Influence of Estrogen on the Progression of Kidney Injury in Murine IgA Nephropathy

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Background: IgA nephropathy (IgAN) is characterized by the deposition of the IgA1-IgG immune complex in glomerular mesangial areas. The progression of IgAN in patients is more prominent in men than women. Grouped ddY (gddY) mice are a useful animal model for IgAN patients. However, the mechanisms underlying the gender difference in IgAN has not been clearly understood.

Method: We divided female gddY mice into 4 groups as follows: 4 weeks and 14 weeks control mice (4wFC, 14wFC), 14 week-old ovariectomy mice (14wOvx), and 17β-Estradiol (E2) replaced ovariectomy mice (14wO+E2). Levels of the urinary albumin creatinine ratio (ACR), serum IgA and IgA-IgG immune complex (IC) concentrations, and renal histopathological findings were examined. In order to elucidate the renal protective effects of E2, we examined estrogen receptors (Erα, Erβ), TGF-β1 mRNA expressions, and the number of F4/80 positive macrophages.

Results: The numbers of sclerotic glomeruli were increased in 14wFC compared with 4wFC. The numbers of advanced sclerotic glomeruli in 14wOvx were reduced by half in 14wO+E2. Urinary ACR and serum IgA and IgA-IgG concentration were increased in 14wOvx and recovered by E2 replacement. Similar changes were observed in the intensity of IgA, IgG, and C3 depositions in glomeruli by immunofluorescence. The numbers of F4/80 positive cells were increased in 14wOvx and recovered to the level of 14wFC in 14wO+E2. Erα mRNA was increased in 14wOvx but Erβ mRNA was increased in 14wO+E2 compared with 14wFC, suggesting exogenous E2 affects through Erα. TGF-β1 mRNA was increased in 14wOvx and recovered to the 14wFC level by E2 replacement.

Conclusion: Estrogen deficiency accelerates the progression of glomerular injury suggesting a contribution to the gender difference in IgAN. Macrophage infiltration, estrogen receptors (Erα, Erβ), and TGF-β1 metabolism may be involved in the progression of IgAN.

Key words: gddY, ovariectomy, Erα, Erβ

Introduction

IgA nephropathy (IgAN) is a disease characterized by the binding of galactose-deficient IgA1 (GdIgA1) or anti-GdIgA1 IgG autoantibody to produce IgA1/IgG immune-complexes (IgA1/IgG IC) that deposit in the renal glomerular mesangial areas1)-3). It is assumed that an upper respiratory tract infection and/or tonsillar mucosal infection impairs immune tolerance and stimulates B cells (CD19+B cells) or dendritic cells through TLR9/MyD88. Mucosal B cells may secrete circulating GdIgA1, which induces renal mesangial IgA deposition and kidney injury4)-6). An immunohistological analysis of renal biopsy tissues shows that mesangial cell proliferation and matrix expansion are associated with glomerular immune deposition of IgA1/IgG IC, C3, and/or IgM co-deposits. These
deposits remain the gold standard for the diagnosis and evaluation of the activity of IgAN\cite{1}-\cite{3}. Molecular approaches in the pathogenesis of IgAN have revealed that GdIgA1 may bind to fibronectin or collagens in the mesangial extracellular matrices, enhance the expression of inducible nitric oxide synthase, and release various mediators of renal injury that are not unique to IgAN such as angiotensin II, aldosterone, proinflammatory and profibrotic cytokines, and growth factors\cite{7}-\cite{10}. The ddY mouse, a spontaneous animal model of IgAN, was first reported in 1985\cite{11}. Our research group has established that early onset IgAN-prone “grouped ddY” (gddY) mice that will 100% develop IgAN within 8 weeks of age and has identified four marker loci (D1Mit216, D1Mit16, D9Mit252, and D10Mit86), which linked with the early onset-phenotype in a genome-wide association analyses. This model shows mild proteinuria without hematuria, glomerular IgA and IgG co-deposition, with a highly variable incidence and degree of glomerular injury due to the heterogeneous genetic background, and a similarity to human IgAN. Furthermore, it has been reported that renal failure worsens earlier in male mice than female\cite{12}. Therefore, we hypothesized that gddY mice can be a useful model for the observation of underlying molecular mechanisms of the sex difference on the progression of IgAN.

Despite of the conflict effects of breast cancer, the potentially protective effects of estrogen on neuron, bone, cardiovascular tissue, kidney, endocrine organ, liver and immune system were reported. The estrogen activates through the estrogen receptors (Erα, β) and estrogen-related receptors signaling pathways and regulates the splice in the target cells to exhibit inflammatory or anti-inflammatory effect on each organ\cite{13}-\cite{15}. It has been reported that E2 also exerts potent anti-oxidative effects that may contribute to a protective effect for the female gender during the course of renal diseases by modulating cell proliferation and the synthesis and degradation of collagen and proteoglycans. In addition, estrogen may indirectly influence these processes by modulating the synthesis and release of vasoactive agents, cytokines, and growth factors, which in turn are capable of altering renal mesangial cells, tubular epithelial cells, and interstitial cell function\cite{16}-\cite{18}.

Several experimental studies with glomerulosclerosis- (GS) susceptible mice have indicated that an estrogen deficiency may alter the balance of renal development and contribute to progression of GS. It was reported that ROP mice develop GS during their life span in response to nephron reduction\cite{19} and that ovariectomized female GS-prone ROP mice decreased not only mRNA expressions of Erα and Erβ, but also ER transcriptional activities in glomeruli and mesangial cells (MCs)\cite{20} \cite{21}. However, E2 replacement to physiological levels could not prevent progression in ovariectomized GS-prone ROP mice that exhibited increased macrophage infiltration, an accumulation of extracellular matrices, predominantly of laminin, and a marked distortion of the glomerular architecture\cite{21}. Moreover, estrogens have generally been shown to have an anti-fibrotic and anti-apoptotic effect\cite{17} \cite{18}, while androgens have been shown to increase prosapoptotic and profibrotic signaling\cite{17} \cite{22} \cite{23}.

The objective of the present study was to determine the E2 replacement state that would have protective effects on the progression of kidney injury in IgAN prone gddY mice, and if it did, by what mechanism. We hypothesized that estrogen deficiency due to ovariectomy may impair the ER balance\cite{20} \cite{21} of kidneys. Since E2 may play a protective role by decreasing serum dihydrotestosterone (sDHT)\cite{24} \cite{25} and inhibiting macrophage infiltration, we measured the serum levels of DHT and counted the number of macrophages in the renal interstitium. Furthermore, we examined whether an E2 deficiency effects TGF-β mRNA expression, resulting in the progression of IgAN-prone gddYmice\cite{17} \cite{18} \cite{20}. These examinations then would partially elucidate the renal protective effects of E2.

Materials and Methods

1. Experimental model design

The experimental protocol of this study was approved by the Ethics Review Committee for Animal Experimentation of the Juntendo University Faculty of Medicine, Tokyo, Japan. The gddY mice were established through the selective mating of early-onset IgA prone ddY mice for more than 20 generations\cite{12}. This resulted in 100% proteinuria, mesangial cell proliferation, and mesangial matrix expansion with gdIgA1 depositions at 8 weeks. The
mice were maintained in specific pathogen free (SPF) room at the animal facility of Juntendo University, Tokyo, Japan.

Fifteen female gddY mice were divided into four groups. The mice of 2 control groups were sacrificed at 4 weeks (4wFC; N=4) and 14 weeks (14wFC; N=4) of age. Ovariectomies were performed on seven of the mice ovariectomy at 6 weeks of age and 3 of them were replaced with estrogen pellets until 14 weeks of age, and then sacrificed (14wO+E2; N=3). Another 4 ovariectomized mice with any therapy were sacrificed at 14 weeks of age (14wOvx; N=4).

2. Ovariectomy via the dorsal route and replacement of E2

Ovariectomies were performed using inhalational anesthetic with isoflurane (Mylan Pharmaceuticals Co, Ltd. Tokyo, Japan). Dorsal incision 1.5 cm long, between the middle of the back and the base of the tail. Incisions of the muscles were made bilaterally. After the peritoneal cavities were accessed, the ovaries were surrounded by a variable amount of fat. The fat tissue was connected with the kidney capsule. Using an electric knife (V-10, BRC, Tokyo, Japan) to separate the fat tissue, we removed the ovaries. The fallopian tubes and blood vessels were ligated with the electric knife. After ensuring that no bleeding was present, the peritoneal cavity was closed using three single catgut stitches (Matsuda Sutures Tokyo, Japan).

In the 14wO+E2 group mice, as mentioned above, we implanted an E2 pellet (0.18 mg, 60-day slow release, 3 mm in diameter, SE-121; IRA, Sarasota, FL, USA) into the subcutaneous tissue of the neck after a dorsal incision using small size tweezers. The doses of E2 are frequently used by researchers and have been claimed to establish physiologically safe concentrations of E2 (4.6–21 pg/ml during oestrus to 57–88 pg/ml during pro-oestrus) and exert a beneficial effect in mice. At 4°C for 5 minutes and stored at ~80°C. These samples were collected at 4 and 14 weeks of age. Serum IgA and IgA–IgG IC were measured using a sandwich ELISA kit (Bethyl Laboratories, Montgomery, TX) using a modified method based on our previous report. Purified rat anti-mouse IgG antisera (BD Biosciences, Pharmingen, San Diego, CA) and horseradish peroxidase-conjugated goat anti-mouse IgA (Zymed Laboratories, San Francisco, CA) were used for immunofluorescence (IF). Serum dihydrotestosterone (sDHT) was measured with a sandwich ELISA kit (Cusabio, Wuhan, China). Albuminuria was defined according to the albumin/creatinine ratio (ACR μg/mg) using a DCA 2000 immunoassay system (Siemens Healthcare Diagnostics, Tokyo, Japan).

4. Histological analysis

Kidneys were removed after perfusion with normal saline. For light microscopy, specimens obtained from the renal cortex were fixed in 20% formaldehyde and embedded in paraffin, and 3-μm sections were collected. These sections were stained with hematoxylin and eosin (H-E), and periodic acid-Schiff (PAS). For the quantitative analysis of glomerular sclerosis (GS), global glomeruli/cross-sections were observed and assigned the following scores based on previous reports, as follows: 0 points, no GS; 1 point, mild GS (approximately 25%); 2 points, moderate GS (approximately ≤50%); and 3 points, severe GS (approximately ≤75%). The sclerosis scores were calculated as follows: [Σsevere glomerular sclerosis number of global kidney section/glomerular number global kidney section].

5. Immunofluorescence (IF) staining

Kidney specimens were mounted in an optimal cutting temperature (OCT) compound (Sakura Finetek, Tokyo, Japan) and then stored at ~80°C. The specimens were cut into 3-μm sections and fixed with acetone at ~20°C for 5 min. A DyLight 594-conjugated goat anti-mouse IgA antibody (ab, 13442, Abcam, Tokyo, Japan), Alexa 488-conjugated goat anti-mouse IgG antibody (Invitrogen AG, Basel, Switzerland), and FITC-conjugated rat monoclonal anti-mouse C3 antibody (11H9 sc-58926 Santa Cruz, California, USA) were used for these IF staining. After overnight an incubation
at 4℃ and a washing with PBS 3 times, the slides were mounted with a mounting medium (Dako, Tokyo, Japan). These samples were imaged using confocal laser microscopy (Olympus Corporation, Tokyo, Japan).

6. Immunohistochemistry
The kidney sections for the immunohistochemistry of F4/80 were fixed with acetone at −20℃ for 5 min. After blocking with 0.3% H2O2 in 70% methanol/PBS at room temperature (RT) for 30 min, they were incubated with a rat monoclonal anti-mouse F4/80 antibody (Cl: A3-1 ab6640 Abcam, Tokyo, Japan) at 4℃ overnight. After washing with PBS 3 times, the slides were incubated with an HRP-conjugated rabbit anti-rat IgG antibody (Dako, Tokyo, Japan) at RT for 30 min. After washing three times with PBS and once with water, the slides were incubated with a DAB substrate solution (Dako, Campintienia, USA) for 5 min, which resulted in a brown staining. After counterstaining with hematoxylin for 5 min and washing with water, we finally mounted them with a mounting medium (Dako, Tokyo, Japan). The sections were observed with a light microscope (Olympus Corporation, Tokyo, Japan). The numbers of F4/80 positive cells were counted in the kidney sections of each mouse at each time point.

7. Real-time polymerase chain reaction (PCR) assays
Total RNA was extracted from the cortex of kidneys with the TRIzol reagent (Invitrogen AG, Basel, Switzerland) and RNeasy mini kit (Qiagen). PCR was performed using a Fast SYBR Green master mix (Applied Biosystems) and a 7, 500 real-time PCR system (Applied Biosystems). The results were quantified using a standard curve generated from the analysis of serial dilutions of a reference cDNA prepared from the kidneys from other gddY mice, and data were normalized to those of GAPDH mRNA. We performed a quantitative real-time PCR to determine the levels of GAPDH, \(\text{Er}^{\alpha}\), \(\text{Er}^{\beta}\), and TGF-\(\beta\)1 mRNAs using the primers (Invitrogen, California, USA) shown in Table-1.
were observed in serum concentration of IgA–IgG IC. The histologic changes in the glomeruli, such as mesangial cell proliferation and mesangial matrix expansion, were observed in 14w of age and estrogen depletion by Ovx increased in the degree of severity, while the replacement of E2 showed a recovery of about half (Figure-1A, B). Increased glomerular deposits of IgG, IgA, and C3 were also observed coincident with renal histopathologic damage appearance, as shown in Figure-1C.

Figure-1  E2 affected IgAN progression in gddY mice
A: Representative histologic appearance of glomerular lesion. Numbers of glomerulosclerosis (GS)/section of each group. B, C: PAS staining and immunofluorescence staining of IgA, IgG, merge by IgA plus IgG and C3. *p<0.05. Original magnification: ×400.

Figure-2  Serum concentrations of DHT (sDHT) in 4wFC, 14wFC, 14wOvx, and 14wO+E2 mice
Increased levels of sDHT were observed in 14wOvx and recovered by E2 replacement in 14wO+E2. Means±SDs are shown *p<0.05.

Figure-3
A: The immunohistochemistry of F4/80. Severe F4/80 positive cells infiltration was seen in 14wOvx mice. Original magnification: ×200 and ×400. B: Numbers of F4/80 positive cells in each group. Means±SDs are shown: *p<0.05. **p<0.01. ***p<0.005.
2. E2 replacement therapy restored sDHT

The concentration of sDHT was increased in 14wOvx and was reversed in 14wO+E2 to the level of 14wFC. The result indicates that the E2 replacement by E2 pellets restored E2 levels after ovariectomy (Figure-2).

3. Interstitial macrophage infiltration

Interstitial infiltrations of F4/80 positive cells, i.e. macrophages, are shown in Figure-3. By immunohistochemistry, F4/80 positive macrophages were diffusely observed in the interstitium in 14wOvx. The increased F4/80 positive macrophages in 14wOvx were markedly reduced by E2 replacement (14wO+E2) to the levels of 14wFC. These results suggest that E2 may play a protective role by decreasing sDHT and inhibiting macrophage infiltration.

4. Ovariectomy (Ovx) increased Erα and TGF-β1 mRNA expression

Erα and TGF-β1 mRNA were increased by Ovx and reversed by E2 replacement (Figures-4A, C). However, Erβ was not changed by Ovx but increased by E2 replacement (Figure-4B).

Discussion

The objective of this study was to examine the pathological roles of estrogen in murine IgA nephropathy (IgAN) and elucidate its underlying mechanisms, since a gender difference is one of the key factors in the prognosis of this disease. Since it has been postulated that female sex hormones have renal protective effects in mice with severe glomerulosclerosis (GS), we divided female gddY mice, i.e. animal model mice for human IgAN, into 3 groups, (1) 14w-old control mice (14wFC), (2) ovariectomized at 6w-old (14wOvx) mice, and (3) E2 replacement after the ovariectomy at 6w-old (14wO+E2) mice and compared the histological changes in their kidneys. As a result, the ovariectomy worsened not only ACR but also GS with immunohistological changes and interstitial macrophage infiltrations, consistent with urinary ACR and serum IgA and IgA-IgG IC concentrations, although statistically significant difference was not recognized the ACR between 14wOvx and 14wO+E2 because of a limited number of mice. Furthermore, E2 replacement recovered some of these, indicating that the estrogen has protective effects on GS in gddY mice. Therefore, it may suggest that an estrogen deficiency contributes to
the development and progression of GS in postmenopausal women\textsuperscript{16-18}. In addition, we found a significant increase of F4/80 positive macrophages in the interstitium in 14wOvx, that was recovered by E2 replacement. This result may further indicated the involvement of macrophages in the renal progression of gddY mice. Since sDHT increased by ovariectomy\textsuperscript{24} 25), as shown in 14wOvx mice, it might also contribute to the progression of kidney injury in this mice model\textsuperscript{17} 22) 23). Thus, these results seemed to be correlated with sDHT and with renal histopathological changes, including F4/80 positive cell infiltration.

Recently, it has been proposed that IgAN may be an autoimmune disease in which the B–cells are aberrantly activated by innate-immune systems and thus secrete nephritogenic IgA and related autoantibodies, which leads to glomerular deposition and subsequent kidney injury\textsuperscript{4-6}. On the other hand, it has been long believed that E2 is not only a sex hormone but also an important regulator of the immune system. E2 exerts its effect through two nuclear receptors, i.e. ER\textsuperscript{α} and ER\textsuperscript{β}, with cooperative-interaction on B-cell maturation and activation playing pro-inflammatory and anti-inflammatory roles in each organ\textsuperscript{13-15}. In this study, we found that mRNA expressions of ER\textsuperscript{α} and ER\textsuperscript{β} were decreased in 14wFC compared with 4wFC. Interestingly, ER\textsuperscript{α} increased by ovariectomy was restored to previous levels by E2 replacement. However, ER\textsuperscript{β} did not change through ovariectomy but increased after E2 replacement. We could not explain the difference at this time, although, ER\textsuperscript{β} exhibits an inhibitory action on ER\textsuperscript{α}-mediated gene expression and in many instances opposes the actions of ER\textsuperscript{α}\textsuperscript{15}.

It has been reported that estrogen alters B-cell development to decrease lymphopoiesis and increase the frequency of B-cells appearance in marginal zone. Both ER\textsuperscript{α} and ER\textsuperscript{β} are required for complete down-regulation of B lymphopoiesis, while only ER\textsuperscript{α} is needed to up-regulate immunoglobulin production in both the bone marrow and spleen\textsuperscript{28} 29). Therefore, it might be a specific change in gddY mice, suggesting abnormalities in their B-cell function.

Since it have reported that the ER\textsuperscript{α} was activated by testosterone and DHT\textsuperscript{30}, not only deficiency of estrogen and progesterone but high DHT may induce high ER\textsuperscript{α} expression\textsuperscript{30-32} that contribute the secretion of IgA1 by B-cells.

In this study, we measured the mRNA expressions of TGF–β1 mRNA, which was slightly increased by ovariectomy and recovered by E2 replacement. These results support the idea that TGF–β1 plays a central role in promoting progressive kidney injury\textsuperscript{13} 22) 23), and estrogen negatively regulates TGF–β1, contributing to renoprotective effects\textsuperscript{17} 18).

In conclusion, an estrogen deficiency by ovariectomy accelerates the progression of the glomerular injury, further suggesting a contribution of gender difference in IgAN. Macrophage infiltration and TGF–β1 may be involved in the process. Since the physiological level of E2 replacement was not enough to prevent the progression of the glomerulosclerosis (GS), other protective factors aside from E2 lost by ovariectomy, may be involved in the gender difference of gddY mice. These mice may provide useful insights into the pathogenesis of IgAN, such as effect of gender differences on the progression of human IgAN.

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Conflicts of interest

The authors declare no conflict of interest associated with this manuscript.

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