Short Communication

Interstitial Fibroblast-Like Cells Express Renin-Angiotensin System Components in a Fibrosing Murine Kidney

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Recently, the renin-angiotensin system (RAS) was implicated in organ fibrosis. However, few studies have examined the localization of RAS components, such as angiotensin II receptors, renin (REN), angiotensinogen (AGTN), and angiotensin-converting enzyme (ACE), in the fibrosing kidney. To localize these components in the fibrosing kidney, we used a murine model of renal fibrosis that shows an enhanced expression of angiotensin II type 1A receptor (AT_{1A}R) and AGTN. Our results indicate that the overall expression of angiotensin II type 2 receptor (AT₂R) and ACE was attenuated in this model, whereas REN expression was unchanged. In addition to tubular epithelial cells that were positive for AT_{1A}R, AT₂R, REN, and AGTN, interstitial fibroblast-like cells expressed AT_{1A}R, REN, AGTN, and ACE in the fibrosing kidney. The interstitial fibroblast-like cells that were positive for AT_{1A}R mRNA were further characterized as positive for the expression of vimentin and transforming growth factor- β 1. These data provide strong evidence for a tubulointerstitial RAS within the fibrosing kidney, and a link between the RAS and renal fibrogenesis. (*Am J Pathol* 2002, 160:765–772)

The activation of angiotensin II receptors by angiotensin II has been reported to contribute to the progression of renal fibrosis.^{1–3} The intrarenal renin-angiotensin system (RAS) was described in the 1980's,⁴ and the kidney contains angiotensin II at a significantly higher level than

serum.⁵ Although the intrarenal RAS may be altered in some diseased states,^{6–12} the localization of each component of the RAS, such as angiotensin II type 1 and type 2 receptors (AT₁R and AT₂R), renin (REN), angiotensinogen (AGTN), and angiotensin-converting enzyme (ACE), in the fibrosing kidney is undefined. In this study, we use immunocytochemical analysis and *in situ* hybridization techniques to identify cells expressing the RAS components in a murine model of advanced interstitial fibrosis of the kidney.

Materials and Methods

Murine Model of Renal Fibrosis

We used 5-week-old mice that are transgenic for transforming growth factor (TGF)- β 1¹³ and that had undergone a subtotal nephrectomy as a model of renal fibrosis. These mice reproducibly develop significant interstitial fibrosis at 12 weeks after surgery. Sham-operated TGF- β 1 transgenic mice were used as controls. We confirmed that the control transgenic mice did not show any significant fibrosis in the kidney by 17 week of age. After 17 weeks, the control mice randomly develop renal fibrosis. At 12 weeks after renoablation, half of each kidney was fixed using 4% paraformaldehyde in phosphate-buffered saline overnight. A portion of each fixed specimen was processed into paraffin blocks for histopathology. The rest of the fixed tissue was rinsed in serial concentrations of sucrose, and then snap-frozen in preparation for immunostaining and *in situ* hybridization. Total RNA was extracted from the homogenates of the other half of each kidney using TRIzol (Life Technologies, Inc., Grand Island, NY) according to the manufacturer's instructions.

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cDNA Preparation

cDNA fragments of murine AT_{1A}R (643 bp), AT₂R (568 bp), REN (363 bp), AGTN (509 bp), and ACE (348 bp) were obtained by reverse transcriptase-polymerase chain reaction (RT-PCR) and subcloned into pBluescript II KS+. The primer sequence for the AT_{1A}R corresponded to 5'-CTGAGACCAACTCAACCCAGA-3' (sense; bp 44 to 64) and 5'-AGGAAGCCAGGATGTTCTT-3' (anti-sense; bp 667 to 686).¹⁴ The primer sequence for the AT₂R corresponded to 5'-TGGAGTTGCTGCAGTTCAAT-3' (sense; bp 112 to 131) and 5'-TCCCGGAAATAAAATGTTGG-3' (anti-sense; bp 660 to 679).¹⁵ The sets of primers for the other components were derived elsewhere.¹⁶ The AT₁R subtypes, AT_{1A}R and AT_{1B}R, exhibited >90% homology in the coding region of the cDNA sequence.¹⁴ Therefore, this AT_{1A}R cRNA probe may detect both AT_{1A}R and AT_{1B}R mRNAs.

RNase Protection Assay

The RNase protection assay was performed according to methods described previously.¹⁷ The cRNA probes were generated from the cDNA fragments described above.

RT-PCR

Because it is expressed at lower levels in the kidney than the other components, AT₂R mRNA was separately detected by RT-PCR based on the method reported previously.¹⁸ The primer set for AT₂R cDNA is described above. The PCR was continued for 40 cycles of denaturation at 94°C for 1 minute, annealing at 57°C for 1 minute, and extension at 72°C for 1 minute. Linearity of the amplification was verified using serial quantities of template.

Immunostaining

Indirect immunofluorescence was performed on 4-μm cryostat sections. Polyclonal rabbit anti-AT₁R antibodies

(Santa Cruz Biotechnology, Santa Cruz, CA), anti-AT₂R antibodies,¹⁹ anti-REN antibodies (a generous gift from K. Murakami, University of Tsukuba, Tsukuba, Japan),²⁰ and anti-ACE antibodies (a generous gift from P. Corvol, College de France, France)²¹ were used as the primary antibodies for detection of the RAS components. For phenotyping interstitial fibroblast-like cells (FbLCs), polyclonal goat anti-vimentin (VIM) antibodies (Sigma, St. Louis, MO), rabbit anti-FSP1 antibodies,²² and anti-TGF-β1 antibodies (LC, which recognizes intracellular TGF-β1),¹³ and fluorescein isothiocyanate (FITC)-conjugated monoclonal mouse anti-α-smooth muscle actin (α-SMA) antibody (Sigma) were used. Rhodamine-conjugated polyclonal donkey anti-goat IgG, anti-rabbit IgG (Chemicon International Inc., Temecula, CA), and FITC-conjugated goat anti-rabbit IgG (American Qualex, San Clemente, CA) were used as the secondary antibodies. Dual-immunostained sections were analyzed using a confocal microscope (MRC600; Bio-Rad Laboratories, Hercules, CA). All of the secondary antibodies had been isolated by immunoaffinity chromatography and were preabsorbed with appropriate sera including sheep serum.

In Situ Hybridization

In situ hybridization was performed using methods described in detail previously,¹⁸ and using the cRNA probes described above. Endogenous alkaline-phosphatase activity in the kidney was intensively inactivated with levamisole (Sigma). Hybridization and the final wash were performed at 45°C overnight and with 2× standard saline citrate for 30 minutes at 45°C, respectively. In this experiment, we used sections lacking digoxigenin-labeled cRNA probes or using sense cRNA probes as a negative control. Using some of these sections, dual immunostaining with anti-α-SMA, anti-FSP1, anti-VIM, and anti-TGF-β1 steps were performed as described above.

Table 1. The Localization of RAS Components in Control and Fibrosing Kidneys

RAS component	Control kidney	Overall change in expression	Fibrosing kidney (Immunostain)	Fibrosing kidney (<i>in situ</i> hybridization)
AT _{1A} R	TEC	↑	TEC	FbLC TEC
AT ₂ R	TEC	↓	TEC*	TEC*
REN	JGA TEC*	↔	FbLC JGA* TEC*	FbLC JGA*
AGTN	TEC	↑	(Unchecked)	FbLC TEC
ACE	GC TEC	↓	FbLC*	(Unchecked)

RAS, renin-angiotensin system; AT_{1A}R, angiotensin II type 1A receptor; AT₂R, angiotensin II type 2 receptor; REN, renin; AGTN, angiotensinogen; ACE, angiotensin-converting enzyme; TEC, tubular epithelial cells; JGA, juxta-glomerular apparatus; GC, glomerular cells; FbLC, fibroblast-like cells.

*Occasional and weak.

Figure 1. **A:** A control kidney taken from a sham-operated TGF- β 1 transgenic mouse (H&E; original magnification, $\times 100$). **B:** A fibrosing kidney taken from a 5/6 nephrectomized TGF- β 1 transgenic mouse at 12 weeks after surgery. Significant glomerulosclerosis and interstitial fibrosis have developed (H&E; original magnification, $\times 100$). **C:** A RNase protection assay of AT_{1a}R mRNA in the fibrosing murine kidney. **D:** A RT-PCR assay of AT₂R mRNA in the fibrosing kidney. **E:** A RNase protection assay of REN, AGTN, and ACE mRNAs in the fibrosing kidney. **F:** A quantitative densitometric analysis of **C** to **E**. AT_{1a}R and AGTN mRNAs were significantly increased, AT₂R and ACE mRNAs were decreased, and REN mRNA was unchanged in the fibrosing kidney. t-RNA and adrenal samples were used as negative and positive controls, respectively, in **C** and **D**. In **C** to **E**, a representative blot selected from three separate experiments is shown, and the densitometric data in **F** were obtained from these three blots.

Statistical Analysis

Values are presented as means $\pm$ SE. Statistical differences between the data were evaluated by the Student's *t*-test, with $P < 0.05$ used as the requirement for significance.

Results

The results of this study are summarized in Table 1. The control, sham-operated, TGF- β 1 transgenic mice showed no significant tubulointerstitial changes (Figure 1A),

whereas 5/6 nephrectomized, transgenic mice showed marked glomerulosclerosis and interstitial fibrosis at 12 weeks after surgery (Figure 1B). In parallel to the progression of parenchymal changes in the fibrosing kidney, RNase protection assays demonstrated that AT_{1a}R and AGTN mRNA expression was significantly increased. In addition, RT-PCR and RNase protection assays revealed that AT₂R and ACE mRNA expression was decreased, respectively (Figure 1; C to F). The AT₁R protein was localized primarily to the tubular epithelial cells, some of which were clearly identified as proximal tubules because of their brush borders (Figure 2A). The AT₂R pro-

tein expression remained focal in the tubular epithelium (Figure 2C). *In situ* hybridization demonstrated that AT_{1A}R mRNA was localized not only in the tubular epithelial cells, but also in the solitary interstitial FbLCs in the fibrosing kidney (Figure 2B). In contrast, AT₂R mRNA was localized focally in the tubular epithelium alone (Figure 2D), which is consistent with the localization of the AT₂R protein (Figure 2C). In the control, sham-operated kidney, AT₁R and AT₂R expression was detected only in the tubular epithelial cells, as previously reported (data not shown).^{23,24} Although the overall expression of REN mRNA in the kidney was constant during the progression of renal fibrosis (Figure 1E), the distribution changed. While REN was localized exclusively in the juxtaglomerular apparatus (JGA) in the control kidney (data not shown), REN expression in the fibrosing kidney was localized primarily in the interstitial FbLCs and, occasionally, in the JGA mostly in the superficial cortex (Figure 2, E and F). AGTN expression was also observed in the interstitial FbLCs, as well as the tubular epithelial cells (Figure 2H). However, ACE expression was restricted to the interstitial FbLCs only (Figure 2G).

To further characterize the interstitial FbLCs expressing AT_{1A}R, a dual-immunostaining method was performed on sections after *in situ* hybridization. We examined three phenotypic markers for interstitial FbLCs; α -SMA, FSP1, and VIM. The interstitial FbLCs expressing AT_{1A}R mRNA were found to be VIM-positive, but negative for FSP1 (Figure 3, A and B) and α -SMA (data not shown). As a control experiment to confirm the co-localization found in this procedure, we examined serial sections in a mirror image. One section was assayed for AT_{1A}R mRNA by *in situ* hybridization, and the other section was examined for VIM immunoreactivity. Our results showed an exact co-localization (data not shown). The overexpression of TGF- β 1 mRNA at the renal level has been demonstrated by Northern analysis in aged transgenic mice with renal fibrosis¹³ and in the present model by RNase protection assay (RPA) (data not shown). These VIM-positive FbLCs that expressed AT_{1A}R mRNA were also positive for TGF- β 1 (Figure 3, C and D), suggesting that these cells produced TGF- β 1 in response to angiotensin II, thus contributing to renal fibrogenesis.

Discussion

In the present study, we have shown that the gene expression and protein localization of the RAS components are altered in the fibrosing murine kidney. RNase protec-

tion assays revealed that AT_{1A}R expression was significantly enhanced compared to that of the control kidney of tg mouse without renal fibrosis. In addition, *in situ* hybridization and immunostaining demonstrated the presence of AT_{1A}R in the tubular epithelial cells and the interstitial FbLCs. The effects of AT₁R may include the induction of hypertrophy and increases in the biosynthesis of TGF- β , monocyte chemoattractant protein-1, osteopontin, and extracellular matrix proteins, all of which may be involved in renal fibrogenesis.^{25,26} In this study, we have shown that VIM-positive FbLCs in the fibrosing kidney expressed the AT_{1A}R gene, and were simultaneously positive for TGF- β 1, which likely contribute to renal fibrogenesis. Although we were unable to detect AT₁R in the interstitium using polyclonal anti-AT₁R antibodies, this may have been because of the tissue fixation method used and/or a low detection efficiency.^{7,23,27,28} These findings are partially consistent with those of previous studies in which the localization of the RAS components in diseased kidneys was examined.⁶⁻¹² However, in these previous studies, areas within the interstitial fibrosis were not examined in detail. Therefore, little is known regarding the RAS components within the interstitial FbLCs.

In the case of AT₂R, RT-PCR showed an attenuated AT₂R expression, and the weak signals in some of the tubular epithelial cells were detected in the fibrosing kidney. Some tubular epithelial cells not apparently yet in significant damage continued expressing AT₂R, while the expression in most of the other cells has subsided during fibrogenesis. The activity of angiotensin II via AT₂R in the tubular epithelium remains to be clarified in this pathological condition.²⁵ Moreover, AT₂R has not been detected previously in rodent renal interstitial FbLCs, either *in vivo* or *in vitro*.^{10,19,23,29} while there is convincing evidence of the expression of AT₂R in human cardiac fibroblasts.³⁰

The localization and expression levels of other components of RAS, REN, AGTN, and ACE, were also found to be uniquely altered in the fibrosing kidney. REN expression is localized in the JGA and the afferent arterioles in the normal kidney, and extends into the glomeruli and/or the proximal tubules in some diseased kidneys.^{4,6,7,11} In this murine model of fibrosing kidney, the localization of REN expression moved from the JGA to the interstitial FbLCs without a significant change in the overall expression level in the kidney. The total expression level of AGTN was significantly enhanced in the fibrosing kidney. Using the *in situ* hybridization method, we detected high levels of AGTN mRNA in the interstitial FbLCs and re-

Figure 2. The localization of RAS components in the fibrosing murine kidney. **A:** Dual immunostaining for α -SMA (green) and AT₁R protein (red). AT₁R protein is localized primarily in the tubular epithelial cells (**arrows**). Some brush borders in the proximal tubules (**arrowheads**) were also positive for AT₁R. However, α -SMA-positive FbLCs were negative for AT₁R (FITC and rhodamine; original magnification, $\times 200$). **B:** *In situ* hybridization for AT_{1A}R mRNA with the anti-sense probe. Not only the tubular epithelial cells (**arrows**), but also the solitary interstitial FbLCs (**arrowheads**) expressed AT_{1A}R mRNA. Nonspecific signals remained on some of the brush-borders (BCIP/NT; original magnification, $\times 150$). **C:** Dual immunostaining for α -SMA (green) and AT₂R protein (red). AT₂R protein remains focally localized in the tubular epithelium (**arrows**). The interstitial FbLCs, including α -SMA-positive FbLCs, were negative for AT₂R (FITC and rhodamine; original magnification, $\times 200$). **D:** *In situ* hybridization for AT₂R mRNA with the anti-sense probe. AT₂R mRNA is also localized focally in the tubular epithelium (**arrows**) (BCIP/NT; original magnification, $\times 150$). **E and F:** Immunostaining (**E**) and *in situ* hybridization (**F**) for REN. REN expression is detected in the interstitial FbLCs (**arrows**), and, occasionally, in the juxtaglomerular apparatus (**arrowhead**) (FITC; original magnification, $\times 200$ in **E** and BCIP/NT; original magnification, $\times 100$ in **F**). **G:** Immunostaining for ACE. ACE expression is detected in the interstitial FbLCs alone (**arrows**) of the fibrosing kidney (FITC; original magnification, $\times 200$). **H:** *In situ* hybridization for AGTN. In addition to the tubular epithelial cells (**arrows**), the interstitial FbLCs can express AGTN mRNA (**arrowheads**) (BCIP/NT; original magnification, $\times 100$). **I:** Negative control: *in situ* hybridization with the sense probe (BCIP/NT; original magnification, $\times 150$).

Figure 3. The characterization of the AT_{1A}R-positive interstitial FbLCs. **A:** *In situ* hybridization for AT_{1A}R mRNA with the anti-sense probe (BCIP/NT; original magnification, ×200). **B:** Dual immunostaining for FSP1 (green) and VIM (red) on the same section of **A**. The interstitial FbLCs expressing AT_{1A}R (arrows) in **A** are negative for FSP1, but positive for VIM in **B** (arrows) (FITC and rhodamine; original magnification, ×200). **C:** *In situ* hybridization for AT_{1A}R mRNA with the anti-sense probe (BCIP/NT; original magnification, ×150). **D:** Dual immunostaining for TGF-β1 (green) and VIM (red) on the same section of **C**. The interstitial FbLCs positive for AT_{1A}R mRNA (arrows) in **C** are also positive for TGF-β1 and VIM in **D**. These dual-positive cells yield a yellow immunofluorescence (arrows). TGF-β1 expression in the interstitial FbLCs is significantly greater than that in the tubular epithelial cells (FITC and rhodamine; original magnification, ×150).

duced levels in the tubular epithelial cells. The proximal tubular epithelium expresses AGTN in the normal kidney and in some diseased kidneys.^{7,31} However, in the fibrosing kidney, the interstitial FbLCs may produce AGTN rather than the proximal tubular epithelial cells because of significant degeneration of the latter. These dynamic

changes in REN and AGTN suggest that the enzyme-substrate reaction occurs in the fibrosing kidney.

ACE is up-regulated in the proximal tubular epithelium and in the vasculature in some diseased kidneys,^{7,9,11} whereas in the normal kidney ACE is positive in the endothelial cells of vessels including interlobular arteries,

afferent and efferent arterioles, and glomerular capillaries.⁴ This observation in the diseased kidneys is in contrast to our finding in the fibrosing kidney. We did not determine the ACE expression in the middle of the course of renal fibrosis in this model, which may change biphasically. In the kidney with advanced fibrosis, *de novo* synthesis of ACE by the interstitial FbLCs seems to replace the expression by the proximal tubular epithelium in parallel to its degeneration.

As described in this study, the interstitial FbLCs in the fibrosing kidney are highly heterogeneous. These phenotypically and functionally different FbLCs seem to be derived from a variety of precursor cells (eg, resident fibroblasts, infiltrating mononuclear cells originated from bone marrow stromal cells, transdifferentiated tubular epithelial cells, and vascular smooth muscle cells and pericytes surviving vessel attenuation) and play roles in concert to generate renal fibrosis.^{17,22,32} In the present study, although the intrarenal level of angiotensin II was not determined, we have shown that each component of the RAS was expressed by the interstitial FbLCs. These results suggest that, at least within the interstitium, angiotensin II may be generated and, in turn, activate some FbLCs in the fibrosing kidney. From these data, therefore, it would be agreed that the development of renal fibrosis is slowed by inhibiting the RAS using ACE inhibitors and AT₁R antagonists, even though the systemic RAS is suppressed as in hyporeninemia in diabetics.¹

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