Change in intrarenal Ghrelin expression in immune complex-mediated glomerular disease in dogs

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ABSTRACT. Ghrelin is a peptide hormone that is mainly produced by the stomach. The kidney is a major source of local ghrelin, and maintaining body fluid balance is considered a critical role of renal ghrelin. However, there are no reports on renal ghrelin in small animal medicine. The present study investigated the intrarenal localization of and change in ghrelin expression in dogs with immune complex-mediated glomerulonephritis (ICGN). Ghrelin immunoreactivity (IR) was observed in the distal tubules of normal kidneys. Ghrelin IR was weak in ICGN kidneys, and the quantitative ghrelin IR score was significantly lower in ICGN kidneys than in normal kidneys. In cases of ICGN, plasma creatinine concentrations showed a positive correlation with the ghrelin IR score.

KEY WORDS: canine, ghrelin, glomerular disease, immune complex-mediated nephritis, immunohistochemistry

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Ghrelin, a peptide hormone, was first discovered in rat and human stomachs [8]. It plays several important roles including growth hormone release and appetite stimulation [9, 10, 12]. Various organs other than the stomach also synthesize local ghrelin [4, 5]; the kidney is a major source of local ghrelin. The intrarenal localization of ghrelin was first investigated in our study of rodent kidneys [18]. We found that it was primarily distributed in the distal tubules. A similar distribution has since been observed in the human kidney [2]. In diseased human kidneys, ghrelin immunoreactivity (IR) in renal tubules is decreased during proliferative glomerulopathy [3]. However, the intrarenal expression of ghrelin in normal and diseased canine kidneys has not been reported.

Glomerular disease is a common form of kidney disease in dogs and is divided into three major categories based on the pathological evaluation of renal biopsies: Immune complex-mediated glomerulonephritis (ICGN), non-ICGN and amyloidosis [1]. ICGN garners the greatest clinical interest as it has the highest prevalence among these categories [16]. The purpose of the present study was to clarify the intrarenal localization of ghrelin in normal canine kidneys and to evaluate how it changes in ICGN.

Normal kidney samples were obtained from clinically healthy male Beagles (n=6, 2–3 years old), who were euthanized after use in other surgical experiments. The stomach was chosen as a positive control tissue for ghrelin IR. Experiments were performed in accordance with the Guidelines for Animal Experimentation of Kagoshima University, Japan. Samples were fixed in 10% neutral buffered formalin and embedded in paraffin according to the routine procedure. ICGN samples (n=12) were obtained from renal biopsies of clinical cases in dogs. All cases were clinicopathologically diagnosed as protein-losing nephropathies. The owners provided informed consent for renal biopsies to definitively diagnose the kidney disease and for research use of the samples after diagnosis. Histopathological diagnosis of renal biopsies was based on light microscopy, transmission electron microscopy and immunohistochemical (immunoglobulin [Ig] G, IgA, IgM and complement C3) analyses.

Immunohistochemistry to detect ghrelin was performed using 3-μm paraffin sections. The procedure was as follows: (1) deparaffinization and rehydration; (2) antigen retrieval treatment by microwave heating in 10 mM citrate buffer (pH 6.0) with pre-warming for 5 min, heating for 10 min and cooling for 20 min; (3) incubation with 3% H2O2 for 30 min; (4) washing in 10 mM phosphate buffered saline (PBS, pH 7.4); (5) blocking with 0.25% casein (Sigma-Aldrich Corp., St. Louis, MO, U.S.A.) in PBS for 30 min; (6) incubation overnight at 4°C with goat anti-ghrelin antiserum (C-18) (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) diluted 1:1,000 in blocking solution; (7) washing in PBS; (8) incubation for 30 min with biotinylated horse anti-goat IgG (H+L) (Vector Laboratories, Burlingame, CA, U.S.A.) diluted 1:200 in blocking solution; (9) washing in PBS; (10) incubation with peroxidase-conjugated streptavidin (KPL, Gaithersburg, MD, U.S.A.); (11) washing in PBS; (12) immunosignal detection using a 3,3′-diaminobenzidine (DAB) system (DAB-buffer tablet; Merck KGaA, Dormstadt, Germany); and (13) termination of the reaction with distilled water. Sections were counterstained with Mayer’s
hematoxylin. For the negative control sections, normal goat IgG (Santa Cruz Biotechnology) was used instead of the primary antibody. Specificity was tested using affinity pre-absorption of primary antibody with 10 µg/ml synthesized ghrelin peptide (Santa Cruz Biotechnology).

Ghrelin IR in tubules was evaluated using a point-counting method described in a previous report [11]. Digital images were captured at 200× magnification and prepared (approximately seven images/section). Then, 300 circles per image (approximately 2,100 circles/section) were created using Photoshop software (Adobe Systems, San Jose, CA, U.S.A.). The circles containing glomeruli and large vessels were considered exclusion points, and those containing ghrelin IR were considered positive points. The percentage of ghrelin positive points per evaluation points (total points − exclusion points) was calculated. To avoid interference from interstitial fibrosis, which decreases the tubular area, the percentage of ghrelin IR points was divided by the percentage of interstitial fibrosis, which was determined using the same point-counting method. The calculated value was used as the score for intrarenal ghrelin IR. The glomerulosclerosis score was also evaluated in a semi-quantitative manner as previously reported [14]. The glomerular diameter served as an index of glomerular hypertrophy and was measured using an ocular micrometer.

The difference between normal and ICGN kidneys in terms of the ghrelin IR score was evaluated using the Mann-Whitney U test. Relationships between the ghrelin IR score and extent of renal tissue damage (glomerulosclerosis, glomerular diameter and interstitial fibrosis) in ICGN samples were evaluated with the Spearman rank correlation coefficient. Relationships between the ghrelin IR score and clinicopathological data, such as the plasma concentrations of urea, creatinine (pCre), phosphorus, total protein and albumin, urinary protein/creatinine ratio (UP/C), urine specific gravity (USG) and systolic blood pressure (SBP) measured using the Doppler method, were also evaluated with the Spearman rank correlation coefficient. All analyses were performed using the PASW software program for Windows (IBM SPSS Statistics, Armonk, NY, U.S.A.).

In the normal stomach, which works as a positive control, numerous cells with ghrelin IR were observed in the gastric glands (Fig. 1A). These cells were considered ghrelin-producing gastric endocrine cells, because the localization of immunopositive cells in the canine stomach was consistent...
RENAL GHRELIN IN GLOMERULONEPHRITIS

Table 1. Correlations between the ghrelin immunoreactivity score and data from clinicopathological and histomorphometrical analyses

| Blood chemicals     | Creatinine | Urea nitrogen | Total protein | Albumin | Phosphorus |
|---------------------|------------|---------------|---------------|---------|------------|
|                     |            | (n=12)        | (n=12)        | (n=11)  | (n=10)     |
|                     | 0.594*     | 0.106         | 0.292         | 0.055   | 0.503      |
| Blood pressure and Urinalysis |           |               |               |         |            |
| SBP (mmHg)          |            |               |               |         |            |
|                     | 0.524      | 0.166         | 0.175         |         |            |
|                     | (n=8)      | (n=12)        | (n=11)        |         |            |
| Tissue damage       |            |               |               |         |            |
| Glomerulosclerosis  | 0.063      | 0.472         | 0.289         |         |            |
| (n=12)              |            | (n=12)        | (n=12)        |         |            |
| Glomerular diameter | 0.472      |               |               |         |            |
| (n=12)              |            |               |               |         |            |
| Interstitial fibrosis | 0.289     |               |               |         |            |
| (n=12)              |            |               |               |         |            |

Values represent the Spearman rank correlation coefficient. * Statistical significances were defined as P<0.05. SBP, systolic blood pressure; UP/C, urinary protein/creatinine ratio; USG, urine specific gravity.

In conclusion, the present study demonstrated that intrarenal ghrelin IR is distributed in the distal tubules in dogs. Although the ghrelin IR score was lower in the ICGN kidneys than in normal kidneys, this score was positively correlated with the pCre level in ICGN cases. These findings might be correlated with a renoprotective effect of ghrelin, and further investigation into this potential function in kidney disease is necessary.

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