Association between the Intrarenal Renin-Angiotensin System and Renal Injury in Chronic Kidney Disease of Dogs and Cats

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ABSTRACT. The association of renin and angiotensin II, which are potent components of the renin-angiotensin system, with the severity of chronic renal disease was investigated immunohistochemically in dogs and cats. Immunoreactivities of renin and angiotensin II were evaluated quantitatively, and their correlations with the degrees of glomerulosclerosis, glomerular hypertrophy, interstitial cell infiltration and interstitial fibrosis were statistically analyzed. Immunoreactivities for renin were detected in afferent arteries in both dogs and cats. The score of renin-positive signals showed no correlation with plasma creatinine concentration or any of the histopathological parameters, except for the diameter of glomeruli in dogs. Immunoreactivities for angiotensin II were detected in tubules (primarily proximal tubules) and interstitial mononuclear cells in both dogs and cats. The score of tubular angiotensin II correlated with glomerulosclerosis and cell infiltration in cats but not in dogs. The score of interstitial angiotensin II correlated with plasma creatinine concentration, glomerulosclerosis, cell infiltration and fibrosis in dogs and with glomerulosclerosis and cell infiltration in cats. In conclusion, the results of the study suggest that intrarenal renin-angiotensin system is correlated with the severity of kidney disease, with the underlying mechanism differing between dogs and cats.

KEY WORDS: angiotensin II, canine, feline, kidney disease, renin.

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Kidney disease, particularly chronic kidney disease (CKD), has increased together with longevity in dogs and cats. Previous studies have indicated that the pathological mechanisms of the incidence and progression of CKD differ between dogs and cats. Previous investigations on the histopathological differences between canine and feline kidney disease show that tissue damage appears to be prominent in the glomerulus in dogs and in the tubulointerstitium in cats [16, 22]. The results of our recent study demonstrate these species-specific histopathological features [29], and we suggest that the mechanisms for development of myofibroblasts, which play critical roles in renal fibrosis, differ between canine and feline CKD. Although many other species-specific mechanisms have been hypothesized to be involved in the pathological differences between canine and feline CKD, most of them remain unclear.

The renin-angiotensin system (RAS) is a major physiological mechanism that regulates hemodynamics including systemic blood pressure and renal glomerular capillary pressure. However, activation of the RAS is a major risk factor for the progression of CKD. Hypertension and increase of angiotensin II (Ang II) induced by an enhanced systemic RAS cause malignant hemodynamic changes directly or indirectly [26]. RAS-induced glomerular hypertension may

contribute to glomerular hypertrophy and glomerular sclerosis. Moreover, the tissue-specific local RAS has been found to have a pathological role in inducing fibrotic changes [6, 14]. Local RAS refers to *in situ* synthesis of all components for Ang II generation, and the kidney is an example of an organ capable of local RAS-dependent fibrosis [23].

The relationship between RAS enhancement and renal disease progression has been demonstrated in laboratory rodents [4, 18, 25], and the presence of this mechanism in human kidney disease is suggested in a recent report [10]. Excessive Ang II generation due to RAS activation induces renal damage via extremely complex molecular mechanisms. Ang II activates certain transcription factors such as nuclear factor κ B and activator protein 1 via type-1 (AT1) and/or -2 (AT2) receptors [15]. Activation of these transcription factors induces upregulation of growth factors (e.g., transforming growth factor β), cytokines (e.g., interleukin 6), chemokines (e.g., monocyte chemoattractant protein 1), and others (e.g., plasminogen activator inhibitor 1), and activation of these mediators leads ultimately to renal fibrosis [15]. However, this relationship has not been fully investigated in small animals, and whether induction of RAS components in the kidney is actually related to the severity of kidney damage remains unclear.

Therefore, in the present study, kidneys of dogs and cats with various stages of CKD were investigated histopathologically and immunohistochemically, and the relationships between renin induction, Ang II induction, and severity of renal lesions were statistically analyzed.

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MATERIALS AND METHODS

Samples: Kidney samples from 23 dogs and 13 cats were used in the study. The kidney samples from all cats and 20 dogs were collected during postmortem examination performed at Kagoshima University, Japan. The remaining 3 samples were obtained from clinically healthy beagles that were euthanized after being used in other surgical experiments. The experiments in this study were performed in accordance with the Guidelines for Animal Experimentation of Kagoshima University, Japan. The dogs ranged in age from approximately 6 months to 20 years and the cats from 6 months to 17 years. Plasma creatinine (pCre) concentration was measured in all cases as a routine clinical laboratory test, and the mean value of the last pCre was 230 $\mu\text{mol/l}$ (median, 62; interquartile range, 53–336) in dogs and 230 $\mu\text{mol/l}$ (median, 133; interquartile range, 71–354) in cats. According to the International Renal Interest Society system of categorizing CKD on the basis of pCre [1], 10 dogs and 5 cats were in stage 1 ($<125 \mu\text{mol/l}$ in dogs, $<140 \mu\text{mol/l}$ in cats), 1 dog and 2 cats were in stage 2 (125–179 $\mu\text{mol/l}$ in dogs, 140–249 $\mu\text{mol/l}$ in cats), 5 dogs and 2 cats were in stage 3 (180–439 $\mu\text{mol/l}$ in dogs, 250–439 $\mu\text{mol/l}$ in cats), and 4 dogs and 2 cats were in stage 4 ($\geq 440 \mu\text{mol/l}$ in dogs and cats). No renal injuries were detected in the remaining 3 dogs (healthy beagles) and 2 cats (a 6-month-old cat with feline infectious peritonitis and a 6-month-old cat with a traffic accident). No cases of acute renal failure were included in the present study.

Tissue preparation: The kidney samples were fixed in 10% neutral-buffered formalin for 24 hr at 4°C. The samples were routinely embedded in paraffin and cut into 3 μm sections, and the sections were stained with periodic acid Schiff (PAS) and Masson's trichrome stains. Immunohistochemical staining was done as follows: The samples were deparaffinized and rehydrated. Antigen retrieval was performed by heating the samples in 10 mM citrate buffer (pH 6.0) in a microwave oven under the following conditions: prewarming, 5 min; heating, 10 min; and cooling, 20 min. The samples were treated with 3% H_2O_2 in distilled water for 30 min, washed in 10 mM phosphate-buffered saline (PBS; pH 7.4), blocked with 0.25% casein in PBS for 60 min, and incubated overnight at 4°C with anti-Ang II rabbit polyclonal antibody (1:4,000 dilution; Peninsula Laboratories, San Carlos, CA, U.S.A.) or anti-renin rabbit polyclonal antibody (1:10,000 dilution; supplied by Dr. Murakami, University of Tsukuba, Japan). The samples were washed in PBS, incubated for 30 min with biotinylated goat anti-rabbit IgG (Vector Laboratories Inc., Burlingame, CA, U.S.A.) diluted at 1:200, washed again in PBS, and then incubated for 15 min with peroxidase-conjugated streptavidin (DakoCytomation, Glostrup, Denmark). Finally, the samples were washed in PBS and tested for immunoreactivity with a 3,3'-diaminobenzidine (DAB) system (DAB Buffer tablet; Merck, Darmstadt, Germany); the reaction was stopped by rinsing in distilled water. The sections were counterstained with Mayer's hematoxylin or PAS-hematoxylin. For the negative control sections, non-immunized rabbit immunoglobulin (IgG) (R&D Systems,

Minneapolis, MN, U.S.A.) was used instead of primary antibody. Antigen retrieval treatment was performed for Ang II detection but was not required for renin detection.

Quantitative analysis: Randomized morphometric analysis was performed according to previously described methods. (i) Diameter of renal glomeruli: Glomeruli at the vascular pole or urinary pole were selected, and the widest diameter of the glomerular capsule was measured [27]. Atrophic glomeruli were excluded from the quantitation. (ii) The extent of glomerulosclerosis was determined using a semiquantitative scoring system described previously [20]. Briefly, approximately 50 glomeruli were examined per animal, and the severity of the sclerotic lesions was graded from 0 to +4 (0, normal; +1, up to 25% sclerosis; +2, 26–50% sclerosis; +3, 51–75% sclerosis; +4, 76–100% sclerosis). The glomerulosclerosis score for each animal was then evaluated in the following manner: If from a total of 100 glomeruli, 10 were grade +1, 20 were grade +2, 15 were grade +3 and 5 were grade +4, the final score was calculated as follows: $[(1 \times 10/100) + (2 \times 20/100) + (3 \times 15/100) + (4 \times 5/100)] \times 100 = 115$. (iii) The degrees of interstitial fibrosis and cell infiltration were also evaluated using a similar semiquantitative scoring system. Briefly, approximately 20 non-overlapping cortical fields (magnification, 200 $\times$) were examined per animal, and the severity of each parameter was graded from 0 to +4 (0, normal; +1, mild; +2, moderate; +3, severe; +4, very severe). The score of each parameter was then determined for each animal. For example, in the case of interstitial fibrosis, if out of the 20 fields examined, 5 exhibited grade +1 changes, 3 grade +2, 2 grade +3, and 1 grade +4, the final score of interstitial fibrosis was calculated as follows: $[(1 \times 5/20) + (2 \times 3/20) + (3 \times 2/20) + (4 \times 1/20)] \times 100 = 105$. (iv) Degree of Ang II immunoreactivity: Positive signals in tubules and those in interstitial mononuclear cells were evaluated separately using a similar semiquantitative scoring system. Evaluation of tubular Ang II was performed in the cortex and not limited to specific tubular segments. Distribution of the positive signals in tubules was graded from 0 to +4 (0, no signals; +1, up to 25% tubules exhibiting positive signals; +2, 26–50% tubules positive; +3, 51–75% tubules positive; and +4, 76–100% tubules positive). (v) Quantitation of renin-positive arterioles was performed according to a previously described procedure [17, 28]. Briefly, the renin index was defined as the ratio of the total number of renin-positive arterioles to the total number of glomeruli and expressed per 100 glomeruli.

Statistical analysis: The Pearson's correlation coefficients and their *P*-values between all the parameters were evaluated using the PASW software program for Windows (IBM SPSS Statistics, Armonk, NY, U.S.A.).

RESULTS

Distribution of renin: In dogs, immunoreactivities for renin were observed in 22 of 23 animals. Positive signals were limited to the juxtaglomerular afferent arterioles in all cases (Fig. 1A and 1B). In cats, immunoreactivities for renin were observed in 12 of 13 animals, and positive

Fig. 1. Immunohistochemical detection of renin in the kidney. A. Dog kidney with stage 1 chronic kidney disease (CKD). B. Normal dog kidney. C. Cat kidney with stage 2 CKD. D. Normal cat kidney. Positive signals are observed in the vascular walls of the afferent arterioles. In dogs, signals are limited to the narrow regions adjacent to the glomeruli (arrows). In cats, signals are more widely distributed in the afferent arterioles. Counterstain: Mayer's hematoxylin. Bars: 50 μ m.

signals were observed in the vascular walls of the afferent arterioles. These reactions in cats were broadly distributed compared with those in dogs (Fig. 1C and 1D). Although signal intensity differed among animals, localization of renin immunoreactivity was similar in normal and CKD cases in both dogs and cats. No positive signals were observed when non-immunized rabbit IgG was used instead of the primary antibodies in both dogs and cats (data not shown).

Distribution of angiotensin II: In dogs, immunoreactivities for Ang II were observed in all 23 samples. Ang II-positive signals were observed in proximal tubular cytoplasm, exhibiting a granular pattern (Fig. 2A and 2B). Varying degrees of positive signals were detected in the proximal tubules, except in the beginning of the convoluted part. In cats, immunoreactivities for Ang II were observed in 10 samples, and mild and focal signals were observed in proximal straight tubular and distal tubular or collecting duct cells (Fig. 2C). In normal cases, signals for tubular Ang II tended to be mild or absent compared with CKD cases in both dogs and cats (Fig. 2B and 2D), with the exception of 1 dog that showed strong signals. Some interstitial mononuclear cells were also positive for Ang II in 17 dogs and 9 cats with CKD; this reactivity was more prominent in dogs (Fig. 2E and 2G). These Ang II-positive interstitial cells differed from lymphocytes, plasma cells, granulocytes, fibroblasts, and mast cells, and were considered macrophages (Fig. 2E–H). In normal cases, no positive signals were observed in the interstitial cells. No staining was observed when non-immunized rabbit IgG was used instead of the primary antibodies in both dogs and cats (data not shown).

Quantitative findings: The statistical results are summarized in Table 1.

Renin: In dogs, only the diameter of glomeruli correlated with the score of renin-positive signals, whereas the remaining histopathological parameters and pCre did not show significant correlation. In cats, all histopathological parameters and pCre showed no significant correlation with the score of renin-positive signals.

Angiotensin II: Positive signals in tubular cells and those in interstitial mononuclear cells were evaluated separately. In dogs, all histopathological parameters and pCre showed no significant correlation with the score of tubular Ang II. The score of interstitial Ang II, however, was significantly correlated with pCre, glomerulosclerosis, cell infiltration and fibrosis. In cats, the scores of tubular Ang II and interstitial Ang II significantly correlated with those of glomerulosclerosis and cell infiltration.

DISCUSSION

Renin is a key enzyme of the RAS and is produced by specialized juxtaglomerular cells in the normal mammalian kidney [11]. Such juxtaglomerular renin in healthy animals is a component of the systemic RAS. Recent studies of human, rat, and mouse kidneys have demonstrated renin mRNA and protein to be expressed in proximal and in collecting duct cells, and such tubular/ductal renin is considered a local RAS [12, 19, 21]. Upregulation of the local renal RAS has been demonstrated in various kidney diseases, such as diabetic and hypertensive nephropathies [3, 7] and autosomal reces-

Fig. 2. Immunohistochemical detection of angiotensin II in the kidney. A. Dog kidney with stage 1 CKD, cortex. B. Normal dog kidney, cortex. C. Cat kidney with stage 1 CKD, outer medulla. D. Normal cat kidney, outer medulla. E. Dog kidney with stage 4 CKD, cortex. F. Serial section of panel E (HE stain). G. Cat kidney with stage 2 CKD, cortex. H. Serial section of panel G (HE stain). Granular positive signals are detected in proximal tubular cells (A and C), but these signals are weak or absent in normal kidneys (B and D). Signals are more intense and widely distributed in dogs than in cats. Small numbers of mononuclear cells show positive signals (E and G, arrows), and these positive cells resemble macrophages (F and H, arrows). Counterstain: PAS-hematoxylin (A-D) or Mayer's hematoxylin (E and G). Bars: 50 μ m.

sive polycystic kidney disease [13], and enhancement of local RAS activity is regarded as an important pathological event in the progression of CKD in current medicine [24]. In the present study, renin-positive signals were visualized by immunohistochemistry. Although varying degrees of renin-

positive signals were detected in afferent arterioles in dogs and cats, no signals were detected in tubules or collecting ducts in any of the animals. Therefore, we postulated that the changes observed in renin-positive signals by immunohistochemical analysis reflected systemic RAS activity.

Table 1. Correlations between immunohistochemical parameters and pathological parameters

	Dogs			Cats		
	Renin	t-Ang II	i-Ang II	Renin	t-Ang II	i-Ang II
pCre	NS	NS	0.64**	NS	NS	NS
Glomerulosclerosis	NS	NS	0.86**	NS	0.83**	0.67**
Cell infiltration	NS	NS	0.67**	NS	0.67*	0.65*
Interstitial fibrosis	NS	NS	0.74**	NS	NS	NS
Diameter of glomeruli	0.42*	NS	NS	NS	NS	NS

pCre: plasma creatinine concentration. t-Ang II: tubular angiotensin II, i-Ang II: interstitial angiotensin II. Values represent the correlation coefficient. * $P < 0.05$. ** $P < 0.01$. NS: not significant.

In the present study, Ang II-positive signals were successfully identified in canine and feline kidneys by immunohistochemistry, with positive signals clearly detected in tubular cells. These positive cells might reflect the eventual uptake of tubular fluid Ang II, because high concentrations of Ang II in the tubular fluid and endocytosis of Ang II in the proximal tubules have been suggested in rat kidneys [9], and recent studies have demonstrated that such internalization of Ang II is mediated by AT1 and megalin receptors [9]. In contrast, quantitative results indicated local production of Ang II, because no significant correlation was found between the score of tubular Ang II and that of juxtaglomerular renin. This result suggests that the changes in tubular Ang II reflect local, and not systemic, RAS activity in canine and feline CKD.

Ang II-positive signals were also detected in interstitial mononuclear cells, which were considered macrophages from their morphological features. The role of macrophages in renal injury has been extensively studied using a rodent model of unilateral ureteral obstruction (UUO); it was demonstrated that activated interstitial macrophages play a crucial role in UUO-associated tubulointerstitial fibrosis and produce mediators such as transforming growth factor beta and matrix metalloproteinases [1]. Although the pathological role of renal interstitial macrophages has not yet been clarified in canine and feline spontaneous renal fibrosis, Ang II-immunoreactive macrophages, which were observed in the present study, might be associated with local RAS activation in canine and feline CKD. In contrast, endocytosis of Ang II may also be a role of interstitial macrophages, because high concentrations of Ang II in the interstitial fluid have been demonstrated in rat kidneys [9]. Whether Ang II-positive signals in canine and feline kidneys reflect local production of Ang II or the eventual endocytosis of Ang II is difficult to determine by the present immunohistochemical examination; this warrants future immunoelectron microscopic study.

Glomerular hypertrophy in canine CKD is hypothesized to be associated with the enhancement of systemic RAS activity, and a previous study demonstrated a close relationship between enhancement of the systemic RAS and glomerular hypertrophy in laboratory rodents and human patients with CKD [30]. In the quantitative analysis, the diameter of glomeruli in dogs was significantly correlated with the score of renin-positive signals. Increase of glomerular size in the present study reflected compensatory glomerular hypertrophy, because most of the diseased kidneys included atrophic

glomeruli, which were excluded from the quantitation. Therefore, compensatory glomerular hypertrophy in canine CKD might be associated with the enhancement of systemic RAS activity. Increased single nephron glomerular filtration rate and glomerular hypertension are considered possible mechanisms of glomerular hypertrophy; this should be clarified in a future study.

In dogs, although the score of tubular Ang II showed no correlation with all histopathological parameters, that of interstitial Ang II was correlated with glomerulosclerosis, cell infiltration, and fibrosis. In addition, the score of interstitial Ang II showed correlation with pCre. In veterinary medicine, pCre is the most reliable biochemical marker in blood for evaluating renal function, and canine and feline CKD is currently evaluated on the basis of pCre [2]. Therefore, the induction of interstitial Ang II is postulated to be closely associated with the severity of canine CKD. The correlation between the score of interstitial Ang II and that of glomerular sclerosis was a very interesting finding. Previous reports show that in glomerulosclerosis, Ang II is associated with glomerular mesangial cell proliferation and extracellular matrix production [5], as well as with glomerular hemodynamic changes [9]. In the present study, it was suggested that interstitial Ang II induced not the glomerular hemodynamic changes but the glomerular mesangial cell proliferation and extracellular matrix production, because the score of interstitial Ang II showed no correlation with the diameter of glomeruli.

In cats, the scores of tubular and interstitial Ang II significantly correlated with those of glomerulosclerosis but not with pCre. pCre is the most reliable marker for feline CKD, as described earlier [2], and its significance as a prognostic marker of feline CKD has been recently demonstrated [8]. Therefore, the degree of renal Ang II induction is not correlated with the severity of feline CKD, although Ang II induction may contribute to the progression of glomerulosclerosis. This hypothesis is supported by the finding of our present study and the evidence demonstrated in our recent study [29]. Briefly, our recent report demonstrates that the severity of CKD in cats is significantly correlated with interstitial fibrosis but not with glomerulosclerosis, and our present study shows no significant correlation between the score of tubular/interstitial Ang II and that of interstitial fibrosis. Therefore, renal Ang II induction may be a pathological event that is indirectly associated with the severity of CKD.

in cats.

In conclusion, the present comparative study demonstrates that the RAS-mediated pathological mechanisms underlying the severity of CKD differ between dogs and cats. In dogs, the severity of CKD was correlated with renin and Ang II induction in the kidney. In cats, on the other hand, the severity of CKD was not directly associated with renin and Ang II induction in the kidney, although renal Ang II induction might contribute to the progression of glomerulosclerosis. These species-specific differences are of great interest, and further studies are needed to clarify the canine- and feline-specific pathological mechanisms of kidney disease.

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