Interleukin-12 alters the physicochemical characteristics of serum and glomerular IgA and modifies glycosylation in a ddY mouse strain having high IgA levels

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Abstract

Background. We recently developed a ddY mouse strain having high IgA levels (HIGA) that provided a murine model of IgA nephropathy. We additionally showed that administration of interleukin (IL)-12, a potent helper T (Th)1-inducing cytokine, induced an apparent reduction in serum IgA levels. In the present study, we assessed the influence of IL-12 administration on several physicochemical characteristics of nephritogenic IgA molecules in HIGA mice.

Methods. HIGA mice received daily intraperitoneal injections of IL-12 or control injections of phosphate-buffered saline for 3 weeks. Crescent formation and levels of circulating and glomerular IgA were analysed. Moreover, potential changes in charge, size, and glycosylation of serum and glomerular IgA were investigated.

Results. In the IL-12 group, glomerular IgA deposition was faint, although crescent formation was more marked than in the control group. Serum IgA levels in IL-12 mice were significantly lower than in controls. IL-12-treated mice also showed markedly decreased acidic and polymeric IgA both in sera and in glomerular eluate. A lectin-binding study revealed a markedly reduced ratio of sialylated and galactosylated IgA in the sera and in glomerular eluate from HIGA mice kidneys. IL-12 treatment significantly increased sialylation and galactosylation of circulating IgA, although glycosylation of IgA in glomerular eluate remained low.

Conclusions. In HIGA mice showing under-glycosylation, IL-12 administration may lead to changes in the physicochemical characteristics of IgA, and this may occur through a shift to Th1. These results suggest that the Th1 and Th2 balance might play a role in the development of immunopathologic lesions in this model of IgA nephropathy.

Keywords: anionic charge; crescent formation; glycoprotein; helper T cell; IgA nephropathy; polymeric IgA

Introduction

IgA nephropathy (IgAN), the most common glomerulonephritis worldwide, is characterized by strong IgA1 deposition in the glomeruli. The ratio of polymeric to acidic IgA is significantly higher in IgAN than in normal subjects, and mesangial IgA is predominantly polymeric and acidic in nature. Although the mechanisms of IgA deposition are not fully understood, recent studies indicated that IgA deposits are formed not only through classical antigen–antibody interactions, but also by other mechanisms as well. Recent reports revealed that the interaction of IgA with mesangium is partly dependent on the Fc fragment of IgA [1,2]. In human IgAN, several studies have demonstrated that IgA1 has an abnormal pattern of O-glycans. Indeed, IgA1 with reduced glycosylation has an increased capacity for self-aggregation, which might favour the deposition of macromolecular IgA in glomeruli [3]. To explain the pathogenesis of IgAN, a number of malfunctions in IgA production and regulation have been proposed, including high serum IgA, enhanced IgA-specific helper T (Th) cells, and diminished numbers of IgA-specific regulatory T cells. Recently, it was reported that expression of the Th2 cytokines, interleukin (IL)-4 and IL-5, was increased in
Peripheral blood mononuclear cells of IgAN patients [4]. This finding suggests that Th2-predominant immune responses may be the source of pathogenesis in IgAN.

To obtain a spontaneous IgAN-prone model possessing these pathogenic characteristics, an inbred strain designated as hyper IgA (HIGA) mice has been previously established through selective mating of ddY mice. This mouse is characterized by hyper serum IgA and glomerulonephritis with polymeric IgA dominant deposition in the mesangium [5]. Analysis of the immunopathologic background of this mouse revealed that interferon (IFN)-γ production by splenic CD4+ T cells was markedly upregulated from a young age compared with C57BL/6 and BALB/c mice, and that both IL-4 and transforming growth factor (TGF)-β1 production increased with age [6]. These results indicate that this mouse shows up-regulated Th1 as the basic immunological characteristic of the T cells, and an age-associated shift to Th2 dominancy. Our recent studies revealed that administration of IL-12, a potent Th1-inducing cytokine, caused reductions in serum IgA levels [6]. This finding suggests that IL-12 affects the physicochemical characteristics, the clearance, or both, of IgA through a shift to a dominant Th1 response.

In the present study, IL-12-induced physicochemical changes in circulating and glomerular IgA were investigated in HIGA mice. Immunopathological analysis revealed mice treated with IL-12 had a significant reduction in circulating and glomerular IgA that was associated with a marked decrease in the acid and polymeric character of the IgA molecules with modification of the low sialylation and galactosylation. These changes in the quantitative and qualitative characteristics of IgA by IL-12 treatment will provide a tool to further study the pathogenic mechanism of IgA deposition in IgAN.

Materials and methods

Mice

HIGA mice were established by selective mating of high serum IgA ddY mice. BALB/c mice were obtained from SLC Japan (Shizuaoka, Japan) and were compared with HIGA mice. All mice were female, and aged from 31 to 36 weeks old.

IL-12 administration

HIGA mice received daily intraperitoneal injections of recombinant murine IL-12 (a gift from Dr Stan Wolf, Genetics Institute, Cambridge, MA, USA) at a dose of 100 ng/mouse (n = 11) for 3 weeks. Six control mice were injected with the same volume of phosphate-buffered saline (PBS). The mice were sacrificed on the last day. Blood samples were obtained just before IL-12 treatment and on the last day at 3 weeks. All animal experiments were performed in accordance with institutional guidelines, and the Review Board of Kyoto University approved the ethics of this study.

Serum creatinine and urinary examination

Serum creatinine was measured using the Jaffe method. Urinary occult blood and protein were determined semiquantitatively using Multistics (Bayer-Sankyo, Tokyo, Japan) at the time of sacrifice.

Light microscopic study of renal tissues

Renal specimens were fixed in Doubosque-Brazil solution and embedded in paraffin. Cross-sections of central portions of kidneys, containing approximately 100 glomeruli, were stained with periodic acid-Schiff (PAS). The percentages of glomeruli showing crescent formation were determined.

Immunofluorescence study

Staining for IgA and IgG on frozen renal sections was performed by a direct method using fluorescein isothiocyanate conjugated anti-mouse IgA or IgG antibody (Cappel Laboratories, Cochranville, PA, USA) as previously described [6]. The sections were then observed by LSM 410 confocal laser scanning microscopy (Carl Zeiss, Oberkochen, Germany). The grade of deposition was evaluated quantitatively by measuring the intensity of the fluorescence in the glomerular areas with Photoshop 4.0 (Adobe, San Jose, CA, USA) and this was graded from 0 to 255 [6].

Measurement of serum immunoglobulins

IgA and IgG in the sera were measured by a modification of the sandwich ELISA [6] using goat anti-mouse IgA (Cappel Laboratories) or IgG (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA) antibodies as plate-coated antibodies. Serum IgG1 and IgG2a were measured as previously described [6] by a modification of the sandwich ELISA using monoclonal rat anti-mouse IgG1 or IgG2a antibodies (BIOSYS, Compiegne, France) as plate-coated antibodies. The IgG1 to IgG2a ratio was applied to evaluate IL-12-induced changes in Th1 and Th2.

Elution of glomerular IgA from isolated kidneys

The elution of IgA from isolated glomeruli, obtained using a sieving technique, was performed using a 2 h incubation with 0.02 M citrate buffer, pH 3.5 [7]. After centrifugation, the supernatant was obtained and dialysed against PBS (pH 7.4) overnight and then assayed for IgA.

Charge analysis of IgA

The electric charge of the IgA in each of the serum samples and pooled glomerular eluates was analysed by sucrose density gradient isoelectric focusing (IEF) as previously described [7]. After 18 h of focusing at 160 V at 7°C, a 35 µl aliquot was collected from the bottom of each tube, dialysed against PBS, and assayed for IgA. The ratio of acidic IgA percentage before and after treatment was compared between IL-12- and PBS-treated samples.

Size analysis of IgA

Each serum sample and pooled glomerular eluate was fractionated using high performance liquid chromatography (HPLC) with a 7.5 mm × 60 cm SW column (5–1000 kDa
fractionation range; Tosoh, Tokyo, Japan) having a flow rate of 0.5 ml/min [5]. Eighty fractions of each sample were collected in 0.1 M PBS (pH 7.0), containing 0.1 M NaCl. Mouse IgG (150 kDa) and IgM (900 kDa) were used as molecular mass markers. Two series of fractions, from 37 to 48 (polymeric or dimeric IgA, 525–320 kDa), and from 48 to 55 (monomeric IgA, 320–150 kDa), were prepared. The series of fractions were assayed for IgA. The ratio of polymeric IgA percentage before and after treatment was compared between IL-12 and PBS samples.

Measurement of lectin-binding serum IgA

To examine terminal galactosylation and sialylation of the IgA molecule, lectin-binding assays were designed using biotinylated elderberry (Sambucus nigra) bark lectin (SNA; Vector Laboratories, Burlingame, CA, USA) and Ricinus communis agglutinin I (RCA-I; Vector Laboratories), which recognize terminal sialic acid (specifically Neu5Ac2-6Gal) and galactose residues, respectively [8]. Microtiterplates coated with goat anti-mouse IgA were incubated with these

Fig. 1. Effect of IL-12 administration on glomeruli. Light microscopic findings of glomeruli in the PBS and IL-12 groups are shown in (A) and (B), respectively (PAS staining, magnification ×400). After IL-12 administration, prominent glomerular inflammatory lesions with cellular crescent formations, moderate glomerular hypercellularity, and segmentally severe mesangial proliferation were observed (B). The percentage of glomeruli showing crescent formations were counted in cross-sections (2 μm) of kidneys containing approximately 100 glomeruli. Quantitative analysis indicated that the percentage of glomeruli having crescent formations was significantly increased in the IL-12 group (C). Results are expressed as means ± SEM. *P<0.05 vs control group. Immunofluorescence findings showing glomerular IgA deposition in the PBS and IL-12 groups are shown in (D) and (E), respectively (magnification ×400). The severe IgA mesangial deposition decreased with IL-12 administration (E), and quantitative analysis confirmed a significant decrease in glomerular IgA deposition (F). The results are expressed as means ± SEM. *P<0.05 vs control group.
samples and biotinylated SNA or RCA-I was then applied. ALP-conjugated streptavidine (Oncogene Research Products, Darmstadt, Germany) was applied and binding to lectin and IgA were measured. To avoid artificial increases in binding caused by high concentrations, the samples were appropriately diluted to achieve nearly equal IgA concentrations. The ratio of lectin SNA and RCA-I bound IgA to total IgA was also calculated.

**Statistical analysis**

The results are expressed as means±SEM. Statistical significance was determined using the Mann–Whitney U-test (unpaired t-test). Differences in lectin binding IgA among groups were evaluated by one-way ANOVA, followed by Fisher’s protected least significant difference post hoc tests. Differences were considered significant if P-values were <0.05.

**Results**

**Characteristics of HIGA mice treated with IL-12**

There were no differences in body weights nor in spleen or kidney weights between the control and IL-12 groups (data not shown). Although the untreated HIGA mice showed mild proteinuria without haematuria, there were no increases in haematuria or proteinuria (data not shown). IL-12 administration caused slight increases in serum creatinine (0.67±0.25 mg/dl in controls and 0.84±0.65 mg/dl in the IL-12 group). Histologic analysis of IL-12-treated mice revealed prominent glomerular inflammatory lesions that included cellular crescent formations. These mice also had moderate glomerular hypercellularity and segmentally severe mesangial proliferation. Quantitative analysis revealed that the percentage of glomeruli with crescent formations was significantly increased in the IL-12 group (0.78±1.49% in controls and 2.75±1.54% in the IL-12 group, P<0.05; Figure 1A–C).

**Serum IgA and IgG levels after IL-12 administration**

In the present study, we had planned to administer IL-12 to HIGA mice for a longer period than in previous studies [6]. During PBS treatment, the HIGA mice showed a spontaneous increase in serum IgA after 3 weeks. In contrast, IL-12 treatment significantly suppressed this spontaneous elevation of serum IgA (closed circles). The results are expressed as means±SEM. *P<0.005 vs control group.

**Charge and size analyses of serum IgA**

We reported previously that serum IgA from HIGA mice is acidic and has polymeric or dimeric properties [5]. Following IL-12 administration, analysis of IEF fractionation of serum IgA revealed a significant decrease in IgA acidity (in controls, 1.35±0.33; and in the IL-12 group, 0.69±0.36; P<0.05; Figure 3A–C). Moreover, size fractionation showed a significant decrease in polymeric IgA (in controls, 1.08±0.09; and in the IL-12 group, 0.96±0.07; P<0.05; Figure 4A–C).

**Glomerular IgA deposition after IL-12 administration**

Immunofluorescence analysis revealed that glomerular IgA deposition was fainter in the IL-12 group than in the control group (Figure 1D–F). Quantitative analysis confirmed significant decreases in glomerular IgA deposition (83.08±38.20 in controls and 54.74±12.24 in the IL-12 group; P<0.05). In contrast, there were no differences in IgG deposition between the control and IL-12 groups (23.33±5.96 in controls and 27.14±5.91 in the IL-12 group).

**Charge and size analyses of eluted glomerular IgA**

We next evaluated the effect of IL-12 administration on IgA deposition in glomeruli. As shown in Figures 3 and 4, IL-12 administration decreased both acidic (Figure 3D–F) and polymeric IgA (Figure 4D–F) in the glomerular eluate.

**Abnormal glycosylation of serum and glomerular IgA in HIGA mice**

To determine whether a glycosylation abnormality exists in the serum and glomerular IgA in HIGA mice,
we performed lectin-binding assays using SNA to detect sialic acid and RCA-I for galactose residues. Serum IgA from HIGA mice showed significantly lower binding to lectin SNA (Figure 5A) and RCA-I (Figure 5B) than in control BALB/c mice. However, even though glomerular IgA from kidneys of HIGA mice also showed a low lectin binding that was similar to that of serum IgA, SNA binding was not as low as in the sera (ratio in BALB/c sera, 0.95 ± 0.30; in HIGA sera, 0.33 ± 0.23; and in HIGA glomerular eluate, 0.59 ± 0.09). There was no difference in RCA-I binding between serum and eluted glomerular IgA (ratio in BALB/c sera, 0.85 ± 0.40; in HIGA sera, 0.30 ± 0.14; and in HIGA glomerular eluate, 0.38 ± 0.05).

Glycosylation of serum and glomerular IgA after IL-12 administration

To examine the effect of IL-12 on the glycosylation of IgA, we compared the binding of serum IgA to SNA and to RCA-I, both before and after IL-12 administration. As shown in Figure 6, both sialylation and galactosylation were significantly higher in sera IgA after IL-12 administration. In controls, the sialylation ratio in controls was 0.30 ± 0.04 (before) and 0.29 ± 0.04 (after), and in the IL-12 group, 0.30 ± 0.03 (before) and 0.40 ± 0.05 (after; P < 0.0001). The galactosylation ratio in controls was 0.89 ± 0.06 (before) and 0.86 ± 0.09 (after), and in the IL-12 group, 0.86 ± 0.10 (before) and 1.25 ± 0.30 (after; P < 0.0001). In contrast, there were no differences in glycosylation of glomerular IgA deposition between controls and the IL-12 group. The sialylation ratio in controls was 0.34 ± 0.03 and in the IL-12 group was 0.36 ± 0.07, and the galactosylation ratio in controls was 1.04 ± 0.14 and was 1.02 ± 0.06 in the IL-12 group.

Discussion

In the present study, we examined in HIGA mice the physicochemical changes in IgA induced by IL-12, a cytokine that transforms Th cells into the Th1 subtype.
and promotes cellular immunity. Our results revealed that acidic and polymeric IgA in the glomerular eluate and in sera were markedly decreased after IL-12 treatment. Moreover, IL-12 treatment significantly increased sialylation and galactosylation of circulating IgA, although the glycosylation of IgA in glomerular eluate remained low. Not only the relative decrease in quantity but also physicochemical changes of circulating IgA may have affected the decrease in glomerular IgA deposition after IL-12 treatment.

In HIGA mice, there is an age-associated clonal expansion of IgA-producing B cells [5,7]. Furthermore, serum IgA levels and mesangial deposition markedly increase with age and are accompanied by an age-associated shift from Th1 to Th2 cells [6]. In the present study, the significant reduction in total IgA levels induced by IL-12 administration, especially the acidic and polymeric IgA forms in both the sera and glomerular eluate, confirmed a down-regulation of clonal expansion of IgA-producing B cells induced by the Th1 shift in HIGA mice. These specific alterations were observed exclusively in HIGA mice, and not in other strains, including BALB/c and C57BL/6 mice (data not shown).

We observed in HIGA mice significant reductions in sialylation and galactosylation in both circulating and glomerular IgA compared with BALB/c mice. Because IgA levels in HIGA mice are ~10-fold higher than in BALB/c mice [5], it is very likely that the amount of IgA available for reaction with galactosyltransferase and sialyltransferase becomes saturated, producing an under-glycosylation of IgA. We believe that an age-associated shift to Th2 dominancy provides a potent background for this phenomenon. In a recent report [8], stimulation of mouse B lymphoma CH12LX cells with IL-4 plus IL-5 caused a significant reduction in the terminal glycosylation of secreted IgA. To explain these phenomena, this same group suggested that Th2 cytokine stimulation, which accelerates humoral immunity by increasing the production of immunoglobulin, including IgA, causes a decrease in cytoplasmic glycosylation in B cells due to accelerated secretion. They additionally showed that increased production of Th2 cytokines leads to abnormalities in IgA glycosylation.
in a murine experimental model of IgAN [9]. Our lectin-binding assay revealed an increase in galactosylation and sialylation in circulating IgA after IL-12 administration. These data suggest that a shift from Th2 to Th1, induced by IL-12, may have reduced Th2 cytokine levels such as IL-4 and IL-5, producing the accelerated sialylation and galactosylation of IgA. Interestingly, and in contrast to serum IgA, IgA glycosylation abnormalities in the glomerular eluate were not altered by IL-12 treatment. This indicates that highly selective IgA, which remains under-glycosylated even after IL-12 treatment, can be deposited in the mesangium, although the quality is decreased.

In human IgAN, IgA1 has an abnormal pattern of O-glycans with a significant increase in the proportion of IgA1 molecules. Several lines of evidence indicate that in IgAN there is increased binding of GalNAc-specific lectins to circulating IgA1 in IgAN, suggesting an under-galactosylation of IgA1 hinge glycopeptides. These abnormal O-glycans may alter IgA1 clearance via interaction with hepatic asialoglycoprotein receptors (ASGPR), whose ligand is Gal on hinge O-glycans and which contributes to glomerular deposition of IgA. Furthermore, a recent report revealed a markedly higher affinity of mesangial IgA for GalNAc eluted from IgAN patients [10]. There are several differences in IgA molecules between humans and mice. Human IgA is divided into two subclasses, IgA1 and IgA2, whereas in mice there is no evidence for specific subclasses. In addition, human IgA is mostly monomeric, whereas murine IgA is mostly polymeric. Moreover, human IgA has O- and N-glycans, whereas murine IgA has only N-glycans [11]. Therefore, although it is clear that murine data should be
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In humans, under-glycosylation of IgA may represent at least one of the pathogenic factors underlying mesangial IgA deposition, possibly through alteration in the charge of the IgA molecule, in this strain of mice.

Sialylated IgA is representative of the level of anionic and acidic IgA. In humans, there are conflicting reports on IgA sialylation. Hiki et al. [12] found decreased sialylation and galactosylation in both circulating and glomerular IgA1 in IgAN patients. Leung et al. [13] showed that the O-glycan of polymeric-IgA1 from IgAN patients is over-sialylated. Allen et al. [14] reported that although fluorophore-assisted carbohydrate electrophoresis revealed an increased percentage of O-glycans with single GalNAc units, there was no difference in the percentage of sialylated glycans in total circulating IgA between IgAN patients and normal controls. These discrepant observations may arise from the fact that polymeric IgA represents only a small fraction of the total circulating IgA in humans [13]. Thus, it is difficult to clarify whether polymeric IgA is over-sialylated when only total IgA is studied. Although the contribution of sialylation to IgA acidity has not been completely clarified, there is agreement that acidic and negatively charged IgA1 play a role in IgA deposition [15].

It has been reported that under-glycosylated IgA1 favours self-aggregation and induces a significant increase in in vitro adhesion to extracellular matrix (ECM) proteins [3]. To explain this phenomenon, it was suggested that the deletion of sialic acid alters the charge of IgA1 molecules and results in appropriate conditions for localization of IgA1 molecules in glomeruli. This may occur by decreasing the electric repulsion between IgA molecules and the glomerular basement membrane. It is also possible that the novel IgA1 Fc receptor [1,2] in humans causes aggregation of under-glycosylated IgA1 to directly bind to mesangial cells.

However, we found in HIGA mice a marked decrease in acidic IgA despite increases in sialylation after IL-12 treatment. This paradoxical finding indicates that in contrast to human IgA, the change in sialylation is not the main factor determining the charge of IgA molecules in this strain of mouse. Although the mechanisms producing acidic IgA, other than sialylation, remain to be elucidated, it is very likely that the reduced acidification of IgA contributes to the decrease in glomerular deposition after IL-12 administration.

The increased crescent formations in the IL-12 group, despite significant decreases in serum IgA levels and IgA deposition, indicate mesangial IgA deposition may not play a predominant pathogenic role in inducing active inflammatory lesions in this strain. Alternately, it is possible that IL-12-activated Th1 induction mediated the increase in crescent formations. In experimental anti-glomerular basement membrane nephritis, the Th1 immune response and Th1-mediated delayed type hypersensitivity (DTH) are essential for crescent formation [16]. In addition, these lesions were accompanied by increases in CD4+ T cells and macrophages, an effect that is inhibited by Th2 cytokines. We previously reported that IL-12 treatment induces glomerular accumulation of T cells and macrophages in younger Th1 dominant HIGA mice, at an age when IgA deposition had not yet been established [17]. Furthermore, the crescent formations were observed only in the HIGA strain and not in other types of mice (data not shown). These results suggest that IL-12 induces decreases in both circulating IgA and IgA deposition but causes a simultaneous increase in active renal lesion through the Th1 immune response.

Given these effects of IL-12 treatment, we question whether cellular imbalances favouring the Th1 subset provide a detrimental or beneficial effect. The slight, but not statistically significant, worsening of renal function and crescent formation following IL-12 administration suggests a negative effect of this treatment. However, the short duration of the study makes prognosis difficult to determine. Nevertheless, we strongly suspect that further up-regulation of TGF-β after IL-12 administration [6] would accelerate nephrosclerosis during prolonged stimulation by this cytokine.

In summary, IL-12 administration not only decreased the quantity of IgA in sera and glomeruli from HIGA mice, but also changed the physicochemical qualities of under-glycosylated IgA by causing a shift to Th1. The results suggest a potent influence of the balance of Th1 and Th2 on the development of immunopathological lesions in this strain of mice.

Acknowledgements. This study was supported in part by a Grant-in Aid 11671034 from the Ministry of Education, Science and Culture of Japan. We thank Professor Gisho Honda and Dr Toshiaki Makino, for their advice and comments on size analysis by HPLC. We thank Dr Susumu Kobayashi for helpful discussion of the manuscript.

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Received for publication: 8.3.02
Accepted in revised form: 22.7.02