—**Original**—

Tubulointerstitial Injury of Thy-1 Nephritis in Uninephrectomized Stroke-Prone Spontaneously Hypertensive Rats

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Abstract

Thy-1 nephritis was induced in stroke-prone spontaneously hypertensive rats (SHR-SP) with unilateral nephrectomy (UNX) and normotensive same genetic strain Wistar-Kyoto (WKY) rats with UNX to evaluate whether the tubulointerstitial injury in Thy-1 nephritis is accelerated by long-term systemic and intraglomerular hypertension. SHR-SP that underwent UNX at twelve weeks of age were randomly assigned to receive monoclonal anti-thy 1.1 antibody (group SP), and normal saline (group SC). Age-matched normotensive WKY rats served as controls and were given the same dose of monoclonal anti-thy 1.1 antibody after UNX (group WK). In all groups, the blood pressure and renal function were assessed, and morphologic changes of tubulointerstitium were examined by using immunohistochemistry and light microscopy twelve weeks after Thy-1 nephritis induction (in groups SP and WK) and UNX alone (in group SC). In all groups, histological findings, the degree of monocyte/macrophage infiltration, interstitial expression of α-smooth muscle actin (α-SMA), which is a marker for myofibroblasts, and the degree of tubular cell proliferation were examined. In addition, assessments of blood pressure, serum creatinine and BUN levels, and the degree of proteinuria were made. In parallel to glomerular structural damage, interstitial fibrosis with predominant monocyte/macrophage influx, increased interstitial expression of α-SMA and tubular cell proliferation were observed in group SP. A significant increase in serum creatinine and proteinuria were also present in this group. In contrast, the changes observed in group SC were not so evident or extensive as in group SP. The level of proteinuria was lower than that in group SP. No evident tubulointerstitial changes were found in group WK. The results showed that tubulointerstitial injury was prominently progressed in the hypertensive model with Thy-1 nephritis. This suggests that sustained systemic and glomerular hypertension is not only ultimately responsible for the progression of immunologically mediated glomerular injury, but is also responsible for subsequent tubulointerstitial changes. Migration and proliferation of myofibroblasts and intense influx of monocytes/macrophages may contribute to the development of tubulointerstitial fibrosis. (J Nippon Med Sch 2001; 68: 301—309)

Key words: SHR-SP, unilaternal nephrectomy, Thy-1 nephritis, hypertension, tubulointerstitial injury

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Introduction

The mechanisms involved in the progression of renal disease to end-stage renal failure have been stressed independently of the initial pathogenetic factors[1]. Evidence has been provided that there is an association between hypertension and progressive renal damage[1-4]. The spontaneously hypertensive rat(SHR) is a well-studied animal model of human essential hypertension. This inbred strain was developed by selective breeding of the Wistar-Kyoto (WKY) rat stock for higher blood pressure[5]. Although this genetically defined SHR strain spontaneously and consistently develops moderate-to-severe hypertension between 7 and 15 weeks of age, glomerular capillary pressure is normal because of its high pre-glomerular resistance, and the development of renal damage is slow[6,7]. In SHR that undergo unilateral nephrectomy (UNX), preglomerular vasoconstriction can be decreased and glomerular injury induced because of an increase in glomerular capillary pressure[8,9]. The stroke-prone SHR (SHR-SP), a substrain of SHR derived from SHR, is a useful model of human malignant hypertension[10]. Because SHR-SP have more severe hypertension and evident intraglomerular hypertension than SHR, we thought that progressive glomerular damage accompanied by an increase in glomerular capillary pressure might be induced in the UNX SHR-SP. Thus, Thy-1 nephritis, which is characterized by an acute complement-mediated mesangial proliferating glomerulonephritis, was induced in UNX SHR-SP in our previous study. Our results revealed that glomerular injury of Thy-1 nephritis is aggravated by systemic hypertension and subsequent intraglomerular hypertension in this model[11]. However, it is unclear whether tubulointerstitial injuries take a turn for the worse when UNX SHR-SP sustain long-term systemic and glomerular hypertension following Thy-1 nephritis.

To provide evidence for this possibility, we performed a further investigation to focus on tubulointerstitial changes in this model of UNX SHR-SP with Thy-1 nephritis.

Material and methods

All procedures were performed in accordance with the Nippon Medical School Animal Ethics Committee's regulations and recommendations.

Experimental design

Male 12-week-old SHR-SP rats and WKY rats (Sankyo Experimental Animal Supply, Japan) weighing between 240~340 g, were used for the study. The experimental protocol is summarized in(**Table 1**). All animals underwent unilateral nephrectomy of the left kidney (UNX) under ether anesthesia. After the operation, thirteen SHR-SPs were randomly divided into two groups. Group SP (experimental group of SHR-SP) consisted of eight rats given a single injection of 60 µg IgG/100 g weight of monoclonal anti-thy 1.1 antibody (OX-7; Cedarlane Laboratories, Toronto, Ont. Canada) intravenously through the tail vein, and then they were sacrificed 12 weeks after disease induction. Group SC(control group of SHR-SP)consisted of 5 rats which only received normal saline in the same way and were sacrificed 12 weeks after UNX. Group WK (WKY) consisted of eight rats given the same dose of OX-7 in the same way. They were also sacrificed 12 weeks after UNX. Body weight was measured and urine was collected in metabolic cages with free access to water and standard rat chow. Twenty-four hour urine protein excretion was measured. A polyethylene catheter was inserted into the tail artery and systolic blood pressure measurements were made with a Statham pressure transducer connected to a pressure recorder under light ether anesthesia. Blood samples were collected for serologic examination.

Table 1　Experimental protocol

Groups	Rat	(n)	Uninephrectomy	Antibody or Vehicle
Group SP	SHR-SP	(8)	+	OX-7
Group SC	SHR-SP	(5)	+	Saline
Group WK	WKY	(8)	+	OX-7

Morphological and immunohistological examination

The right kidney was removed, fixed in 20% formalin or 4% paraformaldehyde, respectively, and embedded in paraffin. Sections 2 μm thick were stained with hematoxylin-eosin（HE）, periodic acid-Schiff（PAS）, Masson's Trichrome, and periodic acid-methenamine silver（PAM）for light microscopic examination. For each kidney specimen, tubulointerstitium was examined and the severity of the lesion was assessed.

For immunohistochemical examination, sections of the 20% formalin or 4% paraformaldehyde fixed tissue were deparaffinized and processed by the indirect immunoperoxidase technique. Primary antibodies included: a monoclonal anti-PCNA（proliferating cell nuclear antigen）antibody（Dako, Glostrup, Denmark）for the detection of actively proliferating cells; a monoclonal anti-α SMA（smooth muscle actin）antibody （IgG 2 a, Dako, Glostrup, Denmark）for the detection of myofibroblasts; and a monoclonal anti-ED-1 antibody（IgG 1, Chemicon, CA, USA）for the detection of infiltrating monocytes/macrophages.

Quantitative and semiquantitative evaluation of histological findings

Tubulointerstitial injury was defined as inflammatory cell infiltrates, tubular dilatation and/or atrophy, or interstitial fibrosis. Injury was graded according to the extent of cortical involvement on a scale of 0 to 4 described by Shih W, et al[12]. 0 = normal; 0.5 = small focal area of tubular damage; 1 = involvement of less than 10% of the cortex; 2 = involvement of 10 to 25% of the cortex; 3 = involvement of 25 to 75%; 4 = extensive damage involving more than 75% of the cortex.

Proliferating cells or monocyte/macrophage infiltration were evaluated by immunostaining with PCNA or ED-1. For each sample, 40 randomly selected grid fields in cortical tubulointerstitium, measuring 0.065 mm^2 each at a × 400 magnification using a grid in the eyepiece of the microscope, were examined. Large blood vessels and glomeruli in the grid parts were avoided. The numbers of PCNA + or ED-1 + cells were obtained in this evaluation and the mean values were expressed as cells per millimeter squared （per mm^2）± SD.

To evaluate the degree of myofibroblast accumulation, immunostaining for α-smooth muscle actin（α-SMA）was performed. For each sample, 40 randomly selected grid fields in cortical tubulointerstitium were examined at × 400 magnification using a grid in the eyepiece of the microscope. Large blood vessels and glomeruli in the grid parts were avoided . The semiquantitative score was graded from 0 to 4 according to the percentage of positive α-SMA within each tubulointerstitial grid field . The criterion was performed as previously described by Kliem V, et al[13]. 0 = absent staining; 1 =up to 5%; 2 = 6 to 25%; 3 = 26 to 50%; 4 = 51 − 75; 5 = more than 75% showing positive staining. The mean score per sample was calculated.

The extent of interstitial fibrosis was evaluated in tissue sections with Masson's Trichrome according to the method described above . The semiquantitative scoring system was developed on a scale of 0 to 3 as described by Hugo C, et al[14]. 0= normal interstitium and tubules; 1 = mild fibrosis with minimal interstitial thickening between the tubules; 2 = moderate fibrosis with moderate interstitial thickening between the tubules; 3 = severe fibrosis with severe interstitial thickening between the tubules.

Statistical analysis

All values are expressed as mean ± SD. Comparison was made by the Student's t test. The Mann-Whitney two-sample test was used to investigate differences in life span in each group and between groups SP and SC and WK. Statistical significance was defined as p< 0.05.

Results

1．Hemodynamics, proteinuria and renal function measurements

The time course of systemic systolic blood pressure is summarized in **Fig. 1A**. **Fig. 1A** shows that elevated systolic blood pressure was already present in SHR-SP by the time of the initial systolic pressure determination at 12 weeks of age before the experiment. The systolic blood pressure in groups SP and SC was higher than in the age-matched normotensive group WK（P<0.01）, and no significant difference was seen between group SP receiving OX-7 injection and group SC receiving normal saline throughout the study.

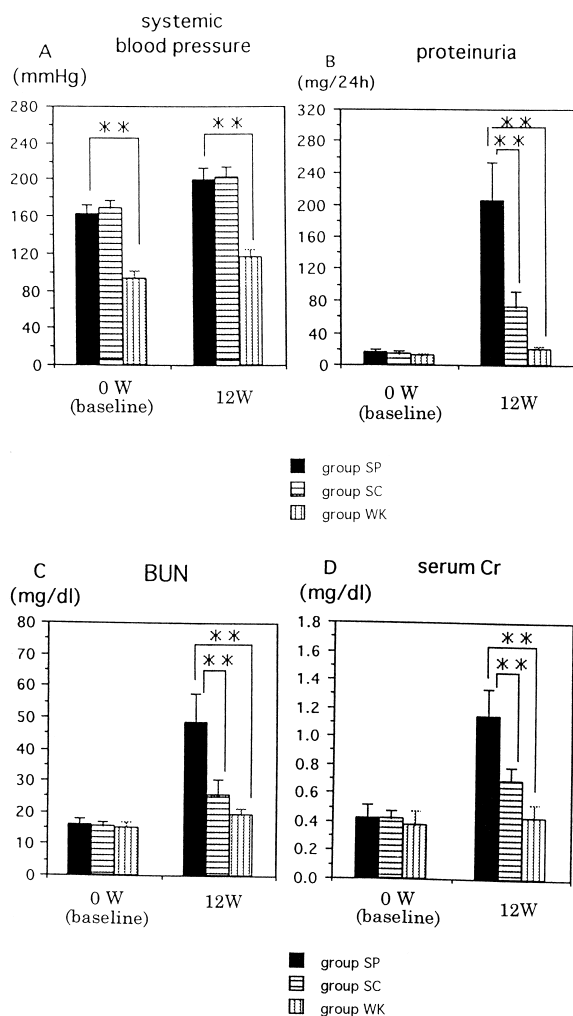The level of urinary protein excretion and renal

Fig. 1 4 Changes in parameters 12 weeks after disease induction. A: systemic blood pressure. B: urinary protein excretion level. C: blood urea nitrogen(BUN)level. D: serum creatinine level in experimental and control groups. Values are expressed as mean ± SD.** P<0.01. Group SP versus group WK and/or group SC.
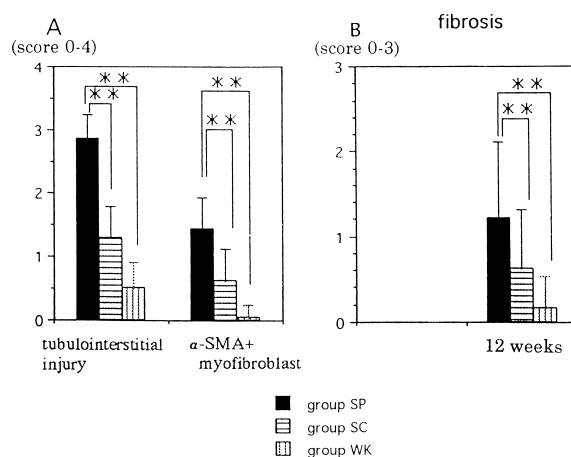

Fig. 2 Semiquantitative evaluation of histological findings. A: tubulointerstitial injury index and myofibroblast accumulation score which is assessed by immunostaining for α-SMA in the cortical tubulointerstitium of the experimental and control groups. B: tubulointerstitial fibrosis score assessed by Masson's Trichrome staining. Values are expressed as mean ± SD. **P<0.01. Group SP versus group WK and/or group SC.

functional changes are summarized in **Fig. 1 B-D**. At the end of the experiment, an evidently elevated level of urinary protein excretion was observed both in group SP and SC. However, the level of urinary protein excretion was significantly higher in group SP than in group SC. Markedly elevated levels of blood urea nitrogen (BUN) and serum creatinine (Cr) were noted in group SP, and this difference was significant.

2. Morphological studies

Twelve weeks after Thy-1 nephritis induction, the glomeruli of UNX SHR-SP were characterized by a marked increment in matrix expansion and wide-

spread focal and segmental glomerulosclerosis. Parallel to the severity of glomerular injuries, predominant tubulointerstitial injuries consisting of tubular atrophy, tubular dilatation, degeneration of the epithelium, tubular cast formation, inflammatory cellular infiltrates, and interstitial fibrosis were observed in group SP rats（**Fig. 2**）. These changes were especially seen in areas with injured and sclerosed glomeruli（**Fig. 3 A**）. Semiquantitative analysis of the extent of interstitial fibrosis detected with Masson's Trichrome staining is shown in **Fig. 2B**. The morphological changes observed in UNX SHR-SP without Thy-1 nephritis showed a mild to moderate increase in mesangial expansion of the glomerulus and scattered focal, and segmental glomerulosclerotic lesions（**Fig. 3B**）due to the progression of vascular damage. The tubulointerstitial injury was less and was not as extensive as in group SP. By contrast, most of the glomeruli and tubulointerstitium of group WK revealed almost their original structure（**Fig. 3C**）12 weeks after Thy-1 nephritis induction, although focal and segmental glomerular proliferative changes still remained.

3. The kinetics of myofibroblast
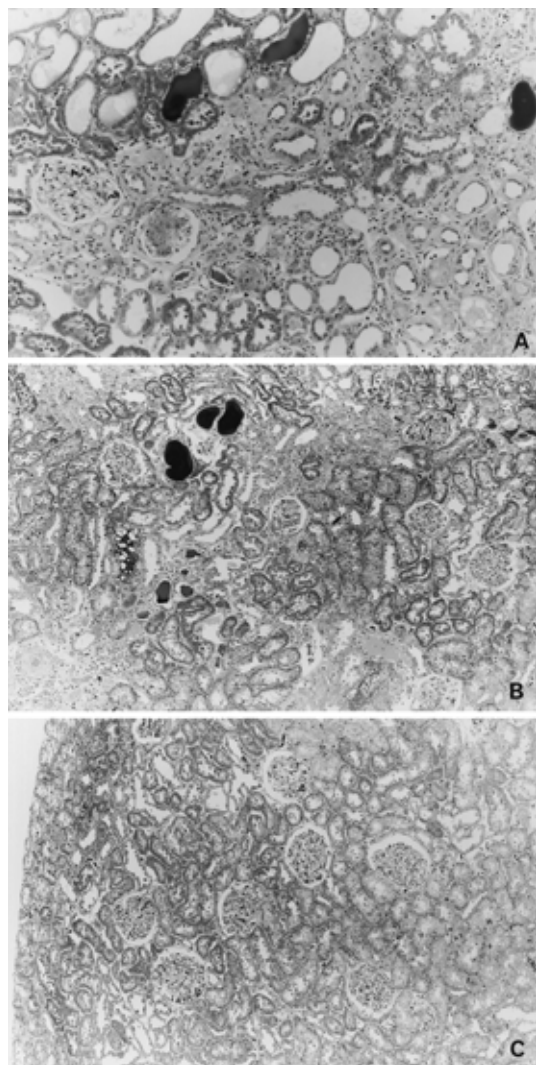
The extent of myofibroblast increase was assessed

Fig. 3 Morphological changes of the glomerulus and tubulointerstitium in group SP（A）, SC（B）and WKY （C）. The sections were stained with Masson's Trichrome stain. Predominant tubulointerstitial injuries and fibrosis with glomerular sclerosis are observed in group SP （A） by 12 weeks. Tubulointerstitial changes are parallel to the severity of glomerular injuries in the tubulointerstitium. A smaller extent of tubulointerstitial injuries and fibrosis with focal and segmental glomerular sclerosis are also observed in group SC （B） due to marked vascular damage. No clear evidence of tubulointerstitial injuries is seen in group WK（C）.（Original magnification for all figures, ×200）.
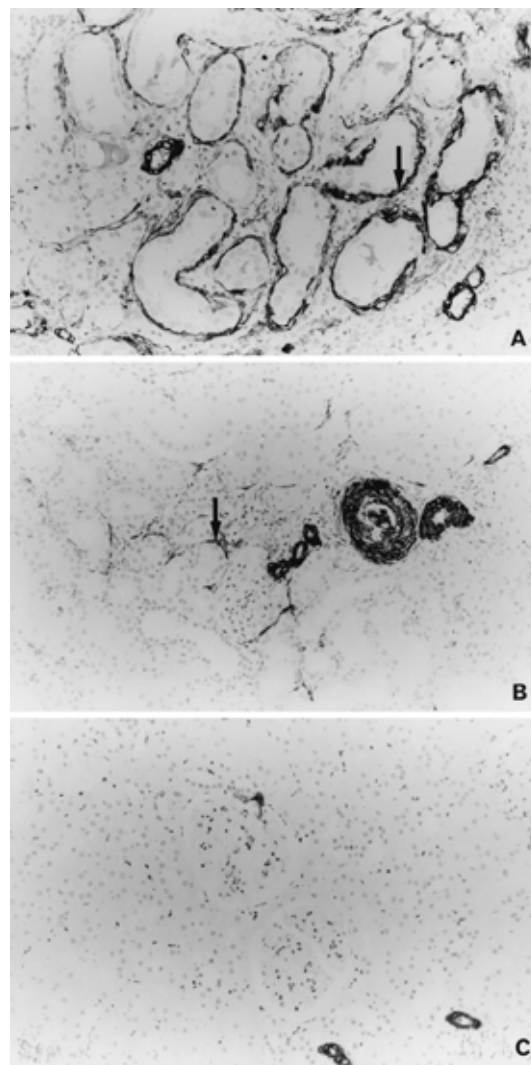

Fig. 4 Immunostaining for α-SMA in the tubulointerstitium in group SP （A）, SC （B） and WKY （C）. Marked increase in α-SMA positive cells （↑） in the peritubular areas （A） is also found in group SP by 12 weeks, which is in keeping with the extent of tubulointerstitial alterations. The increase in α-SMA positive cells （↑） in the peritubular areas （B） is markedly less than in group SP. An absence of definite evidence of α-SMA positive cells in the peritubular areas （C） is observed in group WK at 12 weeks after disease induction, except for the expression of α-SMA in renal arterioles.（Original magnification for all figures, ×200）.

by immunostaining for α-SMA （**Fig. 4**）. The semiquantitative evaluation of myofibroblast accumulation （**Fig. 2A**） showed a marked increase of α-SMA expression in the tubulointerstitium of group SP （**Fig. 4A**）. This was parallel to the extent of interstitial fibrosis. In contrast, no evident expression of α-SMA was detected in the tubulointerstitium of group WK, indicating an absence of myofibroblasts within the tubulointerstitium （**Fig. 4C**）. Although an increase in myofibroblasts was also observed in the tubulointerstitium of group SC （**Fig. 4B**）, the expression of

α-SMA was less and was not as extensive as in group SP. The morphological identification of α-SMA positive cells by light microscopic observation revealed them to be mostly interstitial cells, showing consistent characteristic of myofibroblasts.

4. Interstitial mononuclear/macrophage cell infiltration

The influx of mononuclear/macrophage cells within the tubulointerstitium was evaluated by immunostaining with ED-1. As shown in **Fig. 5A** and **7A**, an evidently elevated interstitial mononuclear/macrophage cell number was observed in group SP, which paralleled the extent of tubulointerstitial injuries and fibrosis, indicating that the degree of interstitial infiltration has a close relation to the development of tubulointerstitial injury and fibrosis. In contrast, no significant interstitial mononuclear/macrophage infiltration was detected in the kidneys of group WK (**Fig. 5 C**). The number of mononuclear/macrophage cells observed in the tubulointerstitium of group SC was significantly lower compared with those in group SP (**Fig. 5B**). The pattern of distribution was focal, and they were mainly localized in a damaged perivascular sheath surrounding hilar arterioles, hilum, and partial periglomerular interstitial areas.

5. Cell proliferative change in the tubulointerstitium

The changes in actively proliferating cells in the renal cortical tubulointerstitium were assessed by immunostaining for PCNA (**Fig. 7B**), a nuclear protein that is increased from the late G_1 to the M phase of the cell cycle. A significant elevation of PCNA positive nuclear staining in the cortical tubulointerstitium was observed in group SP (**Fig. 6**), illustrating evident proliferative activity in the tubulointerstitium 12 weeks after Thy-1 nephritis induction. Although an increased number of PCNA positive tubular and interstitial proliferating cells was also detected in the kidneys of group SC, they were less prominent compared with group SP. PCNA positive cells in the tubulointerstitium of group WK were similar to their baseline values (**Fig. 7B**), illustrating the absence of proliferative activity in the tubulointerstitium of group WK rats 12 weeks after Thy-1 nephritis induction.
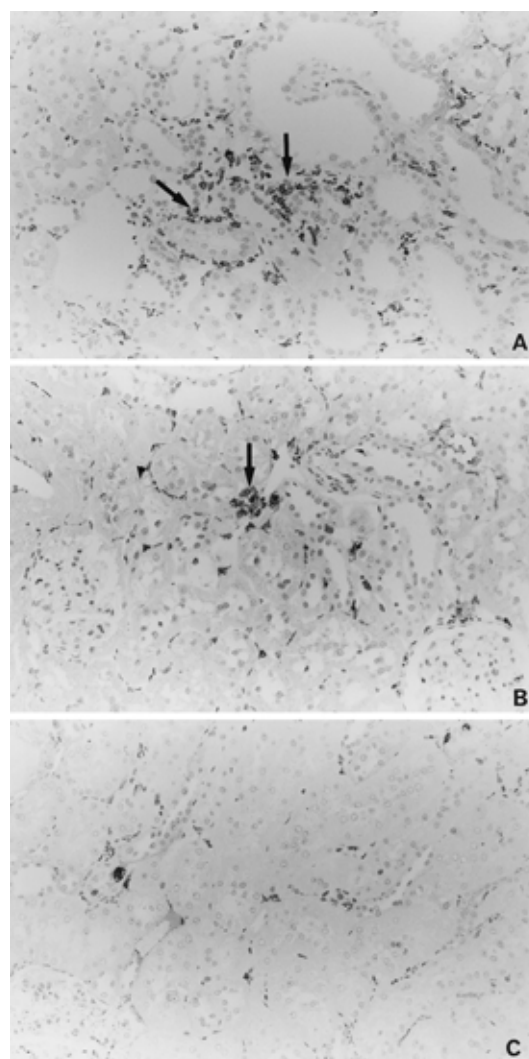

Fig. 5　Immunostaining for ED-1 in the tubulointerstitium in group SP (A), SC (B) and WKY (C). Marked increase in ED-1 positive cells (↑) in the tubulointerstitium is also found in group SP (A) by 12 weeks, which is in keeping with the extent of tubulointerstitial alterations. The number of mononuclear/macrophage cells observed in the tubulointerstitium of group SC (B) was significantly lower than in group SP. Influx of monocytes/macrophages is rarely seen in group WK (C) by 12 weeks. (Original magnification for all figures, ×200).

Discussion

Although the model of experimental mesangial proliferative glomerulonephritis has been widely used for elucidating the inflammatory and proliferative mechanisms of mesangial cells[15-20], the lesions in the one shot antibody model of Thy-1 nephritis are characterized by a self-limited course and reversible histological
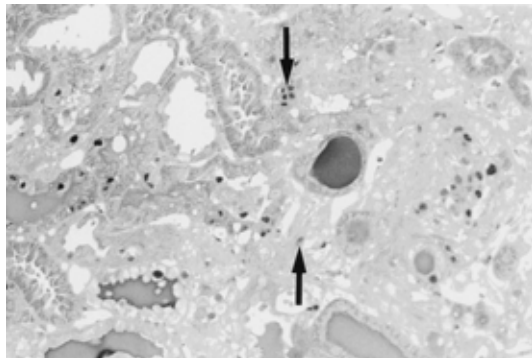
Fig. 6   Marked increase in PCNA positive cells (↑) in the tubulointerstitium is also found in group SP by 12 weeks, which is in keeping with the extent of tubulointerstitial alterations. (Original magnification for all figures, ×200).
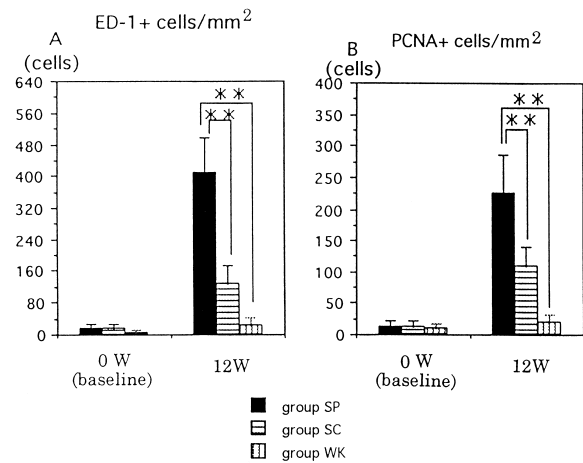

Fig. 7   The number of ED-1 positive macrophages (A) and PCNA positive cells (B) in the cortical tubulointerstitium of the experimental and control groups. Values are expressed as mean ± SD. **P<0.01. Group SP versus group WK and/or group SC.

changes[21, 22]. It is known that many renal diseases progress to end-stage renal failure independent of the initial pathogenetic mechanism[1]. Multiple pathogenic mechanisms have been proposed to be responsible for the process of progressive renal damage leading to end-stage renal failure[23-27]. Being a common accompaniment of most human renal diseases, hypertension has been accentuated as one of the major risk factors in this process of progression[26-30]. The stroke-prone spontaneously hypertensive rat (SHR-SP), a substrain of SHR derived from SHR has been established as a useful model of human malignant hypertension[10]. In our previous study, we compared and analysed two experimental models, in which Thy-1 nephritis was induced in SHR-SP rats with unilateral nephrectomy (UNX) and normotensive UNX WKY rats of the same genetic strain[11]. We focused primarily on comparing the effect of systemic and intraglomerular hypertension on the developing process of glomerular damage following the initial immune-mediated stimulus to the glomerulus. The results of our study revealed that the kidneys of group SP underwent progressive damage, while the kidneys of group WK recovered from the initial episode of immune injury. This suggests that glomerular injury of Thy-1 nephritis is aggravated by the systemic hypertension and subsequent intraglomerular hypertension in this model. Since the glomerular alteration as a primary feature was demonstrated in this experimental model, whether or not the process of tubulointerstitial injury is also accelerated when UNX SHR-SP sustain long-term systemic

and glomerular hypertension following Thy-1 nephritis is unclear. To our knowledge, no studies have been performed that focus on tubulointerstitial changes in experimental glomerulonephritis under systemic and intraglomerular hypertension.

The results of the present study reveal that prominent tubulointerstitial alterations occur in group SP rats. These changes were noted mainly in the areas of injured and sclerosed glomeruli. This indicates that severely injured and sclerosed glomeruli coexist with prominent tubulointerstitial changes. Although the definite pathogenetic mechanisms responsible for the development of these tubuloiterstitial abnormalities in this model are uncertain, one potent possibility is microvascular injury due to the deleterious effect of hypertensive hemodynamic factor. Any sustained increase in the peritubular capillary (PTC) network pressure could adversely affect endothelial, interstitial and tubular cell function[31]. Although group SC also showed glomerular and tubulointerstitial lesions due to the influence of hemodynanamic alterations, the degree of injury was less than and was not as extensive as that of group SP. This suggests that the kidneys of group SP are more susceptible to the influence of hypertensive hemodynanamic alterations. Tubulointerstitial injury with an increase in intrestitial fibrosis would cause oppression and obliteration of the

post-glomerular capillary network and further elevate post-glomerular resistance, which, in itself, may accelerate the progression of glomerular injury, resulting in progressive renal damage and declining renal function.

One significant feature of this study was that evidently increased expression of α-SMA as a marker of myofibroblast was observed in the tubulointerstitium of group SP. The increased expression of α-SMA in the interstitial myofibroblastic cells was in keeping with the extent of tubulointerstitial alterations. Similar changes have been described previously in the 5/6 nephrectomized kidney, a model of progressive renal damage with rapid loss of renal function[13]. This suggests that up-regulated interstitial expression of α-SMA is correlated with the severity of tubulointerstitial injury and fibrosis. The mechanism responsible for the interstitial myofibroblast overproduction is still uncertain, but an intimate correlation between an increase in α-SMA expression and interstitial fibrosis is generally observed in various organs[32,33].

Several cytokines elaborated by circulating cells recruited into the inflammatory site may activate proliferative and migratory fibrogenic responses in resident cells[34]. The resident or recruited fibroblasts, as the progenitors of myofibroblasts, are activated or elaborated to undergo their phenotypic transformation under the induction of some cytokines such as TGF-β, PDGF and extracellular matrix (ECM)[34,35]. Inflammatory cells and their secretory factors can stimulate fibroblast motility and proliferation[34]. Acquiring morphological and biochemical features of the smooth muscle, these fibroblasts are transformed into myofibroblasts, which can be recognized by the expression of α-SMA. Our results demonstrated that a prominent increase in myofibroblast accumulation was consistent with the extent of tubulointerstitial alterations. Therefore, we believe that myofibroblast trasformation is one of the key features in the progression of tubulointerstitial fibrosis.

Another feature of this study was that a prominent influx of monocytes/macrophages was observed in the tubulointerstitium of group SP, while these changes in group SC were not so severe as in group SP and no significant interstitial mononuclear/macrophage infiltration was detected in the kidneys of group WK. Common to all injuries of the kidney is the triggering of interstitial infiltration and tubular damage[35]. As mediators of tubulointerstitial injury, macrophages are recruited into the tubulointerstitium by various chemoattractants and release various cytokines and mediators, which may stimulate the motility and proliferation of fibroblasts and the overproduction of ECM, resulting in tubulointerstitial injury and fibrosis. Our results suggest that the tubulointerstitial influx of monocytes/macrophages in a hypertensive situation plays a major role in the development of tubulointerstitial injury and fibrotic changes.

The present study showed that marked glomerular damage was accompanied by prominent tubulointerstitial injuries and deterioration of renal function in group SP, and the concomitant tubulointerstitial injuries are thought to be the main cause of declining renal function in group SP. It is highly possible that systemic and intraglomerular hypertension is not only ultimately responsible for the progression of glomerular injury, but is also responsible for the subsequent progression of tubulointerstitial changes. Our results indicate that the migration and proliferation of fibroblasts and the intense influx of monocytes/macrophages under hypertensive conditions play the major role in the development of tubulointerstitial injury with fibrotic changes. A mechanism similar to the one in this model is believed to be correlative with the rate of progression to end-stage renal failure in hypertensive humans with glomerulonephritis.

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