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In vitro-generated immune complexes containing galactose-deficient IgA1 stimulate proliferation of mesangial cells

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ABSTRACT

IgA nephropathy (IgAN) patients have elevated serum levels of immune complexes consisting of IgA1 with galactose-deficient hinge-region *O*-glycans (Gd-IgA1) and anti-glycan IgG. These immune complexes deposit in the kidney and activate mesangial cells. To confirm that the activity of these immune complexes depends on the interaction of Gd-IgA1 with anti-glycan IgG, we generated *in vitro* analogous immune complexes using Gd-IgA1 myeloma protein and anti-glycan IgG from cord blood of healthy women. The Gd-IgA1 and anti-glycan IgG from cord-blood serum formed IgA1–IgG immune complexes that resembled those in sera of patients with IgAN. Furthermore, the ability to activate cellular proliferation was dependent on a heat-sensitive serum factor. In summary, we developed a new protocol for *in-vitro* formation of IgA1–IgG immune complexes, thus providing a new tool for studies of the pathogenesis of IgAN.

1. Introduction

IgA nephropathy (IgAN) is characterized by IgA-containing immune complexes in the glomerular mesangium [1,2], with IgA exclusively of the IgA1 subclass [3]. IgA1 can be co-deposited with complement C3 and IgG or IgM or both [1,2]. Mesangial cellular proliferation and expansion of extracellular matrix are typical histological features. These glomerular changes lead to end-stage renal failure in 20–40% patients within 20 years of diagnosis [4–7]. The disease onset and/or recurrent episodes of macroscopic hematuria often coincide with mucosal infections, including those of upper respiratory tract and digestive system [4,8–11].

There is considerable evidence indicating that the mesangial deposits originate from circulating IgA1-containing immune complexes [12–24]. Analysis of the glycosylation of IgA1 in patients with IgAN has provided new insights into the mechanisms underlying immune-complex formation and activation of mesangial cells [21,24–27]. Specifically, circulating immune complexes in IgAN contain IgA1 with galactose (Gal)-deficient hinge-region *O*-linked glycans [21,24,25,28–33] and Gal-deficient IgA1 is the predominant glycosylation variant of IgA1 in the mesangium [34,35]. A relationship between Gal deficiency and nephritis also has been observed in other diseases: Gal-deficient IgA1 [36,37] and IgA–IgG circulating complexes [38] are found in sera of patients with Henoch–Schoenlein purpura who develop nephritis but not in sera of those patients who do not. Also, patients with IgA1 multiple myeloma have high levels of circulating IgA1, but only individuals with the aberrantly-glycosylated variant develop immune-complex glomerulonephritis [39,40].

In IgAN, the aberrant glycans or hinge-region glycopeptides of IgA1 are recognized by naturally occurring IgG and/or IgA1 anti-glycan antibodies and immune complexes are formed [21,24,41–44]. These IgA1-containing immune complexes are of a relatively large molecular mass [26,30,33] and, thus, are not efficiently cleared from the circulation by the liver and tend to deposit in the renal mesangium [45–50].

As only humans and hominoid primates have IgA1 [51], studies of IgAN have been hampered by the lack of appropriate animal models. Alternatively, cultured primary human mesangial cells present a convenient model to evaluate biologic activities of IgA1 complexes [25,26,30,52–64]. Using this model, we and others have demonstrated that immune complexes from sera of patients with IgAN containing galactose-deficient IgA1 activate mesangial cells and induce cellular proliferation [26,30,62,64,65].

Abbreviations: Gal, galactose; IgAN, IgA nephropathy

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Extensive studies of the detailed glycan structures of IgA1, fine specificities of anti-glycan antibodies, and biological activities are hampered by the low quantity of immune complexes isolated from sera of patients with IgAN. Therefore, to address this shortcoming, we developed a new protocol for *in-vitro* production of biologically active IgA1-containing immune complexes. We used cord-blood serum with high levels of anti-glycan IgG to bind to IgA1 myeloma proteins with Gal-deficient hinge-region *O*-glycans that mimic the aberrant IgA1 eluted from glomeruli of patients with IgAN [24,34,35,66–68] to form immune complexes. The advantage of cord-blood serum is that it contains almost exclusively IgG, with only trace amounts of IgM or IgA. Our results showed that formation of the biologically active immune complexes required Gal-deficient IgA1, anti-IgA1 IgG antibody, and a heat-sensitive serum factor. Future studies to identify this factor(s) and describe detailed mechanisms of immune-complex formation will help in designing a better therapeutic target in patients with IgAN.

2. Materials and methods

2.1. Serum samples

Cord-blood serum samples were collected from five pregnant women (one Caucasian and four African Americans) at the time of delivery. The donors did not have any evidence of renal disease as determined by normal values for serum creatinine concentration and urinary protein/creatinine ratio, and the absence of hematuria by urinalysis testing. Serum samples were obtained from three biopsy-proven IgAN patients and a healthy control. The UAB Institutional Review Board approved the study and informed written consent was obtained from each participant before collecting the blood samples.

2.2. IgA myeloma proteins

Two polymeric IgA1 (Mce and Gou) and one polymeric IgA2 (Fel) myeloma proteins were prepared from plasma of patients with IgA multiple myeloma [24,69]. Both IgA1 myeloma proteins had Gal-deficient hinge-region *O*-glycans [24,29].

2.3. Fractionation of immune complexes

Serum samples from patients with IgAN or healthy controls (0.5 ml) were filtered using 0.45 μm filter (Pall Corporation, Ann Arbor, MI, USA) and fractionated on a calibrated Superose 6 column (600 × 12 mm; Amersham Biosciences Corporation, Piscataway, NJ, USA) in phosphate-buffered saline (PBS). For cord-blood serum supplemented with IgA, a mixture of 160 μl cord-blood serum, 80 μg IgA myeloma protein and PBS to a final volume of 500 μl was incubated at 4 °C overnight, filtered and fractionated on a calibrated Superose 6 column. Fractions containing proteins of molecular mass apparently over 700 kDa were collected and every two fractions were pooled and added to the cultured mesangial cells [70]. IgA and IgG–IgA complexes were determined in the fractions by ELISA [25,71].

2.4. Cell cultures

Human mesangial cells were purchased from Cambrex Bio Science Walkersville, Inc. (Walkersville, MD, USA) and cultured, as described [25]. Cells from passages 3 or 4 were maintained in RPMI 1640 supplemented with 20% fetal calf serum (FCS), L-glutamine (2 mmol/l), penicillin G (100 U/ml) and streptomycin (0.1 mg/ml) in humidified 5% CO_2 atmosphere at 37 °C. Twenty-four-well tissue-culture plates were seeded with the mesangial cells for proliferation experiments. At 85%–95% confluence, the mesangial cells were serum-starved in a medium containing 0.5% FCS for 24 h before experiments [70].

2.5. Proliferation assays

Proliferation of the cultured human mesangial cells was measured by ^{3}H-thymidine uptake, as described [26,70]. Serum from healthy control or patient with IgAN or cord-blood serum was supplemented with IgA proteins and incubated at 4 °C overnight (unless otherwise stated). Then, the samples were filter-sterilized, mixed with culture medium containing 0.5% FCS, added in duplicates to mesangial cells, and the cells were incubated for 20 h at 37 °C in 5% CO_2 atmosphere and then analyzed [26,70]. The culture medium supplemented with 10 ng/ml platelet-derived growth factor (PDGF; R&D Systems, Minneapolis, MN, USA) was used as a positive control. Medium alone served as a negative control. Average values were calculated from duplicates for each serum fraction and expressed relative to the negative control (*cpm* of sample/ *cpm* of the control) as relative proliferation. Alternatively, the data were expressed as Δ*cpm*, calculated as *cpm* value of each sample from which the value measured for the control sample was subtracted.

2.6. ELISA

For IgA detection, polystyrene microtiter plates (Nalge Nunc International, Rochester, NY, USA) were coated overnight with 1 μg/ml goat anti-human IgA (Jackson ImmunoResearch Labs, West Grove, PA, USA) [25,71]. After washing and blocking with 1% bovine serum albumin (BSA; Sigma Chemical Company, St Louis, MO, USA) in PBS containing 0.05% Tween-20, serial 2-fold dilutions of duplicate samples and standard serum (The Binding Site, Birmingham, United Kingdom) in blocking solution were incubated overnight at room temperature. The bound IgA was detected by incubation with biotin-labeled goat anti-human IgA (BioSource International, Camarillo, TX, USA) for 3 h at 37 °C, followed by 1-h incubation with horseradish peroxidase-conjugated ExtrAvidin (Sigma). o-Phenylenediamine–H_2O_2 (Sigma) was used as substrate for peroxidase, and color development was stopped with 1 M sulphuric acid. The absorbance at 490 nm was measured using an automated ELISA reader (Bio-Tek Instruments Winooski, VT, USA). The concentrations were calculated based on calibration curves generated from standard serum. The results were expressed in μg/ml.

For measurement of IgG–IgA complexes, 50-fold-diluted fractions were applied on ELISA plates coated with goat anti-human IgG (Jackson ImmunoResearch Labs) and detected with biotin-labeled goat anti-human IgA (BioSource) and developed, as described above. Internal controls were included.

2.7. Depletion of IgA and IgG, and isolation of IgG

To remove IgA1 from serum of a patient with IgAN, serum was adsorbed on immobilized jacalin (1-ml bed volume; EY Laboratories, San Mateo, CA, USA), a lectin specific for *O*-glycans on IgA1. IgG was depleted from serum or cord-blood serum using GammaBind Plus Sepharose (Amersham Biosciences Corporation), using 1 ml of the sample mixed with the same amount of binding buffer (0.01 M sodium phosphate, 0.15 M NaCl, 0.01 M EDTA, pH 7.0). The flow-through was concentrated on Amicon Ultra-4 PL-50 Centrifugal Filter Devices (Millipore, Billerica, MA, USA) to a volume of 1 ml and used as IgG-depleted serum. To isolate IgG, the GammaBind Plus Sepharose column was washed with 5 ml binding buffer and the bound IgG was eluted with acidic buffer (0.5 M acetic acid adjusted to pH 3.0 with ammonium hydroxide) and the pH was adjusted to neutral with Tris–HCl buffer. Then, the IgG samples were desalted on a PD-10 column (GE HealthCare, Chalfont St. Giles, United Kingdom) equilibrated with PBS, and concentrated on Amicon Ultra-4 PL-50 Centrifugal Filter Devices (Millipore) to 1 ml volume. Purity of these samples was assessed after separation by SDS-PAGE under reducing conditions by silver staining, and by Western blotting with IgA- or IgG-specific antibodies.