Different Effects of Castration and Estrogen Administration on Glomerular Injury in Spontaneously Hyperglycemic Otsuka Long-Evans Tokushima Fatty (OLETF) Rats

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Key Words

Castration • Estrogen • Glomerulosclerosis • Diabetes mellitus • Rat

Abstract

Aim: Non-insulin-dependent diabetic mellitus model rats, Otsuka-Long-Evans-Tokushima-Fatty (OLETF), develop diabetic nephropathy presenting with mesangial expansion leading to glomerular sclerosis and thickening of the glomerular basement membrane (GBM), especially in elderly males. The effects of sex hormones and castration on the incidence of diabetes mellitus (DM) have been studied in this strain rat. However, there have been no detailed studies on the effects of castration and sex hormone in the development of diabetic nephropathy. **Methods:** In this study we examine the effect of castration or estrogen on the development of glomerular injury in OLETF rats. Thirty male OLETF rats and 10 male long-Evans Tokushima Otsuka (LETO) rats as a normal control were used. OLETF rats were divided into three groups: group 1 received sham-operation, group 2 was castrated at 6 weeks, and group 3 was administered 0.1 mg estrogen subcutaneously once a month from 6 weeks to 58 weeks of age and LETO rats were assigned

to group 4. Body weight, urinary protein and fasting blood glucose, serum albumin and other serum constituents were investigated every 12 weeks from 12 weeks to 60 weeks of age. In groups 1–3, glucose tolerance test was performed at 38 weeks. Each group was studied morphologically at the end of the experiment (60 weeks of age). **Results:** Castration attenuated proteinuria and glomerular sclerosis accompanied by an amelioration of glucose tolerance, a decrease in mesangial expansion and an attenuation of the GBM thickening. In contrast, although estrogen equally ameliorated glucose tolerance and attenuated the mesangial expansion and the GBM thickening, estrogen failed to attenuate proteinuria and glomerulosclerosis. A significant increase in glomerular tuft volume, and serum levels of growth hormone, total cholesterol and triglycerides was observed in the estrogen-treated rats as compared with the castrated rats. **Conclusion:** Besides the mechanisms involved in the development of diabetic nephropathy, other mechanisms may be involved and contribute to the development of glomerulosclerosis in the estrogen-treated rats, leading to a difference in glomerular injury between the castrated and estrogen-treated OLETF rats.

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Introduction

A spontaneously diabetic rat with polyuria, polydipsia, and slight obesity was discovered in an outbred colony of Long-Evans (LE) rats, which had been purchased from Charles Rive Canada (St. Constant, Quebec) in 1982 and subsequently maintained at the Tokushima Research Institute, Otsuka Pharmaceutical (Tokushima, Japan). A diabetic strain was established by selective breeding in 1990 and has been named the Otsuka-Long-Evans-Tokushima-Fatty (OLETF) strain [1]. From the same LE stock, a control line, Long-Evans Tokushima Otsuka (LETO) has also been established [1]. This non-insulin-dependent diabetes mellitus model OLETF rats show a sex difference in the incidence of diabetes mellitus. Almost 100% of male OLETF rats develop diabetes at 25 weeks of age, whereas only 30% of the female are affected after 60 weeks.

The sex difference and the role of sex hormones in the development of diabetes have been investigated [2–4]. However, there have been no detailed studies on the effects of castration and sex hormone in the development of diabetic nephropathy because of a lack of typical models of a spontaneous diabetic nephropathy rat, although the effects of sex hormones and castration on the incidence of diabetes mellitus (DM) have been studied in this strain rat [5, 6]. Because the diabetic glomerular lesions have been reported to occur in elderly OLETF rats [7], in this study we examine the effect of castration or estrogen on the development of glomerular injury in elderly OLETF rats.

Materials and Methods

Experimental Design

Thirty male OLETF rats and 10 male LETO rats were obtained from Tokushima Research Institute, Otsuka Pharmaceutical. They were fed standard rat chow (Clea Japan Inc., Tokyo, Japan) containing 0.39% NaCl and 24.8% protein, and OLETF rats were divided into three groups of 10 rats. At 6 weeks of age, group 1 (control OLETF) received sham-operation and group 2 (Cast) received castration. Group 3 (Est) received estrogen treatment and LETO rats were assigned to group 4 (LETO). Estradiol valerate, purchased from Japan Schering Co., was diluted in sesame oil to a concentration of 1 mg/ml and the vehicle or the selected dose with 0.1 ml of volume was given subcutaneously once a month from 6 to 58 weeks of age according to the method reported before [8]. The estrogen dose was determined based on our previous study [8].

Blood pressure (BP), body weight (BW), urinary protein, fasting blood glucose, serum albumin (Alb), total cholesterol (Tchol), blood urea nitrogen (BUN) and serum creatinine (Cr) were investigated every 12 from 12 weeks to 60 weeks of age. Systolic BP was measured

in conscious rats by the tail cuff method, with warming for 10 min at 37 °C before measurement. At 60 weeks of age, 10 rats of each group were exsanguinated from the aorta and trunk blood was collected. The study was performed in accordance with the guidelines of the Animal Experimental Guide of Saga Medical School.

Twenty-four-hour urinary protein was measured by the pyrogallol red method [9]. Fasting blood samples were obtained via percutaneous puncture of the tail vein. Serum protein was measured by the Biuret method [10] and the protein fraction was analyzed electrophoretically. BUN was measured by the urease indophenol method and serum creatinine by using Jaffe's method [11]. Serum cholesterol was determined by an enzymatic method [12]. Blood glucose was measured by the glucose dehydrogenase method [13]. Serum testosterone and growth hormone (GH) were determined by employing a radioimmunoassay [14].

At 38 weeks of age, glucose tolerance tests were performed. The rats were fasted overnight followed by intraperitoneal glucose injection (2 g/kg body weight). Whole venous blood was obtained from the tail vein at 0, 30, 60, 90 and 120 min after the injection.

Renal Histological Examination

Animals were sacrificed at 60 weeks of age. The abdominal aorta was catheterized retrogradely. Firstly, the right kidney for electron microscopy was removed after clamping the right renal artery and vein. Subsequently, the left kidney for morphometric analysis was flushed with saline and perfused directly for 3 min at a pressure of 100 mm Hg with 10% neutral buffered formalin and removed. Coronal slices were embedded in paraffin for light-microscopic study. Sections of 2 µm in thickness were stained with hematoxylin and eosin, periodic acid-Schiff reagent (PAS) and periodic acid-methenamine silver. Histological evaluation was done by a pathologist (S.A.) blind to the origin of the samples. The degree of glomerular sclerosis was evaluated semiquantitatively after Raij et al. [15]. At least 100 glomeruli were examined. The severity of the lesions was graded from 0 to 4+ according to the percentage of glomerular sclerosis, where 1+ = 25%, while 4+ = 100%. An injury score was then obtained by multiplying the severity of damage (0 to 4+) by the percentage of glomeruli with the same degree of injury. The extent of injury for each individual tissue specimen was then obtained by adding the scores. Tubulointerstitial change including tubular dilatation, atrophy, proteinaceous casts, interstitial edema and mononuclear cell infiltrates was graded from 0 to 4+ according to the method described previously [16].

Mesangial expansion was determined by the automatic image analyzer, using the modification of the method described previously [17]. Ten glomeruli with cross-section through their vascular poles was randomly chosen from 5 rats of each group. We measured the total glomerular area and the PAS-positive staining glomerular area and then calculated the percentages of PAS-positive areas with respect to the total glomerular areas (fractional mesangial volume).

Glomerular tuft volume (GV) was determined as described by Weibel [18] with a slight modification. Histological sections were projected on a video image (enlargement $\times 200$), using a light microscope and a drawing tube. Outlines of the capillary tufts on the video image were traced manually with an overlay board; these areas were measured using an image analysis system (Videoplan, Kontron Bildanalyse, Munich, Germany). Mean glomerular random cross-section areas (A_m) were determined in at least 50 glomeruli encountered on a serpentine course between the cortex and the medulla in each animal. Thus no glomerulus was counted more than once and all levels of

Fig. 1. Changes in body weight in control (○), castrated (●), estrogen-treated (□) OLETF rats and LETO rats (■). ^a $p < 0.05$, ^b $p < 0.01$, ^c $p < 0.001$ vs. control OLETF.

cortex were evenly sampled. Mean volume of capillary tufts was calculated using the equation derived by Weibel [18]:

$$V = (\beta/\alpha)(Am)^{3/2},$$

where $\alpha = 1.1$ is the size distribution coefficient and $\beta = 1.38$ is the shape coefficient for spheres (the assumed shape of glomeruli).

Tissues for electron microscopy were fixed with 3% glutaraldehyde, washed in cacodylate buffer, postfixed in osmium tetroxide, dehydrated in graded alcohols and embedded in Epon 812. Ultrathin sections were double-stained with uranyl acetate and lead citrate and observed with an electron microscope. Glomerular basement membrane (GBM) thickness was measured in 5 rats of each group by the orthogonal intercept method described by Jensen et al. [19]. In brief, a grid with eight evenly spaced intersecting lines (four horizontal and four vertical) was placed over each photomicrograph and the GBM measurement was made at each point that a line on the grid intercepts an endothelial-GBM interface. The width was measured on a line orthogonal to the edge of the GBM at the endothelial side of the intercept. A mean of 100 measurements was obtained on 2–3 glomeruli. Actual GBM thickness was obtained by the harmonic mean multiplied by 8/3 p [19].

Statistical Analysis

Data were analyzed using StatView J-4.5, a commercial statistics program (Abacus Concepts, Inc. Berkeley, Calif., USA). Data were first analyzed for distribution. Results were expressed as arithmetic mean values $\pm$ SD. The other data were tested by one-way or two-way analysis of variance (ANOVA) and the method of Bonferroni/Dunn for multiple comparisons was applied if the variance ratio (F) reached statistical significance ($p < 0.05$).

Results

Body Weight

The effect of castration or estrogen on growth rate is shown in figure 1. BW in the control OLETF rats was increased until 36 weeks of age, but thereafter growth rate was stunted and reduced. BW in Cast rats or Est rats was also significantly increased, though lower than that of controls before 36 weeks, but thereafter growth rate was not stunted and significantly increased as compared with that of controls until 60 weeks of age. LETO rats grew with age without any reduction of BW, though growth rate was stunted significantly as compared with that of Cast or Est rats throughout the experimental period.

Blood Pressure

The systolic blood pressure was measured at five points during the experiment and the mean BPs of five points of control OLETF rats, Cast rats, Est rats and LETO rats were as follows: 163 ± 12 , 158 ± 13 , 173 ± 19 and 148 ± 18 mm Hg, respectively. There were significant differences in BP among the four groups, when compared using two-way ANOVA with repeated measure. BPs in LETO rats were significantly lower than those of other three groups ($p < 0.05$ for each) and BPs in Cast rats were significantly lower than those of Est rats ($p < 0.05$). There were no significant differences between control OLETF rats and Cast rats or Est rats.

Fig. 2. Changes in urinary protein excretion rates in control (○), castrated (●), estrogen-treated (□) OLETF rats and LETO rats (■). When compared with two-way ANOVA, significant differences are observed between control OLETF rats and castrated OLETF rats or LETO rats ($p < 0.001$ for each).

Proteinuria

Urinary protein excretion rates are shown in figure 2.

Control OLETF rats became more proteinuric than LETO rats from 36 weeks of age and its excretion rates reached a mean value of 271 ± 53 mg/kg/day at 60 weeks of age. Castration significantly reduced the urinary protein excretion rates to levels of LETO rats (Cast rats 29 ± 23 mg/kg/day, LETO rats 14 ± 2 mg/kg/day at 60 weeks), while estrogen treatment did not influence its rates which were almost equal to those of control OLETF rats throughout the experimental period (Est rats 300 ± 139 mg/kg/day at 60 weeks).

Blood Glucose and Glucose Tolerance Test

Fasting blood glucose levels were measured at five points during the experiment and the mean blood glucose levels of five points of control OLETF rats, Cast rats, Est rats and LETO rats were as follows: 186 ± 29 , 199 ± 25 , 194 ± 52 and 137 ± 27 mg/dl, respectively. There were significant differences in blood glucose levels among the four groups, when compared using two-way ANOVA with repeated measure. Blood glucose levels were higher in control OLETF rats, Cast rats and Est rats than LETO rats ($p < 0.001$ for each), but no differences were observed among the three OLETF groups. As shown in figure 3, castration and estrogen administration equally ameliorated glucose tolerance ($p < 0.03$, control OLETF rats vs. Cast rats or Est rats).

Fig. 3. Glucose tolerance test at 38 weeks of age in control (○), castrated (●) and estrogen-treated (□) OLETF rats. When compared with repeated measure ANOVA, significant differences are observed between the control OLETF rats and the castrated or estrogen-treated OLETF rats ($p < 0.05$ for each).

Organ Weight and Serum Constituents

As shown in table 1, castration reduced significantly kidney weight (KW) to levels of the LETO rats and tended to reduce heart weight (HW). Estrogen treatment slightly reduced KW, but did not influence HW. Castration resulted in higher albumin levels and a lesser impairment

Table 1. Serum constituents at the end of the experiment (60 weeks of age)

Group	n	KW g	HW g	Albumin g/dl	T chol mg/dl	TG mg/dl	BUN mg/dl	GH ng/ml	Testosterone ng/dl
G1: control	10	2.20±0.18	2.16±0.40	3.7±0.4	133±23	199±42	22.0±7.0	16.9±2.3	19.9±11.1
Gs: Cast	10	1.31±0.26	1.86±0.27	4.3±0.5	106±29	158±66	13.9±2.3	21.0±6.8	<5
G3: estrogen	10	1.90±0.27	2.31±0.30	3.9±0.4	215±39	280±81	19.0±6.1	46.4±10.8	9.5±4.5
Ge: LETO	10	1.36±0.10	2.09±0.50	4.3±0.2	103±5	n.d.	15.9±2.5	n.d.	n.d.
p G1 vs. G2		<0.001	NS	<0.01	NS	NS	<0.01	NS	<0.005
G1 vs. G3		<0.01	NS	NS	<0.001	<0.001	NS	<0.001	<0.01
G1 vs. G4		<0.001	NS	<0.01	NS		<0.05		
G2 vs. G3		<0.001	<0.05	NS	<0.001	<0.001	NS	<0.001	<0.01
G2 vs. G4		NS	NS	NS	NS		NS		
G3 vs. G4		<0.001	NS	NS	<0.001		NS		

TG = Triglycerides; NS = not significant; n.d. = not done.

of renal function with regard to BUN levels as compared with those of control OLETF rats, though no significant difference in serum creatinine levels was observed among the four groups (data not shown). In contrast, estrogen administration did not exert any beneficial effects on either albumin or BUN levels which were almost equal to those of control OLETF rats and significantly increased serum Tchol and triglycerides levels. Castration or estrogen treatment significantly reduced serum testosterone levels, to a greater extent in Cast rats than in Est rats. Castration did not influence serum GH levels, while estrogen treatment significantly increased those levels.

Pathology

Histological abnormalities included focal and segmental glomerular hyalinosis and sclerosis with increased mesangial matrix substance, compatible with features of diabetic nephropathy, in which the obliteration of glomerular capillary lumina and adhesions to Bowman's capsule were frequently observed in control OLETF rats, but typical mesangial nodular lesions were rarely encountered. The results of morphological analysis are presented in figure 4. Castration or estrogen administration significantly attenuated mesangial expansion close to level of LETO rats. Fractional mesangial volume (fig. 4A) was significantly bigger in control OLETF rats than in Cast, Est and LETO rats ($14.4 \pm 1.8\%$ in control OLETF rats vs. $9.8 \pm 0.5\%$ in Cast rats, $10.7 \pm 1.3\%$ in Est rats and $8.9 \pm 1.4\%$ in LETO rats, $p < 0.001$ for each). There were no significant differences in this respect among Cast, Est and LETO rats. The results of GV appear in figure 4B. Castra-

tion significantly reduced the mean GV to levels close to those of LETO rats ($1.86 \pm 0.26 \mu\text{m}^3 \times 10^6$ in control OLETF rats, $1.34 \pm 0.18 \mu\text{m}^3 \times 10^6$ in Cast rats and $1.23 \pm 0.08 \mu\text{m}^3 \times 10^6$ in LETO rats), while estrogen administration did not reduce the GV ($1.88 \pm 0.24 \mu\text{m}^3 \times 10^6$ in Est rats), which was equal to those of control OLETF rats.

The electron microscopic examination revealed no electron-dense deposits in any of four groups even at 60 weeks of age. As shown in figure 4C, castration and estrogen administration significantly reduce the thickness of the GBM (548 ± 52 nm in control OLETF rats vs. 470 ± 38 nm in Cast rats or 482 ± 34 nm in Est rats) to levels close to those of LETO rats (461 ± 14 nm in LETO rats).

The incidences of glomerulosclerotic lesions were $16.7 \pm 5.2\%$ (control OLETF rats), $3.3 \pm 2.8\%$ (Cast rats), $27.1 \pm 17.8\%$ (Est rats) and $0 \pm 0\%$ (LETO rats), respectively. In control OLETF rats, mild tubulointerstitial changes (mean score 1.2 ± 0.4) were noted in association with glomerular sclerotic changes. Castration significantly attenuated glomerular injury (sclerosis index, SI, 5.7 ± 3.9 , $p < 0.001$) and tubulointerstitial changes (mean score 0.4 ± 0.5 , $p < 0.01$). In contrast, estrogen treatment failed to attenuate both glomerular injury (SI, 39.8 ± 22.1) and tubulointerstitial changes (mean score 1.5 ± 0.8) which were almost equal to those of control OLETF rats (fig. 4D). There were no significant differences in SI and tubulointerstitial changes between control OLETF rats and Est rats. In LETO rats as a normal control, neither glomerulosclerotic nor tubulointerstitial lesions were observed.

Fig. 4. Morphological data at 60 weeks of age.

We investigated the association of SI with GV, serum GH and Tchol levels in all three OLETF groups. There was no significant correlation between SI and GH levels ($r = 0.103$, $p = 0.667$), while a statistically significant correlation was found between SI and the GV ($r = 0.532$, $p < 0.01$) or Tchol ($r = 0.611$, $p < 0.005$).

Discussion

Testosterone has been shown to reduce glucose tolerance [20] and probably thereby to promote the development of DM [5, 6], whereas estrogen reportedly suppressed or delayed the development of DM [21]. In fact, in OLETF rats orchiectomy significantly reduces the de-

velopment of DM in males, whereas ovariectomy enhances its development in females [5, 6]. In this experiment, castration or estrogen treatment significantly improved glucose tolerance and reduced serum testosterone levels, which suggested that each treatment might suppress or delay the development of DM.

Mesangial expansion and thickening of the GBM are the hallmarks representing histologically the initial stage of diabetic nephropathy [22]. The occurrence of diffuse mesangial lesions seems to be major contributing factor to the development of glomerular sclerosis found in the late stage of diabetic nephropathy [23]. The OLETF rat has been reported to exhibit these typical findings of diabetic nephropathy such as mesangial lesions and focal/segmental sclerosis and thickening of the GBM [7]. In this study, castration or estrogen treatment equally improved glucose tolerance, but exhibited a different effect on glomerular injury. Castration attenuated proteinuria, glomerular sclerosis, mesangial expansion, and GBM thickening. On the other hand, estrogen treatment did not attenuate proteinuria or glomerular sclerosis despite having ability to attenuate mesangial expansion and GBM thickening. These findings suggest that besides the mechanisms involved in the development of diabetic nephropathy, other mechanisms may contribute to progression of glomerulosclerosis in the estrogen-treated OLETF rats.

In contrast to the present study, castration in rats with streptozotocin diabetes decreased blood pressure, and attenuated GBM thickening, but failed to affect albuminuria and mesangial expansion [24]. Although the reason for unexpected dissociation between markers of injury is unknown, the dissociation may depend on gender or species of experimental animals.

In this study, a significant correlation between the GV and SI was found. Because a close correlation between glomerular hypertrophy and the incidence of glomerulosclerosis has been reported [25], this finding suggests that the failure of attenuating effect of estrogen treatment on glomerular injury may be related to non-attenuating effect on glomerular hypertrophy. Because an association between GH and glomerulosclerosis has been proposed and shown by the experiments using transgenic mice expressing these hormones [26–28], this different effect of castration and estrogen on glomerular injury may be related to a significant difference in serum GH levels between the castrated OLETF rats and the estrogen-treated OLETF rats. A significant increase in GH levels was observed in the estrogen-treated OLETF rats. These results suggest that glomerular hypertrophy is probably related to increased GH levels in the estrogen-treated OLETF rats, similar to

what has been observed previously in uninephrectomized Sprague-Dawley rats [29]. In addition, we have recently reported that the somatostatin analogue attenuates estrogen-induced augmentation of glomerular injury in spontaneous hypercholesterolemic female Imai rats associated with a reduction of glomerular hypertrophy [30]. On the basis of these results and findings, it is possible that glomerular hypertrophy may be responsible for renal damage [25].

Negative effect of estrogen have been reported in the Cohen diabetic rat, another genetic model diabetes [31, 32]. The finding is very similar to what has been found in the present study. Although the exact mechanism is unknown, estrogen seems to exert a dual effect on glomerular injury in this experiment; firstly attenuating effect on diabetic nephropathy by suppressing plasma glucose, evidenced by a reduction of the GBM width and an attenuation of mesangial lesions and secondly a conversely augmenting effect by enhancing glomerular hypertrophy. As a whole, the effect of estrogen may depend on the balance of its attenuating and conversely augmenting effects. In this experiment, enhanced glomerular hypertrophy would cancel the estrogen's attenuating effect on glomerular injury.

The estrogen-treated OLETF rats showed higher levels of serum total cholesterol and triglycerides than did the control OLETF rats, and higher blood pressure than the castrated OLETF rats. Because these three parameters are important factors in influencing the progression of glomerular injury [33–35], and estrogen is reported to accelerate the development of renal disease in female obese Zucker rats [35] and in female albuminemic rats [36] in association with an increase in triglycerides and cholesterol, the contribution of these factors to glomerular injury observed in the estrogen-treated OLETF rats should be considered.

In conclusion, although castration and estrogen treatment equally improved glucose tolerance, each had a different effect on glomerular injury; attenuating effect of castration but not of estrogen. Besides the mechanisms involved in the development of diabetic nephropathy, other mechanisms concerning GH, blood pressure, serum cholesterol and triglycerides may be at least involved and contribute to the development of glomerulosclerosis, leading to a difference in glomerular injury between the castrated and the estrogen-treated OLETF rats.

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