage of positive cells at the terminal uridine nick 3' end labeling test (GalNAc exposing molecules, $P < 0.05$; Neu5Ac2,6GalNAc, $P < 0.01$) and higher expression of the tumor suppressor protein p53 (Neu5Ac2,6GalNAc, $P < 0.01$; Figure 6).

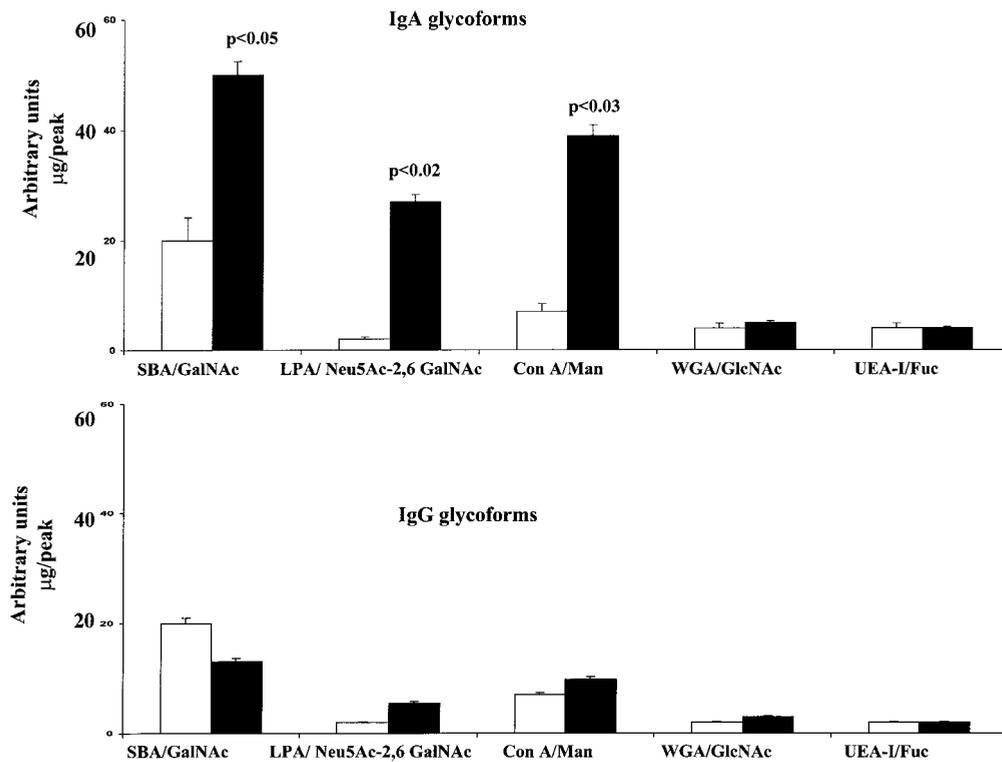NOS activity significantly increased after incubation with

Figure 3. Measurement of IgA and IgG glycoforms isolated in patients with IgAN (■) and healthy control subjects (□) by lectin affinity chromatography and purified by HPLC (see Figure 2 for abbreviations). The concentrations of total IgA and IgG in the HPLC peak/s eluted between 5 and 25 min and corresponding to 250 to 500 kD were expressed in arbitrary units (μg/peak), calculated as IgA or IgG content (μg/ml) $\times$ peak area (mm^2). Patients with IgAN showed, in comparison with control subjects, increased levels of IgA glycoforms with affinity for SBA, LPA, and Con A. No significant difference from patients and control subjects was found in IgG glycoforms content.

each IgA glycoform from patients with IgAN (Figure 6), particularly those bearing Neu5Ac2,6GalNAc residues ($P < 0.01$) and, to a lesser extent, those rich in Man ($P < 0.03$) and GalNAc ($P < 0.05$). No modulation of MC reactivity was observed after incubation with macromolecules isolated by HPLC of glycoproteins eluted by WGA and UEA-I affinity chromatography columns (data not shown).

Discussion

Our study adds further evidence that patients with IgAN have an altered glycosylation of circulating IgA. Moreover, it reports for the first time the modulation of MC functions directly induced by abnormal IgA glycoforms isolated from sera of patients with IgAN.

We used an isolation procedure that ensured the isolation of fractions of serum IgA provided with different sugar exposures. Meanwhile, the method used allowed the optimal respect for the native structure of this Ig. It is known that the isolation of IgA from the other immunoglobulins has some technical difficulties. Affinity chromatography with anti-IgA antibodies cross-linked to Sepharose needs a strong treatment to elute IgA—using a highly acidic glycine buffer—likely altering the sugar residues. Hence, most of the studies on IgA glycosylation pattern in IgAN avoided this method, using instead the lectin Jacalin coupled with agarose (5–10). Jacalin has specificity for Galβ1,3GalNAc (35) and, hence, binds correctly glycated

IgA1 O-glycans. However, the use of Jacalin to purify IgA from serum of patients with IgAN introduces an important bias, as only IgA1 reacting with Gal bound to GalNAc are isolated. Because it has been reported that serum IgA in patients with IgAN has an impaired binding to Jacalin (36) and there now is substantial evidence that circulating IgA1 in these patients has reduced terminal Gal (3), we reasoned that by using Jacalin for serum IgA purification from patients with IgAN, potentially relevant amounts of aberrantly glycosylated IgA could escape isolation and further analysis. Therefore, we tried a different approach, using an affinity chromatography procedure with lectins cross-linked to Sepharose, which allows the interaction with selected sugars expressed on the surface of the IgA molecules that may be modified in case of carbohydrate chain truncation. This method detected small amounts of IgG in equal amounts in patients with IgAN and control subjects. Moreover, the presence of other glycoproteins of similar molecular weight expressing the sugar recognized by each lectin or of fibronectin bound to IgA glycoforms (20) cannot be excluded. However, it is theoretically unlikely that these molecules might be responsible for the different MC activation induced by fractions isolated from patients and control subjects.

The procedure used allowed the analysis of circulating macromolecular IgA (250 to 500 kD) in patients with IgAN, which are thought to be involved in the formation of mesangial
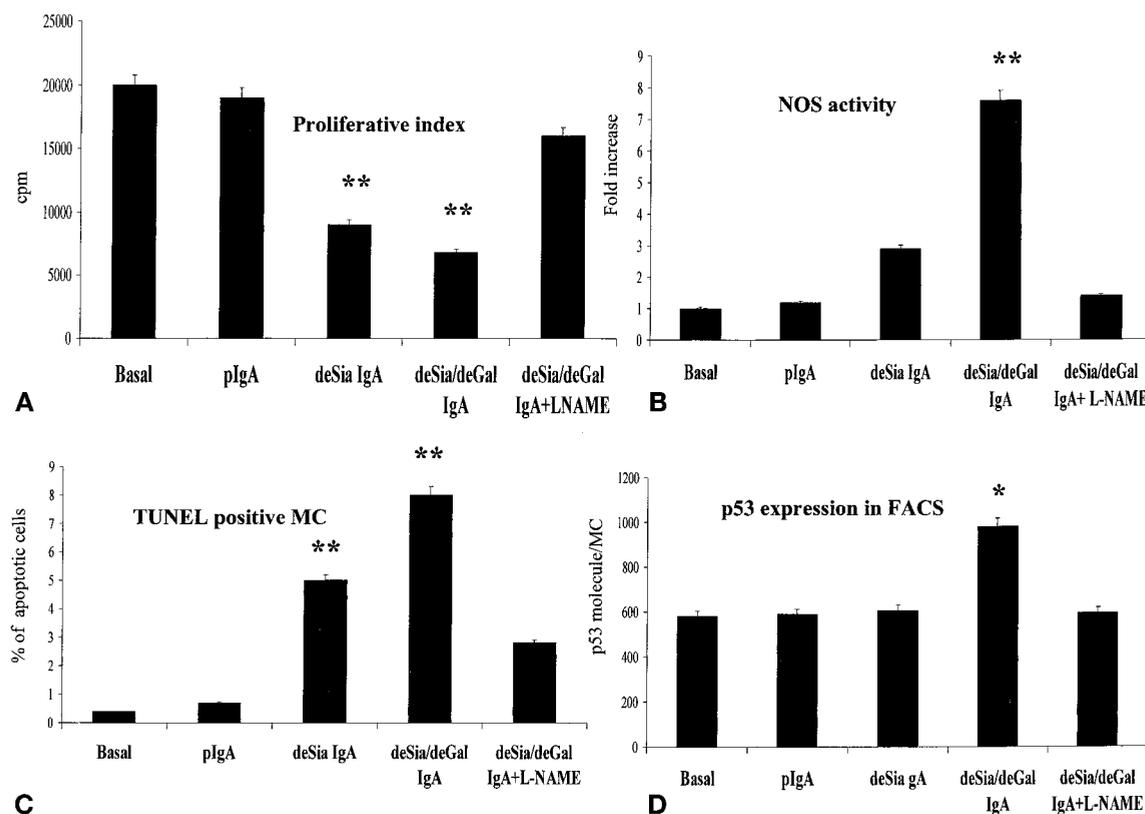
Figure 4. Reactivity of *in vitro* desialylated (deSia) and desialylated/degalactosylated (deSia/deGal) IgA compared with normal polymeric IgA (pIgA) and unconditioned cells (Basal) with cultured human mesangial cells (MC). (A) Proliferative index (^{3}H-timidine incorporation). (B) Nitric oxide synthase (NOS) activity ([^{3}H]citrulline production from [^{3}H]arginine). (C and D) Apoptosis: terminal uridine nick 3' end labeling (TUNEL) (C) percentage of positive cells; p53 quantification (D) by FACS analysis. The last columns report the values obtained conditioning MC with deSia/deGal IgA in the presence of N-nitro-L-arginine methyl ester (L-NAME). Columns represent the mean ± SEM of three experiments, each done in triplicate. *, $P < 0.02$ *versus* values in unconditioned cells (basal); **, $P < 0.01$ *versus* values in unconditioned cells.

deposits (37). Actually, experimental studies have demonstrated that the injection of only macromolecular IgA can lead to IgA deposits, whereas monomeric IgA and polymeric IgA are not nephrogenic *per se* (38). Moreover, recent evidence suggests that in patients with IgAN, the defect in Gal mostly involves polymeric IgA1 forming circulating macromolecular immune complexes (10).

We proved that patients with IgAN have a significant increase in macromolecular IgA with high exposure of GalNAc and Neu5Ac2,6GalNAc, in agreement with the hypothesis of a defective activity, either congenital or acquired, of β1,3 galactosyl-transferase, as demonstrated in a functional assay in circulating B cells (17). Moreover, we detected an increased exposure of Man residues in some IgA glycoforms, which is compatible with a truncation of N-linked glycan chains. N-linked glycans have been demonstrated to regulate the nonimmune lectin-like reaction between IgG and IgA in rheumatoid arthritis, which leads to the formation of circulating macroaggregates (39). The removal of sialic acid and N-linked glycan chains greatly increased the complement-binding properties of both IgA1 and IgA2 (28). However, no N-linked glycosylation defect has been described in IgAN, and differences in isolation steps between the previous reports (5–10) and our method might account for it. Of interest, Rifai *et al.* (40) recently

indicated that altered N-linked glycosylation but not O-glycans affect the recognition of IgA by the asialoglycoprotein receptor and may account for the elevated serum IgA seen in IgAN.

The present study reports that some IgA glycoforms isolated from patients with IgAN can affect several MC functions. Moreover, similar effects were reproduced by using *in vitro* deSia and deGal normal polymeric IgA.

IgA glycoforms isolated from IgAN patients exposing GalNAc or GalNAc bound in 2,6 to Neu5Ac or, to a lesser extent, those exposing Man, as well as *in vitro* deSia and deGal normal IgA were found to modulate several MC functions, decreasing the proliferation rate, increasing apoptotic death, and enhancing NOS activity. The effect was dose dependent and mediated by the impressive release of NO, because the competitive inhibition of the enzyme NOS by L-NAME completely blunted each reaction.

The amounts of NO produced by MC after incubation with aberrantly glycosylated IgA, previously observed to mediate also a decrease in the repairing factor vascular endothelial growth factor-A (27), were so sustained to enhance the cell active suicide mechanism, leading to apoptotic death. This effect is not surprising, because NO, provided with an uncoupled electron, acts as a free radical, enhancing apoptosis via p53 activation (41–43). Indeed, we demonstrated in the present
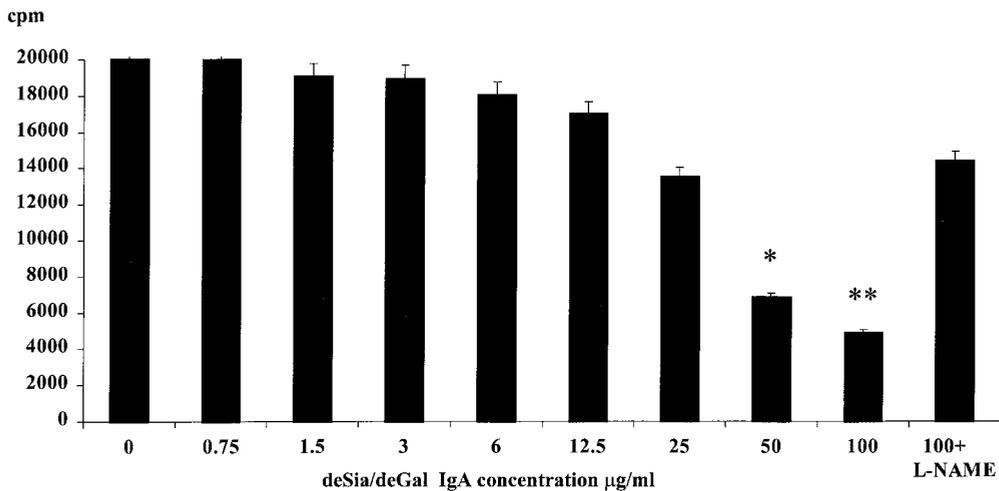
Figure 5. Dose-dependent (ranging from 0 to 100 μg/ml) antiproliferative effect of deSia/deGal IgA on cultured MC. The last columns report the values obtained conditioning MC with deSia/deGal IgA in the presence of L-NAME. Columns represent the mean $\pm$ SEM of three experiments, each done in triplicate. **, $P < 0.01$ *versus* values in unconditioned cells.
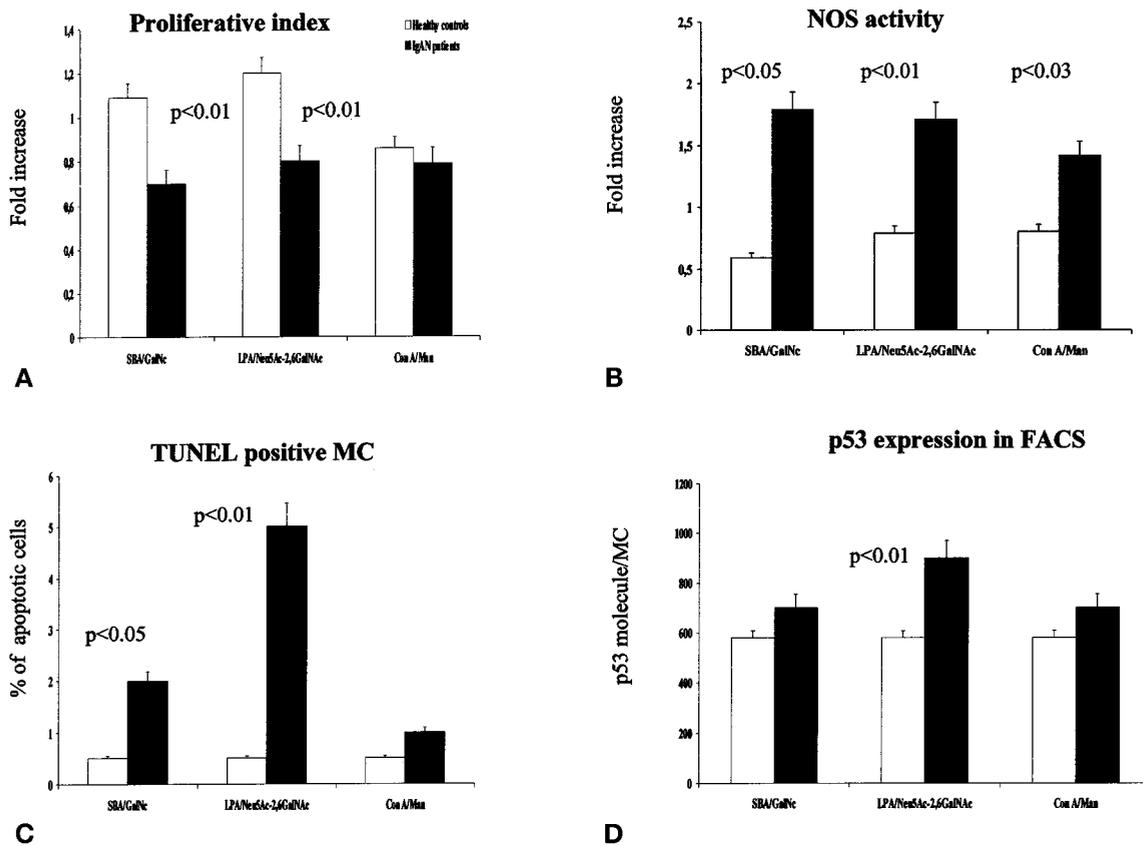

Figure 6. Reactivity of IgA glycoforms isolated by affinity chromatography and HPLC in patients with IgAN and control subjects with cultured human MC (see Figure 3 for abbreviations). (A) Proliferative index (^{3}H-timidine incorporation). (B) NOS activity ([^{3}H]citrulline production from [^{3}H]arginine. (C and D) Apoptosis: TUNEL (C) percentage of positive cells; p53 quantification (D) by FACS analysis. Proliferation rate and NOS activity data are expressed as fold increase in each condition over basal values. Columns represent the mean $\pm$ SEM of three experiments, each done in triplicate. ■, patients with IgAN; □ healthy control subjects.

study an increased transcription of p53 protein after incubation with IgA glycoforms isolated from sera of patients with IgAN.

The above findings sound amazing for a renal disease characterized by increase in mesangial cellularity in association with IgA deposits. Indeed, it is easy to speculate that deposited IgA enhance MC proliferation. However, we must consider the complicated network of factors that interplay during the development and the evolution of IgAN.

The experimental conditions adopted in our study are extremely simplified in comparison to reality, as MC are incubated only with a single stimulus (deglycosylated IgA) at a time. *In vivo*, during the development of the IgAN, several mechanisms are likely to operate contemporarily, and the cytokine-mediated enhancement of MC proliferation might coexist with a direct depressive effect dependent from aberrantly glycosylated IgA.

For instance, we reported that IgA from patients with IgAN have an increased binding to some lectins (12) and particularly lectin-like gliadin fractions (13), and selected lectins—alone or combined with serum IgA via carbohydrate binding—are very active in bridging IgA to MC (44) and in modulating MC synthesis and release of proinflammatory cytokines (33).

Chintalacharuvu and Emancipator (18) recently hypothesized that the pathogenetic process that leads to IgAN begins with an increased production of Th2-type cytokines (interleukin-4 [IL-4], IL-5, IL-6, IL-8, and IL-10) and decreased Th1 cytokines (IL-2 and interferon-γ). The major effect of Th2-released cytokines is the enhancement of MC proliferation and lymphomonocyte mesangial infiltration. Meanwhile, the increase in IL-5 and IL-4 not only enhances the synthesis of IgA from B cells inducing a preswitch but also modulates the pattern of IgA glycosylation (18). IgA with altered glycosylation might undergo self-aggregation (19) or reactions with other glycoproteins or lectin-like molecules (37) to form macromolecular IgA provided with binding to matrix and MC to form mesangial deposits (14). Hence, the mesangium in exposed to both Th2 cytokine effects (which induce the enhancement of MC proliferation and lymphomonocyte infiltration) and to IgA deposit consequences. Accordingly, different mediators, including cytokines, chemokines, and vasoactive substances, that enhance either MC proliferation or apoptosis/sclerosis are operating in the meantime or during different phases of the disease in a complex interaction that accompanies the evolution of the renal disease (45).

Massive release of NO in the mesangial area would favor a feedback mechanism that could downregulate the MC proliferation and leukocyte influx (41,46) resulting from other stimuli, such as IL-6 and IL-8 cytokines released after Th2 unbalanced stimulation. The timely sequence of expression of these mediators will determine the balance between proliferation and apoptosis, leading to a variety of histologic expressions and different disease progression rate.

In this scenario, it is striking that aberrantly glycosylated IgA might favor the progression toward sclerosis. Following this hypothesis, the extent of IgA aberrant glycosylation might govern the individual clinical course. Further investigations are needed to explore this hypothesis. At the moment, the present study confirmed the presence of circulating IgA with altered glycosylation pattern in patients with IgAN and demonstrated that these properties can modulate some reactivities of MC, which could play a role in the development and progression of this nephropathy.

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