Yonago Acta medica 2001;41:37–44

Effects of Testosterone Replacement on Renal Function and Apoptosis on Mesangial and Renal Tubule Cells in Rats

Kuniyasu Muraoka

Department of Urology and First Department of Pathology, Tottori University Faculty of Medicine, Yonago 683-0826 Japan

The effect of testosterone replacement in middle-aged rats on renal function and apoptosis of mesangial and renal tubule cells was evaluated. Middle-aged (13-month-old) male Wistar rats were divided into 2 groups, one of which was given high-dose and the other low-dose testosterone replacement for 2 months. Experiments were performed with the testosterone replacement groups and a non-replacement group when they had reached the age of 15 months. Serum levels of total and free testosterone were measured, and renal function was evaluated by glomerular filtration rate (GFR), serum levels of creatinine, blood urea nitrogen (BUN), urinary protein and *N*-acetyl-β-D-glucosaminidase (NAG). Apoptosis was evaluated by counting the number of terminal deoxynucleotidyl transferase-mediated deUTP-biotin nick end labeling (TUNEL)-positive cells in the mesangial and renal tubule cells. Correlations between total and free testosterone and each parameter were evaluated. Also, 12-week-old rats were evaluated in the same manner as the age control. Among 15-month-old rats, testosterone replacement did not affect serum creatinine, BUN or urinary protein, but the GFR was found to correlate negatively with the total and free testosterone level, and the urinary NAG level was found to correlate positively with the free testosterone level to a significant extent ($P < 0.01$). Percentages of apoptosis of mesangial and renal tubule cells were found to correlate positively with the total and free testosterone levels to a significant extent ($P < 0.01$). In conclusion, the results of this study suggested that testosterone replacement affected the deterioration of renal function in middle-aged rats.

Key words: apoptosis; renal function; testosterone

It has been reported that aging females and castrated male rats excrete less protein in the urine than aging male rats (Gafter et al., 1990), and it has been concluded that androgens are a risk factor in the development of glomerulosclerotic injury (Mulronery et al., 1999; Reckelhoff et al., 1997; Neugarten et al., 1996).

In cultured rat mesangial cells, mesangial cells derived from aged rats show an increased rate of apoptosis when compared to those from young rats, and the rate of mesangial cell apoptosis increases as the concentration of testosterone is increased (Singhal et al., 1997).

It is not known what kind of changes occur in renal function and in mesangial and renal tubule cells when aging rats are subjected to testosterone replacement.

In the present study, the effects of testosterone replacement on renal function and on apoptosis of mesangial cells and renal tubule cells was evaluated in rats.

Materials and Methods

Male Wistar rats were obtained (Shimizu Experimental Material Company, Kyoto, Japan). All rats were housed in a temperature-controlled

Abbreviations: BUN, blood urea nitrogen; dUTP, deoxyuridine triphosphate;GFR, glomerular filtration rate; NAG, *N*-acetyl-β-D-glucosaminidase; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling

room (22 ± 1˚C) with 14 h of illumination daily (0600–2000) and given food and water. Experiments were performed when the rats had reached the age of 12 weeks (Group A, $n = 10$) and 15 months. The 15-month-old rats were divided into 3 groups as in the following: rats with no testosterone replacement (Group B, $n = 6$), low-dose testosterone replacement (Group C, $n = 8$) and high-dose testosterone replacement (Group D, $n = 8$).

In the testosterone replacement groups (Groups C and D), rats were anesthetized for surgery by inhalation of diethyl ether. A silastic tube (Kaneka Medix Corp., Osaka, Japan) 3 cm in length and containing 40 mg of testosterone powder (Sigma, St. Louis, MO) was subcutaneously implanted in the backs of the rats. Four silastic tubes (Group C) or 8 silastic tubes (Group D) were implanted in rats 13 months old. Testosterone replacement was continued for 2 months.

In the experiment, 24-h urine collections were performed for fasting rats which were housed in individual metabolic cages, and urine levels of creatinine, protein and *N*-acetyl-β-D-glucosaminidase (NAG) were evaluated. After completion of urine volume for each day, rats were anesthetized with ether, and blood was withdrawn into heparinized syringes by vena cava puncture in order to determine the serum levels of total and free testosterone, creatinine and blood urea nitrogen (BUN), and then bilateral nephrectomy was performed, and the rats were killed. Each kidney was cut free of surrounding tissue, weighed on a Mettler Basbal scale (Delta Range, Tokyo, Japan), cut by a coronal section through the mid-portion of the kidney, and fixed in 10% buffered formalin. Several paraffin blocks were prepared from this section and several 3-μm sections were cut from each paraffin block and stained via terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL). TUNEL staining was performed with an ApopTag in situ apoptosis detection kit (Oncor, Gaithersburg, MD). Semiquantitative analysis was performed by counting TUNEL-positive cells per field at × 400 magnification. The mean number of TUNEL-positive cells in 50 randomly selected fields in each kidney was calculated.

Table 1. Serum concentration of testosterone

	Total testosterone (ng/mL)	Free testosterone (ng/mL)
Group A	2.0 ± 0.4	8.3 ± 1.9
Group B	0.8 ± 0.1	4.2 ± 1.1
Group C	3.7 ± 0.4	14.5 ± 3.6
Group D	5.0 ± 1.1	20.0 ± 6.7

Data are expressed as mean ± SD.
Statistical analysis by Mann-Whitney *U*-test.
Group A versus Group B, $P < 0.05$.
Group B versus Group C, $P < 0.05$.
Group B versus Group D, $P < 0.05$.

Urine level of creatinine, protein and NAG

Urinary creatinine was determined by the the alkaline picrate method, urinary protein was determined by the pyrogallolred method, and urinary NAG was determined by colorimetry by the Special Reference Laboratory (Hiroshima, Japan) .

Serum levels of total and free testosterone, creatinine and BUN

Total and free testosterone levels were assessed by radioimmunoassay using a kit from Nihon DPC Corporation (Tokyo, Japan). The BUN level was assessed by the urease-UV method, and the serum creatinine level was determined by the alkaline picrate method.

Calculation of the glomerular filtration rate (GFR)

The GFR was determined by the creatinine clearance expressed per gram of kidney weight.

Statistical analysis

The data are presented as mean ± SD; Kruskal-Wallis one-way analysis of variance was performed. When a difference found to be significant,

Table 2. Effects of testosterone replacement on renal function

	Serum creatinine (mg/dL)	BUN (mg/dL)	Urinary protein (mg/dL)	Urinary NAG (U/L)	GFR (mL/min/kw)
Group A	0.2 ± 0.1	15.6 ± 1.4	65.8 ± 27.3	5.9 ± 2.3	174.16 ± 37.90
Group B	0.3 ± 0.1	21.1 ± 1.6	327.2 ± 108.9	4.6 ± 7.3	124.40 ± 9.94
Group C	0.4 ± 0.1	20.4 ± 2.0	445.6 ± 181.4	14.0 ± 10.8	104.47 ± 8.22
Group D	0.3 ± 0.1	21.3 ± 2.3	899.1 ± 487.4	11.3 ± 11.3	98.51 ± 17.36

Data are expressed as mean ± SD.
BUN, blood urea nitrogen; GFR, glomerular filtration rate; kw, kidney weight (g); NAG, *N*-acetyl-β-D-glucosaminidase; NS, not significant.
Statistical analysis by Mann-Whitney *U*-test.
Group A versus Group B: serum creaninine, BUN and urine NAG = NS; urinary protein and GFR = $P < 0.05$.
Group B versus Group C: serum creaninine, BUN and urinary protein = NS; urine NAG and GFR = $P < 0.05$.
Group B versus Group D: serum creaninine and BUN = NS; urinary protein, urine NAG and GFR = $P < 0.05$.

the Mann-Whitney *U*-test for 2 independent samples was used to compare each of the 2 groups. Statistical significance was defined as $P < 0.05$.

Results

Serum concentration of testosterone

The serum levels of total and free testosterone in Group B were significantly lower than those in Group A. Among groups of 15-month-old rats, the serum levels of total and free testosterone in Groups C and D were significantly higher than those in Group B (Table 1).

Serum creatinin, BUN, urinary protein, urine NAG and GFR

The serum creatinin, BUN and urine NAG levels of Groups A and B were similar to that of the control group. The level of urinary protein in Group B was significantly higher than that of Group A, and GFR in Group B was significantly lower than that in Group A.

Among groups of 15-month-old rats, testosterone replacement groups (Groups C and D) showed significantly higher levels of urinary protein and NAG, significantly lower values of GFR, but no significant difference in serum creatinin and BUN (Table 2).

Apoptosis of mesangial and renal tubule cells

TUNEL-positive mesangial and renal tubule cells of high-dose testosterone replacement rats (Group D) are shown in Figs. 1a and b. The percentages of apoptotic mesangial and renal tubule cells were similar to those of Groups A and B (Table 3). Among groups of 15-month-old rats, mesangial and renal tubule cells of Groups C and D showed greater percentages of TUNEL-positive cells when compared to those of Group B.

Correlations between urinary protein, urinary NAG, GFR, apoptosis of mesangial and renal tubule cells and total and free testosterone

In the 15-month-old rats, the correlation between the serum concentration of total and free testosterone and each parameter was examined using linear regression analysis. The urinary protein level was not found to correlate with the total and free testosterone levels (Figs. 2a and b). The urinary NAG level was found to correlate positively with the free testosterone level but not with the total testosterone levels (Figs. 3a and b). The GFR was found to correlate negatively with the total and free testosterone levels (Figs. 4a and b). Percentages of apopto-

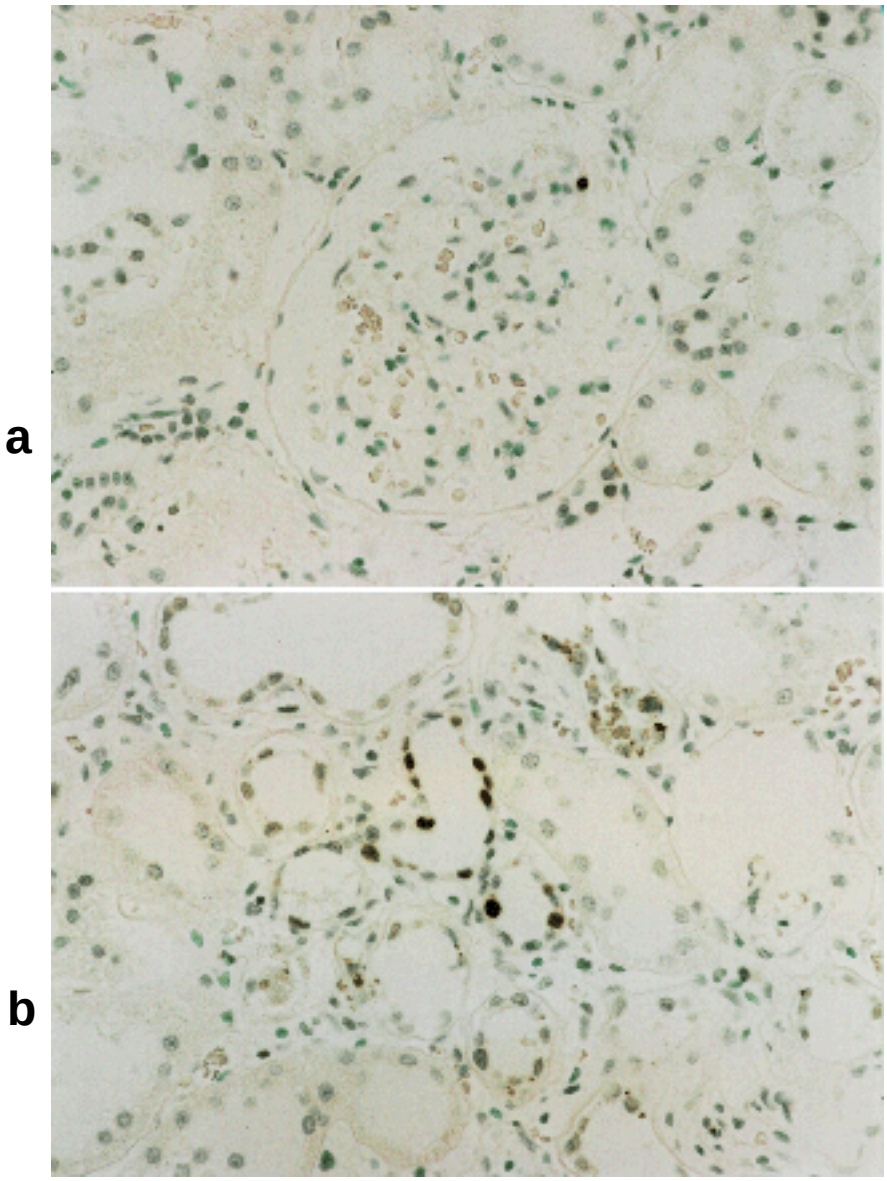

Fig. 1. TUNEL-positive cells of a high-dose testosterone replacement rat at the age of 15 months.
a: TUNEL-positive mesangial cells (original magnification × 400).
b: TUNEL-positive renal tubule cells (original magnification × 400).

sis of mesangial and renal tubule cells were found to correlate positively with the total and free testosterone levels (Figs. 5a, 5b, 6a and 6b).

Discussion

In a previous study involving male Wistar rats, the GFR began diminishing at 16 months (Tanaka et al., 1995). In this previous study, 2 months of testosterone replacement at 13

months old accelerated a reduction of the GFR. The direct effects of testosterone on cellular collagen synthesis have not been extensively studied; however, most available data suggests that it has a stimulatory effect (Fischer et al., 1985; Franchimont et al., 1991). For example, testosterone increases collagen synthesis by vascular smooth muscle cells in culture (Leitman et al., 1994), and administration of testosterone increases the accumulation of collagen and elastin in the aorta of normal and cholesterol-fed animals (Silbiger et al., 1995). It has been suggested that some changes related to collagen may occur along with a significant reduction in the GFR in rats that have undergone testosterone replacement.

Rats that have undergone testosterone replacement exhibited proteinuria. In this study, urine albumin was not detected, and it is not clear what kind of protein was excreted, but it might be that proteinuria evolved from a leak in the basement membrane. Excretion of sex-dependent α2u-globulin (Roy et al., 1966;

Table 3. Effects of testosterone replacement on apoptosis of mesangial cells and renal tubules

	% Apoptotic mesangial cells	% Apoptotic renal tubule cells
Group A	0.96 ± 0.25	0.46 ± 0.21
Group B	1.07 ± 0.36	0.87 ± 0.27
Group C	1.47 ± 0.40	1.23 ± 0.24
Group D	2.05 ± 0.60	1.60 ± 0.35

Data are expressed as mean ± SD.
Statistical analysis by Mann-Whitney *U*-test.
Group A versus Group B, not significant.
Group B versus Group C, $P < 0.05$.
Group B versus Group D, $P < 0.05$.

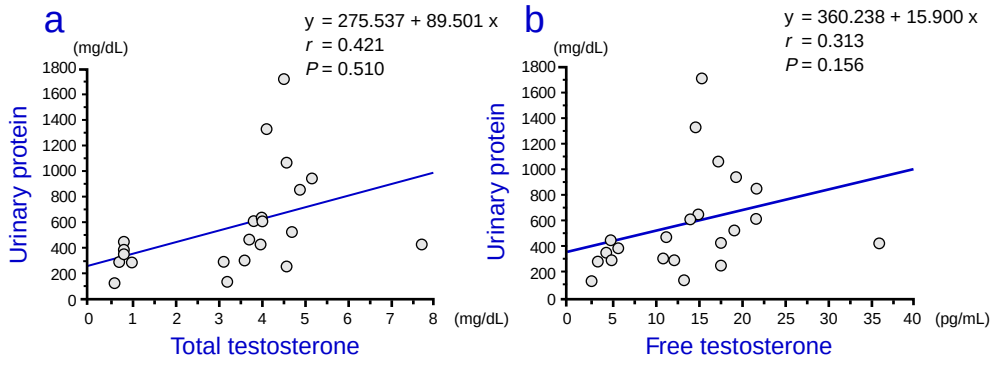

Fig. 2. Correlation between urinary protein and total and free testosterone in 15-month-old rats.

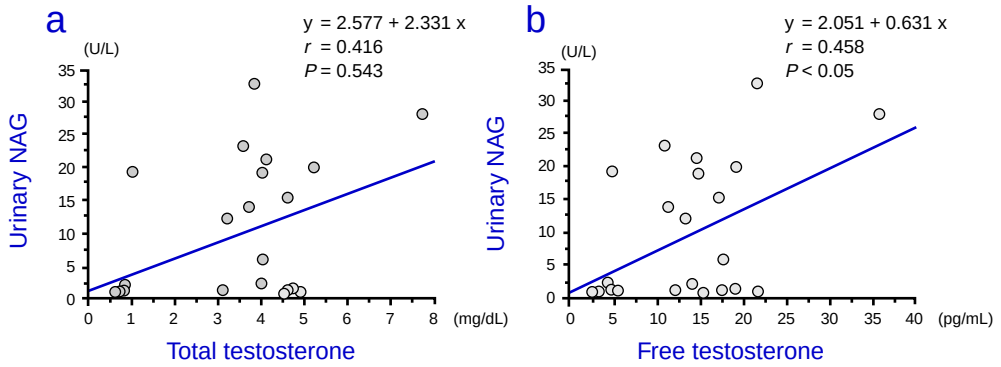

Fig. 3. Correlation between urinary NAG and total and free testosterone in 15-month-old rats. NAG, *N*-acetyl-β-D-glucosaminidase.

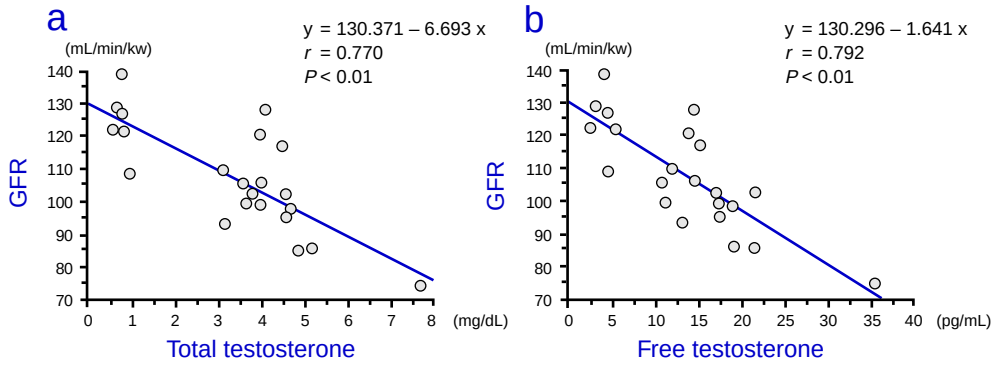

Fig. 4. Correlation between GFR and total and free testosterone in 15-month-old rats. GFR, glomerular filtration rate; kw, kidney weight (g).

Neuhaus et al., 1975) can be abolished by castration of male rats (Irwin et al., 1971). It would seem that sex-dependent globulin could only slightly contribute to proteinuria. It was found that urinary NAG increased and correlated positively with the free testosterone level but not with the total testosterone level. NAG is one of the glycoproteinases in lysosome, and proximal renal tubule epithelial cells contain a lot of NAG. Urinary NAG increases due to an injured proximal tubule. It is believed that free testosterone may injure proximal tubules.

In addition, it was demonstrated that testosterone replacement increased the number of TUNEL-positive cells in the mesangial and renal tubule cells when compared to the control group in middle-aged rats. Cultured mesangial cell apoptosis in male rats increases with the level of testosterone (Singhal et al., 1997). The present study demonstrated that this phenomenon occurred similarly in vivo. In addition, renal tubule cell apoptosis in male rats also increased with the level of testosterone. It is known that cultured mesangial cells from male rats contain nuclear receptors for testosterone (Neugarten et al., 1994). In addition, it is believed that renal tubule cells contain nuclear receptors for testosterone (Davidoff et al., 1980). Free testosterone has true androgen activity, so it is believed that some changes in mesangial and renal tubule cells may occur with a significant increase in the number of TUNEL-positive cells by the direct effect of free testosterone.

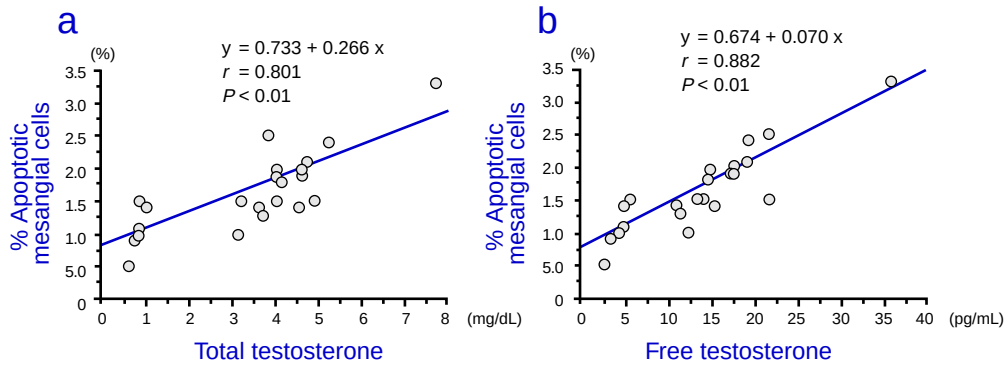

Fig. 5. Correlation between % apoptosis of mesangial cells and total and free testosterone in 15-month-old rats.

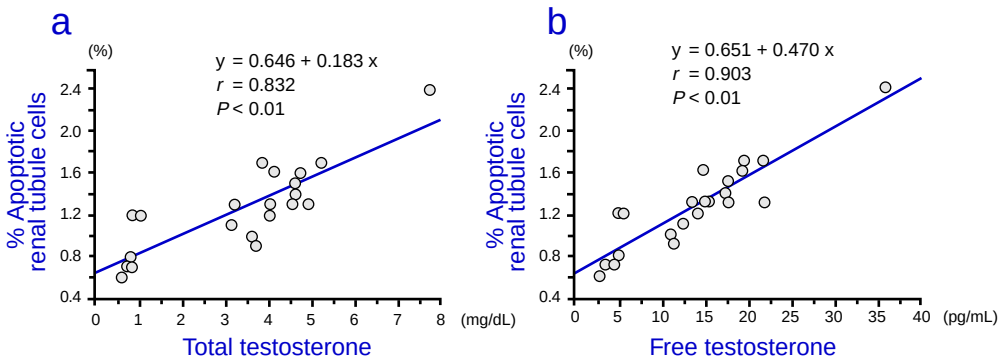

Fig. 6. Correlation between % apoptosis of renal tubule cells and total and free testosterone in 15-month-old rats.

On the other hand, it was surmised that testosterone replacement caused a decrease of gonadotrophic hormone by a feedback mechanism. However, the relation between the gonadotrophic hormone and mesangial and renal tubule cells is not clear. Further, multiple apoptosis signaling pathways have been described, but further studies should be performed to determine the apoptosis pathway for mesangial and renal tubule cells treated with testosterone.

The present study is the first report showing that testosterone replacement in middle-aged rats increased the rate of apoptosis of mesangial and renal tubule cells and the urine level of NAG and decreased the GFR. The concept that androgens are a risk factor for renal dysfunction is supported by the current findings, but further studies are needed to clarify their association with testosterone.

Acknowledgments: The author would like to thank Professor Ikuo Miyagawa of the Department of Urology, Tottori University Faculty of Medicine, for his kind advice and valuable suggestions concerning this study.

References

1 Davidoff M, Caffier H, Schiebler TH. Steroid hormone binding receptors in the rat kidney. Histochemistry 1980;69:39–48.
2 Franchimont P, Bassleer C. Effects of hormones and local growth factors on articular chondrocyte metabolism. J Rheumatol 1991;18:68–70.
3 Fischer GM, Bashey RI, Rosenbaum H, Lyttle CR. A possible mechanism in arterial wall for mediation of sex difference in atherosclerosis. Exp Mol Pathol 1985;43:288–296.
4 Gafter U, Ben Bassat M, Levi J. Castration inhibits glomerular hypertrophy and proteinuria in uninephrectomized male rats. Eur J Clin Invest 1990;4:360–365.
5 Irwin JF, Lane SE, Neuhaus OS. Synergistic effect of glucocorticoids and androgens on the biosynthesis of sex-dependent protein in the male rat. Biochim Biophys Acta 1971;252:328–334.
6 Leitman DC, Benson SC, Johnson LK. Glucocorticoids stimulate collagen and noncollagen protein synthesis in cultured vascular smooth muscle cells. J Cell Biol 1994;98:541–549.
7 Mulroney SE, Woda C, Johnson M, Pesce C. Gender differences in renal growth and function after uninephrectomy in adult rats. Kidney Int 1999;56:944–953.
8 Neugarten J, Silbiger S. Nuclear receptor for estradiol and testosterone in mesangial cells. J Am Soc Nephrol 1994;5:951.
9 Neugarten J, Silbiger SR. Effects of sex hormones on mesangial cells. Am J Kidney Dis 1995;26:147–151.
10 Neuhaus OW, Flory W. The effect of dietary protein on the excretion of alpha2u, the sex-dependent protein of the adult male rat. Biochim Biophys Acta 1975;411:74–86.
11 Reckelhoff JF, Zhang H, Granger JP. Decline in renal hemodynamic function in aging SHR : role of androgens. Hypertension 1997;30(3 Pt 2): 677–681.
12 Roy AK, Neuhaus OW. Identification of rat urinary proteins by zone and immunoelectrophoresis. Proc Soc Exp Biol Med 1966;121:894–899.
13 Silbiger S, Neugarten J. The impact of gender on progressive renal functional impairment. Am J

Kidney Dis 1995;25:515–533.
14 Singhal PC, Reddy K, Franki N, Sanwal V, Kapasi A, Gibbons N, et al. Age and sex modulate renal expression SGP-2 and transglutaminase and apoptosis of splenocytes, thymocytes, and macrophage. J Invest Med 1997;45: 567–575.
15 Tanaka A, Kyoukuwa M, Mori T, Kawashima S. Acceleration of renal dysfunction with aging by the use of androgen in Wistar/Tw rats. In-vivo 1995;9:495–502.

Received December 15, 2000; accepted December 25, 2000

Corresponding author: Dr. Kuniyasu Muraoka