Angiotensin II and IGF-I May Interact to Regulate Tubulointerstitial Cell Kinetics and Phenotypic Changes in Hypertensive Rats

Shuzo KOBAYASHI, Hidekazu MORIYA, Iwao NAKABAYASHI*, Jyunichiro NISHIYAMA*, and Taneo FUKUDA**

Angiotensin II and insulin-like growth factor-I (IGF-I) are known to be actively involved in the pathogenesis of progressive renal injury, particularly in cell proliferation and phenotypic changes that contribute to tubulointerstitial injury. To investigate the possible mechanisms by which angiotensin II type 1 receptor antagonist (AIIA) ameliorates renal injury in a renal ablation model and to determine the contribution of phenotypic changes and IGF-I to morphological changes, we examined 1) whether AIIA attenuated phenotypic changes as markers of β-smooth muscle actin (SMA) and vimentin, 2) whether AIIA altered renal IGF-I expression, and 3) the changes of tubulointerstitial cell kinetics between apoptosis (tested via terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling, TUNEL) and cell proliferation (a test of proliferating cell nuclear antigen, PCNA). Following a sham operation (sham) or 5/6 nephrectomy (Nx), we administered E4177, a potent, selective competitive angiotensin II type 1 receptor antagonist (AIIA), for 10 weeks. In Nx rats, SMA and vimentin expressions developed in injured tubulointerstitium, particularly in hypoperfused scar-adjacent areas, and there was an increase in renal IGF-I expressions. The TUNEL score increased 5-fold and PCNA increased 8-fold, compared with TUNEL and PCNA measurements in sham-operated rats. Renin expression in the juxtaglomerular apparatus was markedly suppressed in the Nx group, although de novo tubular renin expression appeared in Nx, compared with that in the sham group. E4177, both 10 mg/kg (AIIA 10) and 1 mg/kg (AIIA 1), markedly ameliorated renal injury, although blood pressure was less affected in AIIA 1. Both AIIA 10 and AIIA 1 suppressed the neoexpressions of SMA and vimentin in an association with decreased IGF-I expression. Regarding cell kinetics, neither AIIA 10 nor AIIA 1 decreased the TUNEL score; rather, tended to increase, while PCNA was significantly suppressed by AIIA. In conclusion, one of the underlying protective mechanisms of AIIA in this model may be related to the modulations of angiotensin II-induced phenotypic changes and tubulointerstitial cell kinetics through IGF-I.

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Key Words: angiotensin II type 1 receptor antagonist, phenotypic changes, β-smooth muscle actin, IGF-I, apoptosis

Introduction

Several investigators have reported that angiotensin-converting enzyme (ACE) inhibitors (I) or angiotensin II type 1 receptor antagonists (AIIA) (2) lessen progressive renal disease in a remnant kidney model, probably through vasodilatory action on glomerular efferent arterioles, thus leading to a
decreased difference in the hydrostatic pressure across glomerular capillary walls (3–5). In this model, interstitial fibrosis is known to be an important determinant of the progressive renal injury (6) as well as glomerular hypertrophy (7), since interstitial cell proliferation precedes the onset of glomerular cell proliferation and sclerosis (8). Certainly, tubulointerstitial changes, rather than glomerular changes, have been strongly correlated with a decline in glomerular filtration rates (9, 10).

Recent advances have contributed to our understanding of some of the mechanisms underlying tubulointerstitial fibrosis (11). The interactions between resident renal cells and infiltrating cells mediated by autacoids, cytokines, growth factor, and chemokines, are thought to play an important role. Furthermore, it appears that some of these interactions may lead to phenotypic changes in resident renal cells. Phenotypic modulation of these cells consists of their acquisition of new smooth muscle cells and fibroblastic (mesenchymal) characteristics, which is associated with the neoexpression of cytoplasmic cytoskeletal protein (12). In this regard, it is of interest to note that PDGF (13) or angiotensin II (14) induces phenotypic changes in interstitial cells. Johnson et al. have recently hypothesized that essential hypertension may be a type of acquired tubulointerstitial renal disease (15). Under these conditions, angiotensin II would be an important contributor to persistent hypertension and histological injury. In the remnant kidney model, angiotensin II generated in ischemic areas may participate in renal injury as an autocrine and/or paracrine growth factor. Hostetter and his associates studied the importance of the renin-angiotensin-aldosterone (RAS) axis in the rat remnant kidney (16–18) and reported that renin and renin mRNA derived from scar-adjacent tissue were increased (19). This finding may arise from the relative hypoperfusion of those nephrons in a “watershed” zone, as suggested by Meyer and Rennke (20).

Insulin-like growth factor-I (IGF-I) is known to play an important role in compensatory hypertrophy, thus leading to progressive renal injury in the remnant kidney model (6, 21). Although it has been reported that angiotensin II-induced mitogenesis in vascular smooth muscle cells requires the presence of IGF-I and that IGF-I plays a potentially important role as a mediator of the vascular growth responses induced by activation of the renin-angiotensin system (22), little information is available regarding the relationship between IGF-I and angiotensin II in progressive renal injury. Moreover, it has been reported that IGF-I inhibits apoptosis (23), which may play an important role in progressive renal disease in conjunction with cell proliferation (24). Therefore, in the present study, to examine the possible mechanisms in which AIIA limits progressive renal injury in the remnant kidney model, we studied 1) whether AIIA attenuated phenotypic changes as markers of α-smooth muscle actin (SMA) and vimentin (25), 2) whether AIIA altered renal IGF-I expression, 3) whether AIIA altered the changes of tubulointerstitial cell kinetics between apoptosis (tested via terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling, TUNEL) and cell proliferation (a test of proliferating cell nuclear antigen, PCNA), and finally, 5) whether renin expressions were altered by AIIA.

Materials and Methods

Twenty-two male Sprague-Dawley pathogen-free rats, weighing approximately 250 g, were housed individually in cages with a wire meshed bottom at least 10 days prior to the experiments. The animals were handled in accordance with the Guiding Principles in the Care and Use of Animals of our laboratory. During this period, systemic blood pressure was measured twice on separate days by the tail-cuff method (Riken Development Co., Ltd., Tokyo, Japan) while animals were conscious and held with a warmed restrainer. The measurements were repeated 10 times successively on each occasion. The mean of twenty measurements was considered the base-line systolic blood pressure for each animal. When animals were extremely agitated, the measurements were carefully performed again at other times. Seventeen animals underwent a five-sixths nephrectomy (5/6 Nx) by removal of the right kidney and ligature of two of the three segmental branches of the left kidney in a one-step procedure while they were under pentobarbital anesthesia (50 mg/kg body weight, i.p.). In addition, five animals underwent sham nephrectomy (sham). The animals were given a standard diet (Nippon Clea, Japan: 25% casein, 3.44 kcal/g, 0.3% NaCl). E4177, a potent, selective competitive angiotensin II type 1 receptor antagonist, 4-[2-cyclopropyl-7-methyl-3H-imidazo[5,4-β]-pyridin-3-yl]methyl-2-biphenyl-carboxylic acid, was supplied by Eisai Co., Ltd., Tokyo, Japan. The E4177 was dissolved in drinking water at a concentration of 80 mg/l (AIIA 10; n = 6) or 8 mg/l (AIIA 1; n = 6) and administered ad libitum for up to 10 weeks. A nephrectomized group of animals (n = 5) were given water without E4177 (Nx). The amount of water consumed was measured during the mid part of the experiment. Body weight was measured once a week and blood pressure at 5 and 10 weeks. Twenty-four-hour urine specimens were collected at 5 and 10 weeks and assayed for protein by the sulfosalicylic acid method (26).

Ten weeks after renal ablation, all the animals were sacrificed while under pentobarbital anesthesia to study the effects of E4177 on renal morphology and renal function. Blood was drawn from the abdominal aorta, and the left kidney was excised. Coronal sections 3 mm thick were immersed in 4% freshly made formaldehyde solutions in 0.1 M phosphate buffer, and the sections were then used for light microscopic observations. Blood samples were analyzed for creatinine (Cr), blood urea nitrogen (BUN), total protein (TP), total cholesterol (TC), and triglyceride (TG). Creatinine clearance (Ccr) was calculated from urinary creatinine excretion and serum creatinine according to the standard method. Renal and heart weights were measured and expressed as weight per 100 g of body weight.
Morphological Analysis

Fixed coronal sections for light microscopy were embedded in paraffin, and 3-μm-thick sections were stained with periodic acid/silver methenamine and counter-stained with Masson-trichrome (PAM-Masson). Approximately one hundred glomeruli from each kidney were scored in a coded fashion for the presence or absence of significant structural damage as described previously (27). Score 0: normal glomeruli, score 1: mesangial hyperplasia alone, score 2: segmental sclerosis and/or hyalinosis, capillary aneurysms, crescent formations, score 3: global or nearly global sclerosis and/or hyalinosis (obsolescent glomeruli). The percentage of glomeruli having score 2 or 3 was counted as the glomerular sclerosis index (GSI). Thus, glomeruli showing only increased mesangial hyperplasia were not included. Tubulointerstitial alteration was assessed by morphometric analysis using a point counting technique previously reported (27). Morphometry was performed blindly in a coded fashion.

Immunohistochemistry

Immunohistochemistry was performed with the avidin-biotin-peroxidase technique (28, 29). Deparaffinized and rehydrated slides were immersed in 3% aqueous hydrogen peroxide for 30 min to quench endogenous peroxidase activity. Then, to reduce non-specific binding of biotinylated goat anti-rabbit or horse anti-mouse antibody, the slides were placed in 3% normal goat serum or 3% normal horse serum in 0.1 M Tris-buffered saline containing 0.05% Tween 20 (TBS-T) and 0.05% sodium azide. Sections were then incubated with the following antibodies for 1 h at room temperature in a humidified chamber: 1) rabbit anti-rat IGF-I antibody (provided through the National Institute of Diabetes, Digestive, and Kidney Disease, National Hormone and Pituitary Program, by Louis E. Underwood and Judson J. Van Wyk, University of North Carolina, Chapel Hill, USA), diluted 1:500 in TBS-T, 2) monoclonal antibody against vimentin clone V9 (DAKO Japan, Kyoto, Japan), diluted 1:20 in TBS-T, 3) rabbit anti-human polyclonal α-smooth muscle actin antibody (DAKO Japan), diluted 1:40 in TBS-T, 4) rabbit anti-rat renin antibody (provided by Syoukei Kim, Department of Pharmacology, Osaka City University, Osaka, Japan), diluted 1:100 in TBS-T, 5) monoclonal antibody against PC-10 PCNA (DAKO Japan), diluted 1:200 in TBS-T. Following three rinses in TBS-T, secondary link antibody including a biotinylated goat anti-rabbit or horse anti-mouse antibody was applied followed by streptavidin-biotin peroxidase complex (LSAB kit, DAKO Japan) for 10 min (29). Finally, the color was developed by treating the sections with a chromogenic component composed of 0.05% 3-diaminobenzidine (DAB) (Sigma Chemical Co., Poole, Dorset, UK), 0.01% (v/v) hydrogen peroxide, and 0.01 M imidazol made in 0.1 M Tris-HCl buffer (pH 7.4). Tissue sections were lightly counterstained with hematoxylin, dehydrated in an ascending ethanol series and xylene, and mounted with coverslips. The specificity of staining was verified with sections that were treated in a similar manner, except nonimmune rabbit serum or IgG (10 μg/ml) was used instead of specific primary antibody.

In Situ Detection of Apoptosis

In situ detection of apoptosis or DNA fragmentation was performed on tissue sections of rats with an Apoptag Plus in situ apoptosis detection kit (Oncor, USA) or as described elsewhere (30). The method corresponds to the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling TUNEL to detect DNA fragmentation in tissue.

Histochemical Evaluation

Semiquantitative analysis for immunoreactivity was performed in a blinded manner. Immunoreactivity for smooth muscle actin (SMA) and vimentin in tubulointerstitium was graded semiquantitatively as follows and was expressed as the average score in each group: 0, negative; 1+, trace staining in an occasional cortical area; 2+, clearly evident staining in scattered cortical areas; 3+, marked staining in scattered areas; 4+, marked staining found homogenously throughout the entire cortex. Immunoreactivity for IGF-I in cortex was estimated by staining intensity rather than by the extent of distribution for immunoreactivity as follows: 0, negative; 1+, weak; 2+, moderate; 3+, strong staining. As for renin immunoreactivity in juxtaglomerular apparatus (JGA), using a 10× objective lens, we examined about 70 glomeruli in one coronal section and counted all renin-positive areas of JGA. This was expressed as the percentage of renin-positive areas of JGA of total glomeruli examined per a coronal section of one kidney. The evaluation of cell kinetics between apoptosis (TUNEL) and cell proliferation (PCNA) was performed by counting TUNEL-positive or PCNA-positive nuclei in 30 fields per tissue section at a magnification of 200 and was expressed as the total number of positive-staining nuclei per field.

Statistics

Results are expressed as the mean ± SD. Data were analyzed by one-way analysis of variance. Significance level of the difference between the means of individual groups, subjected to the analysis of variance, was established by use of the Student-Newman-Keuls procedure for multiple comparisons, and p less than 0.05 was deemed statistically significant. Nonparametric tests were applied for the glomerular sclerosis index and the tubulointerstitial index, because the distribution is not necessarily normal. The data from groups were first analyzed by using the Kruskal-Wallis H test, and when a probability value was less than 0.05, a Wilcoxon rank sum test was applied to paired groups, for which a value of p < 0.05
Table 1. Serum Biochemical Data 10 Weeks after Ablation

<table>
<thead>
<tr>
<th>Groups</th>
<th>Creatinine (mg/dl)</th>
<th>BUN (mg/dl)</th>
<th>Total protein (g/dl)</th>
<th>Total cholesterol (mg/dl)</th>
<th>Triglycerides (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nx group</td>
<td>0.88 ± 0.09*</td>
<td>32.9 ± 4.4*</td>
<td>7.1 ± 0.6</td>
<td>106.6 ± 8.6*</td>
<td>191.8 ± 24.3*</td>
</tr>
<tr>
<td>AIIA 10 group</td>
<td>0.77 ± 0.05*</td>
<td>33.4 ± 4.3*</td>
<td>6.9 ± 0.4</td>
<td>85.7 ± 10.7**</td>
<td>162.5 ± 77.6*</td>
</tr>
<tr>
<td>AIIA 1 group</td>
<td>0.76 ± 0.12*</td>
<td>28.8 ± 7.0*</td>
<td>6.8 ± 0.3</td>
<td>82.3 ± 15.4***</td>
<td>159.5 ± 41.6*</td>
</tr>
<tr>
<td>Sham group</td>
<td>0.59 ± 0.02</td>
<td>18.6 ± 3.6</td>
<td>6.9 ± 0.2</td>
<td>51.2 ± 5.2</td>
<td>71.6 ± 10.9</td>
</tr>
</tbody>
</table>

*p < 0.05 vs. sham, **p < 0.05 vs. Nx.

Table 2. Urinary Protein Excretion (uPr) and Creatinine Clearance (Ccr)

<table>
<thead>
<tr>
<th>Groups</th>
<th>5 weeks postablation (uPr mg/day)</th>
<th>10 weeks postablation (uPr mg/day)</th>
<th>Ccr/100 g BW (ml/min)</th>
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</thead>
<tbody>
<tr>
<td>Nx group</td>
<td>21.5 ± 7.2*</td>
<td>80.7 ± 28.7*</td>
<td>0.236 ± 0.049*</td>
</tr>
<tr>
<td>AIIA 10 group</td>
<td>14.3 ± 3.8*</td>
<td>23.9 ± 8.2*</td>
<td>0.266 ± 0.040*</td>
</tr>
<tr>
<td>AIIA 1 group</td>
<td>15.3 ± 3.8*</td>
<td>43.7 ± 19.0*</td>
<td>0.250 ± 0.023*</td>
</tr>
<tr>
<td>Sham group</td>
<td>5.3 ± 1.1</td>
<td>6.1 ± 1.4</td>
<td>0.386 ± 0.089</td>
</tr>
</tbody>
</table>

*p < 0.05 vs. sham, **p < 0.05 vs. Nx. There was no statistical difference in these parameters between AIIA 10 and AIIA 1 groups.

Table 3. Organ Growth

<table>
<thead>
<tr>
<th>Groups</th>
<th>Heart/100 g BW</th>
<th>Kidney/100 g BW</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nx group</td>
<td>0.221 ± 0.021*</td>
<td>0.339 ± 0.031*</td>
</tr>
<tr>
<td>AIIA 10 group</td>
<td>0.197 ± 0.010**</td>
<td>0.307 ± 0.030*</td>
</tr>
<tr>
<td>AIIA 1 group</td>
<td>0.205 ± 0.006**</td>
<td>0.306 ± 0.018*</td>
</tr>
<tr>
<td>Sham group</td>
<td>0.185 ± 0.020</td>
<td>0.287 ± 0.015</td>
</tr>
</tbody>
</table>

*p < 0.05 vs. sham, **p < 0.05 vs. Nx. There was no statistical difference in these parameters between AIIA 10 and AIIA 1 groups.

Results

There was no difference in blood pressure among the groups before the operation. All nephrectomized groups showed significant weight loss during the first postoperative week, then gained weight for up to 10 weeks. There was no difference in body weight among the nephrectomized groups. Water consumption (ml) measured at 5 weeks after 5/6 Nx was as follows: 38 ± 4, 42 ± 3.2, 39 ± 4.2, and 15.6 ± 2.8 in the Nx, AIIA 1, AIIA 10, and sham groups, respectively.

As shown in Fig.1, systolic blood pressure in the nephrectomized rats (Nx group) increased significantly 5 and 10 weeks after renal ablation. A high dosage of AIIA (80 mg/l; AIIA 10) significantly normalized blood pressure at 5 and 10 weeks. A low dosage of AIIA (8 mg/l; AIIA 1) did not significantly lower blood pressure at 5 weeks. There was a statistically significant difference in blood pressure both at 5 weeks and 10 weeks between AIIA 10 and AIIA 1. In the Nx group, serum Cr, BUN, TC, and TG were significantly increased at sacrifice (Table 1). Both AIIA 10 and AIIA 1 decreased TC significantly and showed a tendency to reduce Cr and TG (Table 1). Urinary protein excretion increased 5 weeks after ablation in Nx, while both AIIA 10 and AIIA 1 significantly reduced proteinuria (Table 2). Also at 10 weeks after ablation, both E4177-treated groups showed significantly lessened proteinuria. Regarding renal function, Ccr/100 g body weight (BW) was significantly decreased 10 weeks after ablation (Nx group) compared to that in the sham group. Both E4177-treated groups (AIIA 10 and AIIA 1) showed a tendency to increase Ccr/100 g BW (Table 2). The 5/6 nephrectomized rats showed significantly increased heart weight compared with that in sham-operated rats (0.221 ± 0.021 g).
Both drugs significantly suppressed this increase in heart weight, as shown in Table 3. With respect to the kidney, Nx rats showed significantly increased kidney weight, compared with that in sham-operated rats (0.339 ± 0.031 vs. 0.287 ± 0.015 g/100 g body weight). AIIA 10 and AIIA 1 tended to suppress the increase in kidney weight/100 g body weight, although there was not a significant difference (Table 3).

Renal Morphology

Examination of the renal pathology of remnant kidney 10 weeks after ablation revealed glomerular hypertrophy and extensive glomerular damage consisting of global or segmental sclerosis and/or hyalinosis, including capillary aneurysms associated with widespread tubular dilation, cast formation, tubular atrophy, interstitial fibrosis, as well as scattered foci of inflammation (Fig. 2a). Both AIIA 10 (Fig. 2b) and AIIA 1 dramatically lessened these morphological changes. These differences were reflected in both the glomerular sclerosing index and the tubulointerstitial index.

Table 4. Histological Findings

<table>
<thead>
<tr>
<th>Groups</th>
<th>GSI</th>
<th>TII</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nx group</td>
<td>34.9 ± 5.2*</td>
<td>40.9 ± 7.3*</td>
</tr>
<tr>
<td>AIIA 10 group</td>
<td>20.3 ± 4.2**</td>
<td>21.8 ± 4.6**</td>
</tr>
<tr>
<td>AIIA 1 group</td>
<td>24.6 ± 6.3**</td>
<td>28.1 ± 8.0**</td>
</tr>
<tr>
<td>Sham group</td>
<td>0.5 ± 0.8**</td>
<td>4.8 ± 1.6**</td>
</tr>
</tbody>
</table>

*p < 0.05 vs. sham, **p < 0.05 vs. Nx. There was no statistical difference in these parameters between AIIA 10 and AIIA 1 groups. GSI, glomerular sclerosing index; TII, tubulointerstitial index.

As shown in Table 4, both doses of AIIA caused significant decreases in the GSI (0.5 ± 0.8, 34.9 ± 5.2, 20.3 ± 4.2, and 24.6 ± 6.3 % in sham, Nx, AIIA 10, and AIIA 1, respectively) and tubulointerstitial index (4.8 ± 1.6, 40.9 ± 7.3, 21.8 ± 4.6, and 28.1 ± 8.0 % in sham, Nx, AIIA 10, and AIIA 1, respectively).

Immunohistochemistry

Negative control for immunohistochemistry including vimentin, α-smooth muscle actin, renin, and IGF-I, described in the following paragraphs, showed all negative staining.

Vimentin immunoreactivity was shown in glomerular epithelial cells of sham controls (Fig. 3a). There was no immunostaining of renal tubular epithelium or interstitium in sham-operated rats. However, in the remnant kidneys (Fig. 3b), marked immunoreactivity appeared in collecting or distal tubular epithelium and interstitium. Staining in peritubules was much stronger than in tubular cytoplasm (Fig. 3c), but definite granular staining was also seen in the cytoplasm. The localization of vimentin, however, was confined to the injured tubulointerstitium, particularly to fibrotic, periglomerular, and perivascular areas. Vimentin expression was never detected in proximal tubuli or in sclerosed areas of glomeruli. Staining intensity and distribution were more marked in the interstitium where extensive renal injury, particularly tubulointerstitial injury, was detected. We considered this immunoreactivity to consist of phenotypic changes, and the immunoreactivity appeared to parallel the severity of renal injury, particularly tubulointerstitial changes. As shown in Table 5, semiquantitative analysis for immunoreactivity showed that AIIA dramatically normalized vimentin expression (Fig. 3d) in the tubulointerstitium in association with a decrease in renal damage.

Immunoreactivity for SMA was confined to vascular
smooth muscle cells in sham-operated rats (Fig. 4a). As shown in Table 5, in Nx rats, SMA was highly expressed within interstitium (Fig. 4b). Particularly, SMA expression was increased in scar-adjacent areas (Fig. 4c) as well as in the medullary ray. Overexpression of SMA was also prominent in the periglomerular area (Fig. 4d). In addition to interstitium, SMA was expressed within the expanded mesangial area. AIIA treatment suppressed the interstitial SMA expression (Table 5) in parallel with improving tubulointerstitial alterations (Fig. 4e).

Renin immunoreactivity in sham-operated rats was confined to the JGA (Fig. 5a). Although rare, renin expression was also detected in more proximal sites of the JGA. In the Nx group, renin expression in the JGA was clearly reduced. In the scarred tissue of 5/6 nephrectomized kidneys, however, intense renin immunostaining was present along afferent arterioles supplying glomeruli located in the outermost areas of the scarred tissue. In addition to the renin in JGA, it was sometimes found in tubuli of Nx rats (Fig. 5b). Semiquantitative analysis (Fig. 6) for renin expression in JGA showed

**Table 5.** Semiquantitative Analysis of Immunoreactivity for Vimentin, SMA and IGF-I

<table>
<thead>
<tr>
<th>Groups</th>
<th>Vimentin</th>
<th>SMA</th>
<th>IGF-I</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nx group</td>
<td>3.60 ± 0.55*</td>
<td>3.80 ± 0.45*</td>
<td>2.80 ± 0.45*</td>
</tr>
<tr>
<td>AIIA 10 group</td>
<td>1.67 ± 0.52*,**</td>
<td>1.50 ± 0.55*,**</td>
<td>1.50 ± 0.55**</td>
</tr>
<tr>
<td>AIIA 1 group</td>
<td>2.00 ± 0.63*,**</td>
<td>2.17 ± 0.41*,**</td>
<td>1.83 ± 0.75**</td>
</tr>
<tr>
<td>Sham group</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>1.60 ± 0.55</td>
</tr>
</tbody>
</table>

*p < 0.05 vs. sham, **p < 0.05 vs. Nx.
11.4 ± 1.1% in the sham-operated group, while in the Nx group, the renin positive areas were significantly lower (2.8 ± 0.3%, p < 0.05). In animals treated with either dose of AIIA, the renin-positive area was not statistically different from that of Nx rats, while tubular renin was scarcely found.

IGF-I was localized in distal tubules, in the thick ascending limb of Henle’s loop, and in the cortical and medullary collecting ducts, as described previously (28), in both sham controls (Fig. 7a) and all nephrectomized rats. IGF-I immunoreactivity was increased in renal cortex of the Nx group (Fig. 7b), while AIIA normalized this increase (Fig. 7c). Particularly strong staining was evident in the injured cells of the distal tubules and within the fibrosed interstitium. These results were reflected by the semiquantitative analysis shown.
As shown in Table 6, TUNEL-positive cells were few in sham-operated rats, while in the Nx group there was a statistically significant increase. Neither dosage of AIIA affected the total number of TUNEL-positive cells (Fig. 8). In contrast, increased PCNA-positive cells found in the Nx group were significantly suppressed by both AIIA 1 and AIIA 10 (Fig. 9).

Table 6. TUNEL and PCNA Positive Cells

<table>
<thead>
<tr>
<th>Groups</th>
<th>TUNEL</th>
<th>PCNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nx group</td>
<td>1.23 ± 0.25*</td>
<td>13.16 ± 1.85*</td>
</tr>
<tr>
<td>AIIA 10 group</td>
<td>1.58 ± 0.31*</td>
<td>5.67 ± 2.27**</td>
</tr>
<tr>
<td>AIIA 1 group</td>
<td>1.35 ± 0.22*</td>
<td>8.31 ± 1.73**</td>
</tr>
<tr>
<td>Sham group</td>
<td>0.23 ± 0.07</td>
<td>1.55 ± 0.70</td>
</tr>
</tbody>
</table>

*p < 0.05 vs. sham, **p < 0.05 vs. Nx.

Discussion

Using a 5/6 nephrectomized rat model, we demonstrated that the newly developed angiotensin II type I receptor antagonist, E4177, dramatically lessened progressive renal injury. Although AIIA 1 did not decrease blood pressure at 5 weeks postablation, there was a significant reduction in proteinuria. Moreover, there was no difference in glomerular sclerosis, interstitial change, or immunohistochemistry, regardless of the significant difference in blood pressure between the AIIA 10 and AIIA 1 groups. Although we did not have a control antihypertensive group for which blood pressure was lowered by agents other than AIIA, these findings suggest that the favorable effects of this drug are attributable to angiotensin II blockade rather than to the reduction in blood pressure, which is in agreement with other reports (2, 31–34).

We showed in the present study that increased IGF-I expression in renal cortex of rats in the Nx group was normalized in AIIA-treated animals in parallel with decreased compensatory renal growth. We also found evidence that IGF-I also may participate in the progressive renal injury in addition to the involvement of TGF-β (33, 34) or PDGF (35), which is a potent fibrosis-promoting cytokine, as has been reported. Although we (6) and others (21) have already sug-
gested that IGF-I may contribute to the progression of interstitial fibrosis in the remnant model, this is the first report that angiotensin II blockade limits renal injury associated with the suppression of renal cortical IGF-I. We are not certain whether medullary IGF-I expression is altered or not, since IGF-I intensity in normal medulla is too strong to evaluate the intensity semiquantitatively (28). In our study we found no reduction in medullary IGF-I expression in any group. Little information is available regarding the interaction between IGF-I and RAS, in spite of the potential involvement of IGF-I in progressive renal injury, while it is well known that angiotensin II-induced renal injury or hypertrophy is mediated through TGF-β (36). A potential role for IGF-I in vascular growth responses has been reported (37), and IGF-I mRNA transcripts are increased in the hypertensive aortae of abdominally coarcted rats (38). Delafontaine and Lou (22) have shown that transcriptional activation of the IGF-I gene plays an important role in angiotensin II-mediated vascular growth responses, which have potentially important implications for the role of RAS in vascular hypertrophy/hyperplastic responses. Angiotensin II receptor and IGF-I receptor both are widely distributed through the kidney (39). In particular, IGF-I receptor located on the surface of fibroblasts and/or IGF binding protein (IGFBP) in interstitium may play a role in the interaction between IGF-I and angiotensin II.

Fig. 7. IGF-I immunohistochemistry (x200) a) IGF-I was localized in distal tubules, in the thick ascending limb of Henle’s loop, and in the cortical and medullary collecting ducts as described previously (29) in the sham-operated rats. b) The IGF-I immunoreactivity increased in renal cortex of rats in the Nx group. c) AIIA normalized the increased IGF-I in Nx rats.

Regarding compensatory renal growth after renal ablation, it is well known that IGF-I plays an important role (6). Renal growth is likely to depend on the balance between the proliferation or death, through apoptosis, of activated tubulointerstitial cells as well as glomerular cells. Since it is known that IGF-I inhibits apoptosis (23), we evaluated tubulointerstitial cell kinetics. In the Nx group, the TUNEL score increased 5-fold and PCNA increased 8-fold compared with the TUNEL and PCNA measurements in sham rats. AIIA did not decrease the TUNEL score but rather tended to increase it, which may be compatible with known inhibitory effects of IGF-I on apoptosis. There is a possibility that AIIA decreased IGF-I expression through an inhibitory effect of RAS, thus leading to a decrease in the ratio of PCNA-positive to TUNEL-positive cells in AIIA-treated nephrectomized rats. The altered cell kinetics between apoptosis and proliferation in AIIA-treated rats may be ascribed to ischemia-related injury and its improvement through the inhibition of intrarenal RAS. In this regard, it is known that another angiotensin receptor antagonist, candesartan, increases
myocardial apoptosis in rats with ischemic perfusion injury (40). Both results appear to show that angiotensin receptor antagonist promotes tissue remodeling by increasing apoptosis.

Regarding the development of fibrotic change, the tubulointerstitial influx of monocytes/macrophages is thought to be of particular importance (41). Kunico et al. (11) proposed that recruitment of inflammatory cells, cytokines, proliferation of fibroblasts, and matrix deposition may be key events in the fibrotic process. A recent important observation regarding the pathogenesis of tubulointerstitial changes involves phenotypic changes of tubulointerstitial cells consisting of their acquisition of new smooth muscle cells and fibroblastic (mesenchymal) characteristics, namely myofibroblasts associated with the neoexpression of cytoplasmic cytoskeletal proteins. In the present study we examined the expression of α-smooth muscle actin and vimentin, one of the intermediate filament proteins. Vimentin is known to be expressed in acutely and reversibly damaged kidneys, and it also could be regarded as an indicator of the regenerating and proliferating activity of tubular lesions (42, 43). Our results showed that vimentin, which is not expressed in the tubulointerstitium of normal rat kidney, was expressed in the injured tubulointerstitium. It has been reported that in the remnant kidney (8, 44) and nephrotoxic serum nephritis models (45), interstitial myofibroblasts predominate and exceed the number detected within the glomeruli. Our findings are compatible with these observations. Interstitial myofibroblasts expressing α-smooth muscle actin, desmin or vimentin, or a combination of these, have been associated with a poor prognosis in experimental (45) and clinical (46) glomerulonephritis. Taken together, the neoexpressions of vimentin and α-smooth muscle actin clearly appear to be markers of injured tubulointerstitium and reflect the progressive decline of renal function. Of interest is the finding that angiotensin II induces tubulointerstitial injury with phenotypic changes (14). Therefore, the appearance of vimentin and SMA expressions might be due to ischemia in the injured kidney. In the present study myofibroblasts develop more in a hypoperfused area or in the medullary ray, which is known to be susceptible to hypoxia. The angiotensin II antagonist, E4177, inhibited these local proliferations of myofibroblasts, which are associated with the attenuation of renal injury. According to Navar and Harrison-Bernard (47), locally activated angiotensin II may, through hypoperfusion, directly or indirectly induce myofibroblasts in interstitium, leading to intersti-

Fig. 8. a) Cells shown by the TUNEL method are scarcely found in sham-operated rats (× 200). b) In the Nx group, TUNEL-positive nuclei increased (× 200). c) TUNEL-positive cells are detached in tubular lumen (× 400). d) TUNEL-positive nuclei are not affected by AIIA (× 200).
tial fibrosis and increased vascular resistance. We have recently reported that AIIA exerts a potential renal protective effect associated with the inhibition of phenotypic changes in two-kidney, one-clip Goldblatt hypertensive rats (48) as reported by others (49). Moreover, there is a report that tubulointerstitial injury in aging rats may be the consequence of ischemia secondary to perinuclear capillary injury through fibroblast activation and apoptosis (50).

With respect to renin expression, our result showing decreased renin expression in remnant nephron after 5/6 Nx is compatible with previous findings (51, 52). Regarding the renin expression in nephron segments other than JGA, Rosenberg et al. (18) showed that there is glomerular renin synthesis in remnant kidney after 5/6 Nx. More recently, definitive evidence of renin synthesis by proximal tubules has also been reported (53, 54). Tank et al. (55) showed that in the early phase after unilateral Nx, when circulating and JG renin are unchanged, proximal tubular renin gene expression is increased. Therefore, intrarenal shift of renin synthesis must be considered, although JG renin expression is decreased in the Nx group. Our results showing the appearance of tubular renin expression in the Nx group may not be just by pinocytosis of filtered renin but also by renin synthesis. In contrast, tubular renin expression was scarcely found in the AII-treated group.

In conclusion, we demonstrated that a newly developed angiotensin II AT1 receptor antagonist, E4177, dramatically lessened hypertensive renal injury in the remnant kidney model and that this effect was associated with the suppression of phenotypic changes and increased IGF-I, which may be involved in the later process of renal scarring in injured tubulointerstitium. These favorable effects of AIIA may in part be ascribed to the changes of cell kinetics between the balance of apoptosis and proliferation. The development and inhibition of myofibroblasts and the changes of cell kinetics may depend on ischemia-related injury and its improvement. Concerning the direct relationship between intrarenal RAS and IGF-I, additional studies are clearly needed.

References

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