The study on podocyte injury and altered expression of podocyte-associated molecules in canine glomerular diseases

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DV0803 小林 亮介

The study on podocyte injury and altered expression of podocyte-associated molecules in canine glomerular diseases

イヌの腎糸球体疾患における足細胞傷害と足細胞関連分子の発現変化に関する研究

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General Introduction

Glomerular diseases are thought to be the common cause of chronic renal failure in dogs (Macdougall *et al.*, 1986). Non-selective proteinuria, which is major clinical manifestation in glomerular disease induce tubulointerstitial injury, and raise loss of functional nephron (Nangaku, 2004). Thus, early detection and treatment of podocyte and glomerular injury is meaningful for preventing progressive renal disorder in dogs.

Podocyte is the specially-differentiated epithelial cells covers with glomerular basement membrane. The cells have ultrastructure called foot process (FP) and slit diaphragm (SD) in the peripheral. In humans and experimental models, it has been known that podocyte injury profoundly associated with protein leakage from glomerular capillary walls (Pavenstadt *et al.*, 2003). Moreover, molecules express on FPs and SDs called podocyte-associated molecules were mutated or decreased expression in podocyte and glomerular injury (Huh *et al.*, 2002; Kestila *et al.*, 1998). These molecules such as nephrin, podocin and α-actinin-4 have important role to maintain structure and function in podocyte with molecular interaction, and its aberrant involved in podocyte pathogenesis (Boute *et al.*, 2000; Cybulsky *et al.*, 2009). Firstly, Kestila *et al.* positionally cloned nephrin as responsible gene in human congenital nephrotic syndrome in 1998. Afterward, knowledge of podocyte and its specific molecules have been rapidly brought out in the last decade. These molecules are now used as functional markers, and alteration of molecules have also been revealed in acquired glomerular diseases such as minimal change disease, focal segmental glomerulosclerosis, membranous glomerulopathy (Schmid *et al.*, 2003), IgA nephropathy (Mao *et al.*, 2006) and diabetic nephropathy (Langham *et al.*, 2002).

In canine glomerular diseases, the podocyte pathogenesis remains unclear and the expression and change of the molecules have not been elucidated. We thought the analyses of the molecular expression are essential for functional evaluation in podocyte, as well as morphological study by electron microscopy. The aim of the study is to reveal podocyte injury and altered expression and localization of podocyte-associated molecules in spontaneous glomerular diseases in dogs.

In chapter 1, we define protein expression and localization, and mRNA expression of nephrin, podocin, α-actinin-4 and α3-integrin, which have critical role for normal structure and function of SD and FP. We also show length of SD in normal canine glomeruli that have never been examined. In this chapter, we develop the polyclonal canine nephrin antibody, primers of four molecules and glomerular isolation methods in dogs. On the basis of the results in the chapter 1, we analyze the altered expression of

the four molecules with ultrastructural changes in diseased podocyte using canine renal biopsies in chapter 2. In the chapter, we reveal podocyte injury accompanied by ultrastructural changes, altered localization and expression of nephrin, podocin, α-actinin-4, α3-integrin and WT1, reduction of the cell number and increased nephrin mRNA expression. We also show the correlation between urinary/protein ratio and molecular expression score, and WT1 index and molecular expression score.

Chapter 1

Expression of nephrin, podocin, α-actinin-4 and α3-integrin in normal canine glomeruli

Abstract

The biologic features of podocytes that contribute to the pathogenesis of proteinuria have not been investigated in dogs. The aim of this chapter was to investigate the expression and localization of nephrin, podocin, α-actinin-4 and α3-integrin in canine renal glomeruli. Renal cortical tissue was collected from the kidneys of five normal adult beagles. Western blotting and immunofluorescence microscopy revealed specific expression and localization of the four proteins in canine glomeruli. On immunofluorescence, the staining of the molecules was linear pattern, and nephrin and podocin was limited to the glomeruli, while α-actinin-4 was positive in the mesangial area and wall of arterioles, and α3-integrin in the mesangial area and a few tubules. Western blot analysis showed bands of 180 kD (nephrin), 42 kD (podocin), 105 kD (α-actinin-4) and 130 kD (α3-integrin). Expression of genes encoding the four molecules in isolated glomeruli was detected by reverse transcriptase polymerase chain reaction. The results of the chapter will permit future exploration of podocyte injury and its involvement in protein leakage from the capillary wall in canine glomerular diseases.

Introduction

The podocyte is a terminally-differentiated cell that gives rise to cell processes and foot processes (FPs), which cover the outer surface of the glomerular basement membrane (GBM). FP interdigitate with those of adjacent podocytes, and 30–40-nm-wide slits between FPs are bridged by specific intercellular junctions called slit diaphragm (SD). Podocyte-associated molecules are expressed in the FPs and SDs, and play a crucial role in the structure and function of the podocyte.

In human and experimental animal models, it has been revealed that podocyte injury causes development of proteinuria and glomerulosclerosis (Pavenstadt *et al.*, 2003). Podocyte injury is often accompanied by FPs and SDs effacement. In association with these morphological features, a number of molecules including SD components, cytoskeleton-associated proteins or adhesion molecules, are decreased in podocytes. For instance, nephrin, which is a major component of the SD, was identified as a mutated glomerular protein in human congenital nephrotic syndrome (Kestila *et al.*, 1998), and reduced nephrin expression has been related directly to proteinuria in acquired nephrotic glomerular diseases (Furness *et al.*, 1999; Huh *et al.*, 2002). The *NPHS2* gene, which encodes podocin, was identified as the gene responsible for autosomal recessive steroid-resistant nephrotic syndrome (Boute *et al.*, 2000). In the proteinuric state, down-regulation and shift localization of podocin has been observed (Nakatsue *et al.*, 2005). Altered expression of α-actinin-4, which is an actin cross-linking protein expressed in FPs, has been observed in injured podocytes (Cybulsky *et al.*, 2009; Wagrowska-Danilewicz *et al.*, 2006). Decrease in expression of α-actinin-4 is thought to occur with actin aggregation in FPs. α3β1-integrin heterodimers contribute to podocyte-GBM adhesion, and it has been shown that α3-integrin has an essential role in the formation of podocyte FPs in mutant mice (Kreidberg *et al.*, 1996).

Morphological abnormality of podocytes has been observed in domestic animals with proteinuria; however, the association between podocyte-associated molecules and functional or morphological abnormality of podocytes has not been demonstrated. In dogs, glomerular diseases are thought to be the most common cause of chronic renal failure (Macdougall *et al.*, 1986). Glomerular and podocyte damage cause proteinuria and result in tubular and interstitial injury. Therefore, it is important to detect podocyte injury and treat glomerular diseases at an early stage, in order to prevent progressive loss of renal function in dogs. However, the expression or dynamics of podocyte-associated molecules in the canine renal glomeruli have not yet been investigated.

The aim of this chapter was to define the expression and localization of nephrin,

podocin, α-actinin-4 and α3-integrin in the canine renal glomeruli.

Materials and Methods

Animals

Kidney tissue was obtained from four male and one female adult beagle dogs (numbered 1–5), aged between 1 and 5 years. All dogs were confirmed to be healthy by physical examination and no renal dysfunction was revealed by serum biochemistry and urinalysis. These dogs were humanely destroyed in accordance with the guidelines approved by the Animal Research Committee of Azabu University.

Light Microscopy

A portion of the kidney tissue from the five beagles was fixed in 10% neutral buffered formalin, processed routinely and embedded in paraffin wax. Sections (3 μm) were stained with haematoxylin and eosin (HE) and periodic acid-Schiff (PAS). The presence of glomerular or tubulointerstitial lesions was examined by light microscopy.

Transmission Electron Microscopy

To observe podocyte FPs and SDs, renal cortex from three dogs (numbers 1–3) was cut into 1-mm^3 cubes, fixed in 2.5% glutaraldehyde and post-fixed in 1% OsO_4 for 2 h. The fixed specimens were then dehydrated through ascending grades of alcohol and embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate and a JEOL 1210 transmission electron microscope (JOEL, Tokyo, Japan) at 80 kV was used for examination. In each sample, ten examples of SDs in transverse section were examined in order to calculate the length of the SDs.

Antibodies

The polyclonal antibody specific for canine nephrin was prepared by immunizing rabbits with a synthetic peptide. A peptide located in the extracellular region of the deduced amino acid sequence (NCBI database, accession number: XP_541685) was chosen as the immunogen. The sequence of the peptide is RIPRFPRYRLEGDPSRG. Two rabbits were immunized with the peptide conjugated to the carrier protein keyhole limpet haemocyanin (KLH), and the rabbits were boosted four times with antigen. Two weeks after the final immunization the rabbits were anaesthetized and blood was taken by cardiac puncture before the animals were humanely destroyed. For antibody purification, free peptide antigen was coupled to a HiTrap NHS-activated HP column (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's instructions.

Rabbit anti-canine nephrin peptide immunoglobulins were eluted using 100 mM glycine-HCL (pH 3.0). The antibody was used for immunofluorescence microscopy (IF; at a dilution of 1 in 50) and western blotting (1in 500 dilution).

For detection of the other three podocyte-associated molecules the following reagents were used: rabbit anti-human podocin (Sigma, St Louis, Missouri; 1 in 200 dilution for IF), mouse anti-α-actinin-4 (clone 4D10, Abnova, Taipei, Taiwan; 1 in 100 dilution for IF), rabbit anti-human α3-integrin (Chemicon, Temecula, California; 1 in 500 dilution for IF) and mouse anti-vimentin (clone V9, Dako, Glostrup, Denmark; 1 in 50 dilution for IF).

Immunofluorescence Microscopy

Cryostat sections (3 μm) of fresh dog kidney were fixed in acetone for 1 min at -20 °C and washed with cold phosphate buffered saline (PBS, pH 7.2, 0.01M). For nephrin and α-actinin-4 were pretreated by incubation in citrate buffer (pH 6.0) in a microwave for 10 min at 90 °C. After incubation with 4% BlockAce™ (Yukijirushi, Sapporo, Japan) for 10 min at room temperature, sections were incubated with the primary antibodies at 4 °C overnight. Following washing with cold PBS, the secondary antibodies were applied and incubated for 20 min at room temperature, and then washed off with cold PBS. The secondary antibodies were: anti-rabbit immunoglobulin (Ig) G conjugated to fluorescein isothiocyanate (FITC; Cappel, Aurora, Ohio; 1 in 500 dilution), FITC-conjugated anti-mouse IgG (EY laboratories Inc., San Mateo, California; 1 in 100 dilution) or rhodamine-conjugated anti-mouse IgG (Chemicon; 1 in 400 dilution). To characterize the localization of canine nephrin, double immunofluorescence with antibodies specific for nephrin and vimentin was carried out. For examination, a FSX100 fluorescence microscope (OLYMPUS, Tokyo, Japan) with BP filters (BP460-495 for FITC, BP530-550 for rhodamine, OLYMPUS) was used.

Isolation of the Glomeruli

For western blotting and reverse transcriptase polymerase chain reaction (RT-PCR), the glomeruli were isolated by the sieving method as reported (Sugimoto *et al.*, 1994). Briefly, renal cortical tissues was minced in cold PBS and then sieved through 600, 250 and 125 μm pore-size stainless-steel meshes with cold PBS. The material retained on the 125-μm pore-size mesh was of high glomerular purity (Fig. 1) and was collected into cold PBS.

Western Blotting

The renal cortex and isolated glomeruli were homogenized in a Dounce homogenizer in RIPA buffer (consisting of 0.1% sodium dodecyl sulphate [SDS], 1% TritonX-100, 150 mM NaCl, 1% sodium deoxycholate and 10 mM ethylenediaminetetraacetic acid [EDTA] in 25 mM Tris-HCl, pH 7.2) and the protein concentrations of these samples was assayed by the Lowry method (DC Protein Assay, Biorad, Hercules, California). For SDS-polyacrylamide gel electrophoresis (SDS-PAGE), samples were mixed with an equal volume of sample buffer and boiled for 5 min. Proteins (10 μg/lane) were run on 10% polyacrylamide slab gels and transferred to polyvinylidene fluoride membranes. The membranes were blocked with 5% skimmed milk in PBS with 0.1% Tween 20 for 1 h at room temperature and incubated overnight with the primary antibodies (antibody concentrations were one-tenth of those used for IFM) at 4 °C. After washing with 5% skimmed milk in PBS with 0.1% Tween 20, the membranes were incubated with a horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody (DakoCytomation, Glostrup, Denmark; 1 in 1000 dilution) or HRP-conjugated rabbit anti-mouse IgG antibody (DakoCytomation; 1 in 1000 dilution). Immunoreactivity was visualized using an enhanced chemiluminescence system (GE Healthcare).

Reverse Transcriptase Polymerase Chain Reaction

Total RNA was extracted from the renal cortex and isolated glomeruli using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, California). Extracted total RNA was treated with RNase-free DNase (Promega, Madison, Wisconsin) and used in a reverse transcription reaction employing the Superscript III first-strand synthesis system for RT-PCR (Invitrogen Life Technologies). Primer sequences were derived by determining the predicted mRNA sequences of *Canis familiaris* nephrin precursor (NCBI database, accession number: XM_541685), podocin (XM_547443.2) and ACTN4 (encoding α-actinin-4) transcript variant 1 (XM_541640.2). The mRNA sequence of human α3-integrin transcript variant a (NM_002204.2) was accessed as the predicted sequence for the canine molecule was not available on the database.

The primer sequences were as follows: canine nephrin forward, TGGTCCGACTTGTTGTCAGATT; reverse, ACAGTGGAGAGTGGCAGAACTTG; canine podocin forward, GGAGAGAACAGAAATTAAGGATGTAAGG; reverse, TGCGCTTCGGCTTCCA; canine ACTN4 forward, GGACATTCCCAAGATGTTGGAT; reverse, GGTAGAAGCTGGACACATAGGTCAT. α3-integrin forward, TGGTGGGCAAGTGCTACGT; reverse, TGCACATCTCGTTGTGGTAGGT. Amplification of canine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeper and the primers for this reaction

were: forward, GGCACAGTCAAGGCTGAGAAC; and reverse, CCAGCATCACCCCATTTGAT. The PCR reaction with Takara Ex Taq (Takara Shuzo, Ohtsu, Japan) was performed as follows: denaturation at 94 °C for 5 min, 40 cycles of denaturation at 94 °C for 30 sec, annealing at 52 °C (nephrin and ACTN4), 54 °C (podocin), 55 °C (α3-integrin) and 56 °C (gapdh) for 30 sec, extension at 72 °C for 1 min and final extension at 72 °C for 5 min. The amplification products were separated on 2% agarose gels and stained with ethidium bromide. Sequencing analysis was performed on the amplification product of each molecule.

Results

Light Microscopy

Light microscopy revealed that significant renal lesions were not observed in all dogs (Fig. 2).

Ultrastructure

Transmission electron microscopy revealed that FPs were arranged perpendicular to the GBM, and SD connected adjacent FPs in transverse section (Fig. 3). The length of the SD was 379 ± 24.4 Å (mean ± SD). The results evidenced that podocyte had normal FPs and SDs in glomeruli of the dogs.

Localization and Expression of Nephrin, Podocin, α-actinin-4 and α3-integrin

Immunofluorescence microscopy revealed that the immunoreactivity of canine nephrin, podocin, α-actinin-4, α3-integrin in the glomeruli showed a diffuse pattern along the glomerular capillary wall (Fig. 4). The staining of nephrin was linear and didn't merge with that of vimentin, which is considered to be localized in the podocyte cell body and cell processes (Fig. 4a-c). The staining patterns of podocin, α-actinin-4 and α3-integrin were also linear (Fig. 4d-f). The immunoreactivity of nephrin and podocin was limited to the glomeruli, while α-actinin-4 was positive in the mesangial area and wall of arterioles, and α3-integrin in the mesangial area and a few tubules.

Western blot analysis showed bands of the expected size for each molecule in the isolated glomeruli (Fig. 5), and the molecular weights were: 180 kD (nephrin), 42 kD (podocin), 105 kD (α-actinin-4) and 130 kD (α3-integrin). Preimmune rabbit serum for canine nephrin did not generate a band.

Nephrin, Podocin, ACTN4 and α3-integrin Gene Expression

In the renal cortex and isolated glomeruli, mRNA of neprhin (104 bp), podocin (75 bp),

ACTN4 (92 bp) and α3-integrin (84 bp) were revealed by RT-PCR (Fig. 6). Sequencing analysis of the amplification product showed identification to partial sequences of predicted canine nephrin, podocin and ACTN4 (100%), as well as human α3-integrin (96.4%).

Discussion

To confirm the morphological integrity of SD, we observed the ultrastructure of SD in dog. Although detailed characterization of SD in dog has not been reported, observations of its length and structure in this study showed it to be very similar to SD in other mammals (Ichimura *et al.*, 2007; Rodewald and Karnovsky, 1974). Immunofluorescence microscopy revealed distinct expression of nephrin, podocin, α-actinin-4 and α3-integrin in canine glomeruli. The staining pattern of these proteins was diffuse, covering the surface of GBM, and might correspond to the localization of podocytes. There were small differences in the staining patterns of the molecules. We think this may arise from differences in molecule localization in the podocyte. Localization of nephrin, podocin, α-actinin-4 and α3-integrin in rodent and human podocytes has been reported in SD (Holzman *et al.*, 1999), the cytoplasmic face adjacent to the SD (Roselli *et al.*, 2002), FPs (Ichimura *et al.*, 2007) and the plasma membrane attached to GBM as well as the mesangial area (Bains *et al.*, 1997), respectively. Our IF results revealed that the staining pattern and localization of these proteins in canine podocytes were similar to that reported in humans (Kaplan *et al.*, 2000; Koop *et al.*, 2003), rats and mice (Dai *et al.*, 2006; Kanasaki *et al.*, 2008; Nakatsue *et al.*, 2005).

The results of Western blot analysis using isolated glomeruli reflected the distinct localization of each molecule in the glomeruli. We developed an antibody against nephrin that specifically recognized canine nephrin. Using RT-PCR, we also determined the gene expression of each molecule in canine glomeruli.

Several proteins in podocytes have been reported to be closely associated with maintenance of podocyte and SD in humans, rats and mice (Kawachi *et al.*, 2006). The four proteins we investigated in the chapter are regarded as key molecules in development of proteinuria. In fact, nephrin is a signaling receptor molecule, and its C-terminal cytoplasmic domain functionally interacts with other molecules, such as podocin, CD2AP and actin cytoskeleton (Saleem *et al.*, 2002). Podocin is a hairpin-like stomatin family protein and a raft-associated component of the SD. It is thought that nephrin and podocin have crucial roles in the function of SD, through interaction with other signaling molecules in lipid raft-based signaling platforms (Huber *et al.*, 2001;

Schwarz *et al.*, 2001). Moreover, gene mutation of ACTN4 causes autosomal-dominant focal segmental glomerulosclerosis in patients (Kaplan *et al.*, 2000). α-Actinin-4 interacts with the cytoplasmic domain of β1-integrin *via* integrin-linked kinase (ILK). It has been revealed that α-actinin-4 and α3β1-integrin are implicated in the regulation of podocyte adhesion and survival, mediated by integrin signaling (Dai *et al.*, 2006). Therefore, expression analysis of these molecules in the podocyte might be necessary in the pathologic study of canine glomerulonephropathy.

Our results would be invaluable for further analysis of podocyte injury and its involvement in protein leakage from the capillary wall in canine glomerular diseases.

Figures

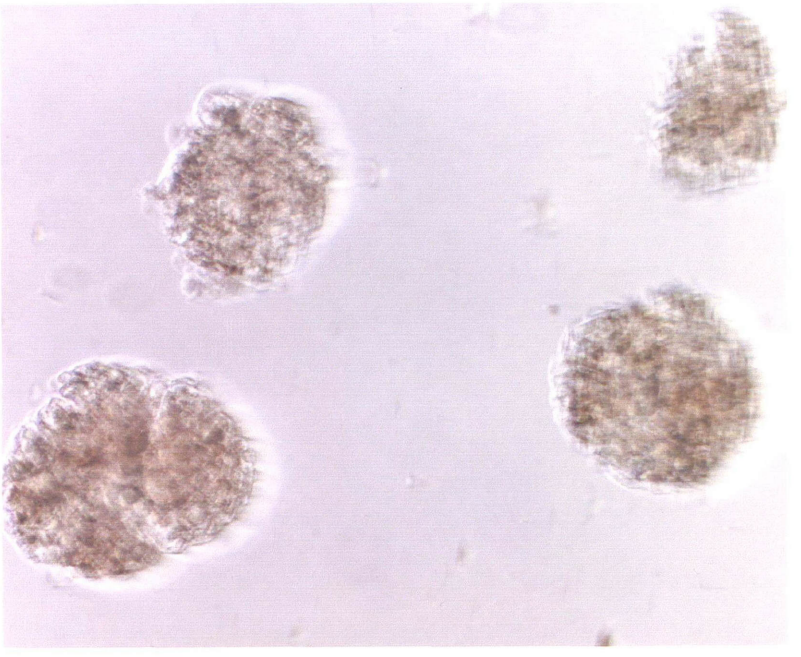

Figure. 1. Isolated glomeruli from a dog. Contamination of tubules and blood vessels are not observed.

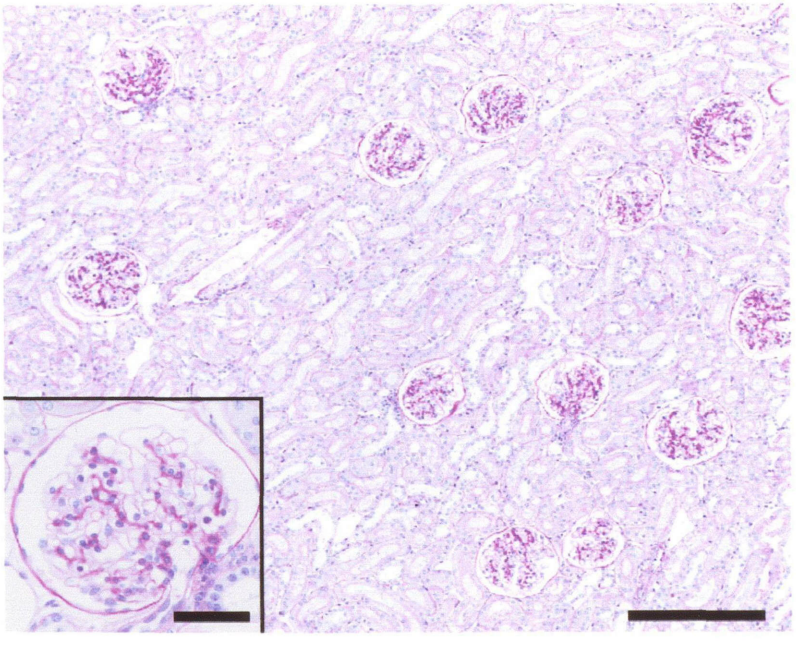

Figure. 2. Kidney from dog No. 2. there are no significant renal lesions. PAS. Bar, 250 μm (inset 50 μm) .

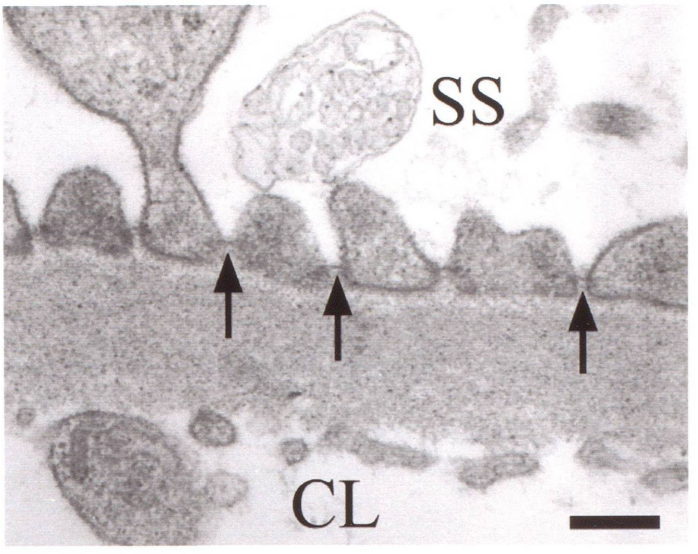

Figure. 3. Transmission electron microscopy of the canine glomerulus. A transverse section of the FPs is shown. FPs are arranged perpendicularly to the GBM and SDs connect adjacent FPs (arrows). The mean length of the SD was 379 ± 24.4 Å. SS, subpodocyte space; CL, capillary lumen. Bar, 200nm.

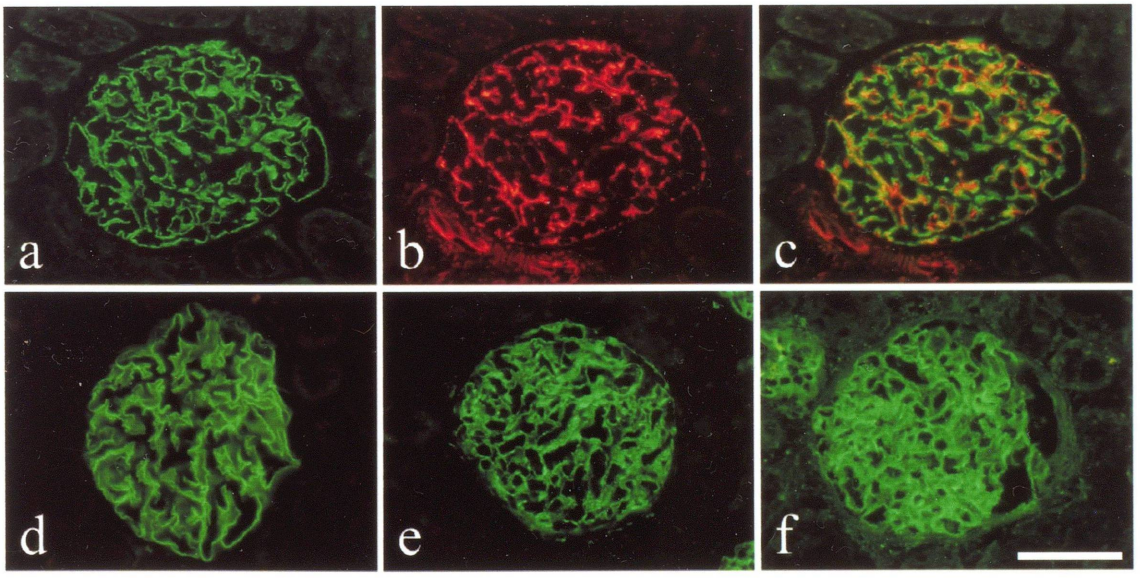

Figure. 4. Localization of the podocyte-associated proteins in the canine glomerulus. (a) neprhin. (b) vimentin. (c) Double labelling for nephrin and vimentin. Localization of nephrin (green) is distinct from that of vimentin (red), which is associated with the podocyte cell body and cell processes. (d) podocin. (e) α-actinin-4. (f) α3-integrin. In (e) and (f), the mesangium of the glomerulus is also labelled. IF. Bar, 50 μm.

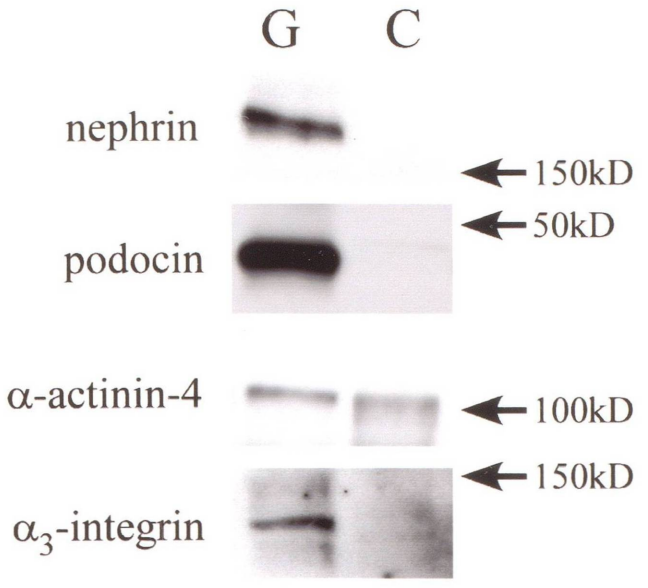

Figure. 5. Podocyte-associated protein expression in canine glomeruli. G, isolated glomeruli; C, cortex. Bands equating to nephrin (180 kD), podocin (42 kD) and α3-integrin (130 kD) are only detected in the isolated glomeruli. A band equating to α-actinin-4 (105 kD) was noted in both the isolated glomeruli and cortical extract.

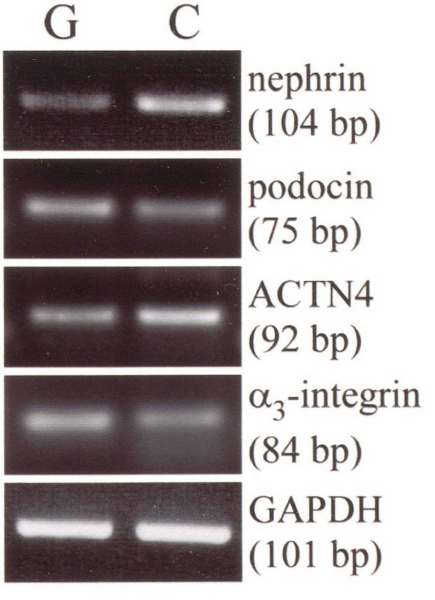

Figure. 6. Expression of mRNA encoding nephrin, podocin, ACTN4 and α3-integrin in extracts of canine glomeruli. G, isolated glomeruli; C, cortex.

The contents of Chapter 1 have been published as Expression of nephrin, podocin, α-actinin-4 and α3-integrin in canine renal glomeruli. J Comp Pathol. 145(2-3):220-225. 2011.

Chapter 2

Podocyte injury and altered expression and localization of podocyte-associated molecules in canine glomerular diseases

Abstract

Glomerular disease is the common renal disease in dogs and the disease may progress to chronic renal failure. Podocyte injury and altered expression of podocyte-associated molecules closely related to protein leakage from capillary walls in humans, mice and rats. However, the pathogenesis of podocyte has not been elucidated in canine glomerular diseases. In this chapter, we reveal altered expression and localization of nephrin, podocin, α-actinin-4, α3-integrin and WT1 with podocyte injury in diseased glomeruli of dogs. Twenty-one canine renal biopsies and renal tissue of 5 adult beagles for comparison were used. The 21 cases were divided into three groups as immune-mediated glomerular disease (IMGD), non-immune mediated glomerular disease (NIMGD) and non-glomerular disease (non-GD). In IMGD and NIMGD, various degree of podocyte injury was confirmed on electron microscopy. Decreasing expression of molecules, especially nephrin, was revealed in all glomerular disease. The localization of nephrin and α-actinin-4 were changed. In correlation tests, the expression score of nephrin and podocin negatively-correlated with urinary protein/creatinine ratio. In membranous nephropathy, molecular depression reflected morphological severity in glomeruli. WT1 positive podocyte/glomerular area was significantly decreased in IMGD and NIMGD and WT1 expression was reduced in injured podocyte. Nephrin mRNA in diseased glomeruli was demonstrated a 26.2-fold higher as compared to normal glomeruli. These results lead us to conclude that podocyte injury with altered expression and localization of podocyte-associated molecules closely involves the pathogenesis in canine glomerular diseases.

Introduction

Glomerular disease is the common renal disease in dogs, which progressively lead to chronic renal failure and end-stage renal disease.(Macdougall *et al.*, 1986; Muller-Peddinghaus and Trautwein, 1977) Regardless of causes and pathogenesis, the glomerular injury induces protein leaking to urine, and persistent non-selective proteinuria commonly raises tubulointerstitial damage through direct or indirect effects.(Nangaku, 2004) Thus, early detection and treatment of glomerular disease is necessary for preventive of loss of renal function in canine renal disease.

In glomerular disease, podocyte injury is profoundly associated with protein leakage from capillary walls. Morphological changes of podocyte such as effacement of slit diaphragms (SDs) and foot processes (FPs) are revealed by electron microscopy in proteinuric state.(Scaglione *et al.*, 2008) In humans, mice and rats, ultrastructural podocyte changes are accompanied by altered expression of podocyte-associated molecules that express in SDs and FPs. These molecules maintain podocyte function and its specific structure with each molecular interaction.(Dai *et al.*, 2006; Otaki *et al.*, 2008) Podocyte injury is raised in various glomerular diseases, and primary or secondary damage are involved in podocyte pathogenesis. For instance, NPHS1 (nephrin gene) and NPHS2 (podocin gene) mutations cause congenital nephrotic syndrome of the Finnish type and autosomal-recessive steroid-resistant nephrotic syndrome in children, respectively.(Boute *et al.*, 2000; Kestila *et al.*, 1998) In acquired human glomerular disease, it has been suggested that decreased expression of podocyte-associated molecules such as nephrin, podocin, a-actinin-4 and podocalyxin involved in development of proteinuria.(Guan *et al.*, 2003; Koop *et al.*, 2003) Furthermore, it has been reported that the molecules were decreased and changed the localization in nephrotic experimental models.(Guan *et al.*, 2004; Nakhoul *et al.*, 2005) On the other hands, podocyte injury also adversely affect on other podocytes,(Matsusaka *et al.*, 2011) endothelial cells,(Hauser *et al.*, 2009) or mesangial cells in glomeruli.(Morioka *et al.*, 2001) Therefore, podocyte injury and altered expression of the molecules closely interact with glomerular injury. In addition, the podocyte is noted as therapeutic target for progressive glomerular disease because podocyte have poor regenerative abilities.

In canine glomerular diseases, podocyte injury and dynamics of podocyte-associated molecules have not been revealed. Recently, ichii *et al.* reported that expression of nephrin and α-actinin-4 were decreased in chronic kidney diseases in dogs.(Ichii *et al.*, 2011) However, it has not been unclosed that alteration of podocyte-associated molecules in early and primary injury of podocyte and glomeruli in dogs. We previously

defined protein and mRNA expression of nephrin, podocin, α-actinin-4 and α3-integrin in normal canine glomeruli.(Kobayashi *et al.*, 2011)

The aim of the study is to reveal alterations of nephrin, podocin, α-actinin-4, α3-integrin and WT1 with podocyte injury in canine glomerular diseases.

Materials and Methods

Renal Biopsies and Controls

The clinical data and pathologic diagnosis of 21 dogs employed in the present study is shown in Table 1. Clinically, the 16 dogs with glomerular disease prominently showed moderate to high degrees of proteinuria in urinalysis, which ranged from 1.4 to 9.8 as urinary protein/creatinine ratio (UPC), or 2+ or 3+ examined by reagent strip. Dogs were of various breeds, aged from 10 months to 12 years, nine males and nine females. These biopsies were surgically carried out by opening the abdominal cavity of the dogs using 16 or 18-gauge Tru-cut-type needles. One third of biopsy tissue was fixed in 10% neutral-buffered formalin for histological examination, another one third was fixed in 2.5% glutaraldehyde for transmission electron microscopy (TEM) and last one third was embedded in optimal cutting temperature (OCT) compound (Sakura Finetek, Tokyo, Japan) for IF, and block embedded in OCT compound was snap-frozen and kept at -80 °C.

The glomerular diseases using in the study including membranoproliferative glomerulonephritis (MPGN, n=2), mesangial proliferative glomerulonephritis (MePGN, n=2), membranous nephropathy (MN, n=7) which were graded stage I to III, glomerular amyloidosis (GA, n=1), minor glomerular abnormality (MGA, n=1), focal segmental glomerulosclerosis (FSGS, n=3). The pathological diagnosis was made based on the light and electron microscopic changes and immune deposition pattern. In all biopsy, Over 10 glomeruli were observed for histological diagnosis on light microscopy. The World Health Organization's classifications of human glomerular diseases were consulted.(Churg *et al.*, 1995) The 21 cases were divided into three groups as immune mediated glomerular disease (IMGD), non-immune mediated glomerular disease (NIMGD) and non-glomerular diseases (non-GD) for comparison. Immune deposition in glomeruli were evidenced by direct immunofluorescence (IF) using antibodies of anti-dog IgG (γ) (Kirkegaard and Perry Laboratories, Gaithersburg, MD, 1:500 dilution), dog C3 antibody FITC conjugated (Bethyl, 1:500 dilution), dog IgA antibody FITC conjugated (Bethyl, Montgomery, TX, 1:200 dilution) and dog IgM antibody FITC conjugated (Bethyl, 1:1000 dilution) for frozen sections. For TEM, glutraraldehyde-fixed tissues were post-fixed in 1% osmium tetraoxide, and embedded

in epoxy resin. Ultrathin sections were double-stained with uranyl acetate and lead citrate, and examined using a JOEL 1210 transmission electron microscope (JOEL, Tokyo, Japan) at 80 kV. Ultrastructural changes were investigated in 19 cases except for No. 9 and 16 in which glomeruli were existed in specimens. Podocyte injury of variable degree such as effacement of FPs and SDs, aggregated actin-cytoskeleton, increased microvilli on cell surface, vacuoles in cytoplasm and hypertrophy of cell body were noted in all 14 cases with glomerular disease. Normal podocyte structures were confirmed in 5 cases of non-GD group.

As controls, the renal cortex tissues of 5 adult beagles (two male and three female, aged 1 to 7 years) were collected. The dogs were confirmed to be healthy by physical examination and no renal dysfunction was revealed by serum biochemistry and urinalysis, and humanely destroyed in accordance with the guidelines approved by the Animal Research Committee of Azabu University. The renal tissues were immediately collected, which were fixed in 10% neutral-buffered formalin or 2.5% glutaraldehyde, or embedded in OCT compound for frozen sections.

Semi-quantitative Analyses of Protein Expression of Nephrin, Podocin, α-actinin-4 and α3-integrin

The protein expression and localization of nephrin, podocin, α-actinin-4 and α3-integrin were examined by IF. The protocol was following: the frozen sections cut at 3 to 4 μm were fixed with acetone at -20°C for 5 min, and washing with cold PBS. For nephrin and α-actinin-4, sections were pretreated by incubation in citrate buffer (pH 6.0) in a 400 W microwave at 90°C for 10 min. After incubation with 4% BlockAce^{TM} (Yukijirushi) for 10 min at room temperature, the primary antibodies were applied and reaction was forwarded at 4°C overnight. The primary antibodies were anti canine-nephrin (we prepared the antibody in previous report(Kobayashi *et al.*, 2011)), anti-human podocin (Sigma, St Louis, Missouri; 1:200 dilution), mouse anti-α-actinin-4 (clone 4D10, Abnova, Taipei, Taiwan; 1:100 dilution), rabbit anti-human α3-integrin (Chemicon, Temecula, California; 1:500 dilution). Following washing with cold PBS, the secondary antibodies were applied and incubated for 30 min at room temperature, and then washed off with cold PBS. The secondary antibodies were anti-rabbit IgG conjugated to fluorescein isothiocyanate (FITC; Cappel, Aurora, Ohio; 1:100 dilution) or FITC-conjugated anti-mouse IgG (EY laboratories Inc., San Mateo, California; 1:100 dilution). For examination, a FSX100 fluorescence microscope (OLYMPUS, Tokyo, Japan) with BP filters (BP460-495 for FITC, BP530-550 for rhodamine, OLYMPUS) was used.

Expression of nephrin, podocin, α-actinin-4 and α3-integrin was evaluated semi-quantitatively using the formula described by Macconi et al.(Macconi *et al.*, 2000) In particular, expression score of individual glomerulus was determined as follows: 0, globally and severe decreased expression; 1, globally and moderate or segmentally (25 to 75%) decreased expression; 2, globally and mild or segmentally (fewer than 25%) decreased expression; 3, normal expression. The number of glomeruli using the evaluation in the dogs was 4 to 50. The conclusive score in each molecule was calculated as the mean $S = [(0 \times N_0) + (1 \times N_1) + (2 \times N_2) + (3 \times N_3)] / (N_0 + N_1 + N_2 + N_3)$ that N_i ($i = 0$ to 3) is the number of glomeruli in each sections.

Counting Analysis of WT1 Positive Podocyte Numbers

WT1 protein is specifically express on the nucleus of podocytes in mature renal tissue.(Mundlos *et al.*, 1993) To investigate the number of WT1 positive podocyte, immunohistochemistry (IHC) were done as follows: the paraffin embedded sections cut at 3 to 4 μm were deparaffinized and pretreated with pepsin solution (Nichirei, Tokyo, Japan) at 37 °C for 20 min. After washing with PBS, endogeneous peroxidases were blocked with 0.3% hydrogen peroxide in methanol for 20 min. Blocking was conducted with 4% BlockAce™ (Yukijirushi) for 10 min at room temperature, and anti-WT1 antibody (clone 6F-H2, Dako; 1:100 dilution) were applied and incubated at 4°C over night. Following washing with PBS, peroxidase-conjugated anti-mouse IgG (Nichirei, Tokyo, Japan) was applied. The sections were developed with diaminobenzidine (DAB) and counterstained with methyl green. The number of WT1 positive podocytes was counted and glomerular area was calculated for each glomerulus in 21 renal biopsies (all glomeruli in each specimen) and normal 4 beagles (each 50 glomeruli). Each index was represented by the number of WT1 positive podocytes per glomerular area. WT1 index were compared among IMGD, NIMGD, non-GD and normal dogs, and correlation between WT1 index and molecular expression score were analyzed by Spearman's correlation tests.

Correlation Test between UPC and molecular score

To investigate a role for selective filtration of podocyte specific molecules in glomeruli, we compared expression scores of nephrin, podocin, α-actinin-4 and α3-integrin with UPC in 18 cases of renal biopsies excepted for 3 cases (No, 5, 12, 14) which were not measured UPC. Spearman's correlation tests were used to assess.

Quantitative Analysis of Nephrin mRNA (NPHS1) Expression

To compare gene expression of nephrin in glomeruli between cases with glomerulonephropathy and normal dogs, the laser microdissection (LMD) and the 7500 Real-time PCR system (Applied Biosystems, Foster City, CA) were used. We extracted 100 glomeruli from each biopsy specimen for applicable determination on real-time PCR in 16 cases of glomerulonephropathy, however, adequate number of glomeruli could not collected in 9 cases (Nos. 2, 3, 7-9, 11, 12, 14 and 16). In particular, acquired numbers of frozen section (8 μm) were mounted on glass slides covered with PEN foil (2.5 μm thick; Leica Microsystems, Wetzlar, Germany) and fixed with 95% ethanol for 30 sec at room temperature, and hydrated using graded alcohol. The sections were stained with 0.05% toluidine blue/diethylpyrocarbonate (DEPC)-treated water for 30 sec, and dehydrated using graded ethanol and xylene. In the LMD system, the glomeruli were manually dissected from the sections. RNA extraction using the RNeasy Plus Micro Kit (Qiagen, Hilden, Germany) was done and total RNA were dissolved in 14 μl RNase-free water. The first-strand complementary DNA (cDNA) for quantitative PCR were synthesized form 12 μl of the total RNA solution using a Sensiscript RT kit (Qiagen), 1 μM random primer (Invitrogen), and 10 U of RNasin ribonulease inhibitor (Promega) in a 20 μl final volume.

Quantification of NPHS1 and 18S rRNA mRNA expression was done using TaqMan Universal PCR Master Mix (Applied Biosystems), with sample cDNA in a final volume of 25 μl per reaction. Sequence of primers and probe was follows: canine NPHS1 forward, GCTGGTGTGTTTGGCCATT; reverse, CGGCCGCGGTTCAGT; probe TCAAGGACTCCCGGACG. The probe was designed at exon-exon junction for preventing amplification of DNA contamination. Human 18S rRNA primers and probe were designed by Applied Biosystems and supplied as Taqman Gene Expression Assays Mix containing a 20x mix of unlabeled PCR forward and reverse primers, as well as Taqman MGB probe (Assay ID: Hs99999901_s1). The PCR reaction of nephrin was carried out in duplicate of for each sample and mean values of the gene expression were calculated as the ration to those of 18S rRNA.

Statistical Analyses

Results were expressed as the mean ± SD. In semi-quantitative analysis and counting analysis of WT1 positive podocyte, differences among groups were identified using one-way ANOVA test. Comparisons between glomerular disease and controls in analysis of nephrin mRNA expression were performed by the Mann-Whitney's *U*-test. A *P*-value of < 0.05 was considered to be statistically significant difference.

Results

Expression and Localization of Nephrin, Podocin, α-actinin-4 and α3-integrin in Diseased Glomeruli

The expression and localization of nephrin, podocin, α-actinin-4 and α3-integrin were changed in 16 cases with glomerular disease. In comparison to normal dogs, the each expression was decreased to various degrees in both IMGD and NIMGD with podocyte injury while there are no differences in non-GD (Fig. 1-8). Semi-quantitative analyses showed alteration of the four molecules in each group (Fig. 9). Generally, nephrin expression was most reduced, and the decreased expression of each molecule was more severe in NIMGD than in IMGD. Nephrin, podocin and α-actinin-4 expression were significantly decreased in NIMGD compared to normal dogs whereas decreased expression of nephrin and podocin were significant in IMGD. Additionally, staining pattern of nephrin and α-actinin-4 were characteristically changed in IMGD and NIMGD. Nephrin pattern were changed to discontinuous granular pattern from normal linear pattern in Nos. 1, 2, 5, 9-11 and 14-16 (Fig. 1, 5). On the other hand, in Nos. 1, 5, 6, 9, 11, 14 and 15, aggregated pattern of α-actinin-4 was observed on a portion of glomerular tufts, although expression of α-actinin-4 was diffusely decreased in a typical (Fig. 3, 7).

Characterization of molecular changes in specific glomerular disease

The molecular alteration in some glomerular disease reflected the severity or features of the disease. In 7 cases of MN, degree of molecular depression had relation to severity of morphological changes in glomeruli. For instance, nephrin expression of No. 7 in which light and electron microscopic changes were sparse (stage I) had only mild decreased expression of nephrin (Fig. 10-12). On the other hand, it was observed that nephrin expression was completely depressed in glomeruli of No.11 that diagnosed MN stage III-IV (Fig. 13-15). The nephrin expression in No. 13 (MGA) was globally decreased and shifted to pseudolinear or finely granular pattern (Fig. 16-18). In FSGS, molecular expression was disappearance in sclerotic area (Fig. 19, 20), and also, that was decreased and changed localization in non-sclerotic glomeruli (Fig. 21, 22).

WT1 Positive Podocyte numbers and reduction of WT1 expression in injured podocyte

WT1 positive podocyte/glomerular area was calculated in 21 cases of renal biopsies and normal dogs (Fig. 23). The index was significantly decreased in IMGD and NIMGD relative to normal dogs and that of NIMGD was most severely degraded. The difference between non-GD and normal dogs was not statistically significant. WT1

index were significantly correlated with molecular expression score (Table. 2., nephrin and podocin; $P = < 0.0001$, α-actinin-4; $P = < 0.003$, α3-integrin; $P = 0.013$). Moreover, reduction of WT1 signals in some podocytes was also observed, which was to be high frequency in injured glomeruli (Fig. 24, 25).

Correlation between UPC and scores of nephrin and podocin

In correlation tests, scores of molecules tends to negatively-correlated with UPC, and it was particular significant in nephrin ($P = 0.003$) and podocin ($P = 0.001$) (Fig. 26, Table. 3).

Expression of Nephrin Gene (NPHS1) in Diseased Glomeruli and normal Dogs

As described above, nephrin expression was most decreased in the molecules we examined and nephrin score was negatively-correlate with UPC. These results lead us to investigate mRNA expression of nephrin in diseased glomeruli. We quantitatively compared nephrin expression in 100 glomeruli between 7 cases with glomerular disease and normal dogs (Fig. 27). As the results, nephrin expression demonstrated a 26.2-fold higher in diseased glomeruli as compared to normal glomeruli.

Discussion

We revealed altered expression and localization of nephrin, podocin, α-actinin-4, α3-integrin and WT1 in various canine glomerular diseases in this chapter. In the dogs, the decreased expression of nephrin and podocin were notable in both IMGD and NIMGD. In human acquired glomerular diseases, a number of podocyte-associated molecule showed decreasing of various degree depend on the glomerular diseases.(Goode *et al.*, 2004; Koop *et al.*, 2003; Schmid *et al.*, 2003) Nephrin and podocin are recognized the molecule constructing the SDs and it has been revealed that dysfunction of the SDs accompanied by decreasing of the molecules cause protein leakage from capillary walls in humans and experimental models.(Boute *et al.*, 2000; Kawachi *et al.*, 2000) In the chapter, decreasing expression of nephrin and podocin are accompanied by the loss of SDs observed in TEM, and the changes occurred from early stage of the glomerular disease as well as humans. Nephrin might especially be the pivotal role for pathogenesis of both IMGD and NIMGD in dogs, and it could be sensitive marker for podocyte injury.

The staining pattern of nephrin and α-actinin-4 were characteristically altered in some cases. It has been reported that linear pattern of nephrin was shifted to granular pattern, which accompanied by effacement of FPs and SDs, and immunoelectron microscopic

study revealed redistribution of nephrin into the cytoplasm.(Wernerson *et al.*, 2003) In addition, dephosphorylated nephrin lost interaction with podocin and endocytosed into cytoplasm in proteinuric state.(Quack *et al.*, 2006) In the study, granular pattern of nephrin was observed with effacement of slit diaphragm on TEM in several canine glomerular diseases. We thought alteration of nephrin pattern in canine diseased glomeruli involved in dysfunction of SDs. Meanwhile, α-actinin-4, which is an actin cytoskeleton linking protein, maintains podocytes adhesion to glomerular basement membrane with integrins.(Dandapani *et al.*, 2007) Interaction between α-actinin-4 and F-actin regulate cytoskeletal dynamics and specific structures of podocyte.(Michaud *et al.*, 2006; Weins *et al.*, 2007) The aggregated patterns on the partial glomerular tuft in our cases were accompanied by fusion of foot processes and actin aggregation. Thus, we thought the changes of α-actinin-4 are associated with reconstructing actin-cytoskeleton and foot processes in injured podocytes.

The molecular expression in several glomerular diseases was characteristically changed. The severity of morphological changes had mutual relation to molecular expression in MN. It has been reported that the decreased expression of nephrin and podocin correlated with the histological severity in human glomerular disease.(Perysinaki *et al.*, 2011) Our data suggested that the progression of canine MN were accompanied by podocyte injury and its molecular depression, which might be involved in protein leakage from capillary walls. In a case of MGA, nephrin pattern were changed to pseudolinear or a finely granular pattern as well as human MGA. In human, MGA corresponding to histology of minimal change disease (MCD), and it has been thought that the nephrin pattern we observed might be attributed to global decreasing the number of SDs in glomeruli of MCD.(Kim *et al.*, 2002) Altered expression of nephrin not only sclerotic area but non-sclerotic glomeruli in FSGS evidenced that podocyte was severely and diffusely injured in the canine glomerular disease.

To analyze the number of podocyte, counting analysis of WT1 positive cells have generally been used in humans and experimental models.(Dai *et al.*, 2010) We measured WT1 positive podocyte number per glomerular area in the dogs and the results suggested that podocyte number was reduced in IMGD and NIMGD. The reduction of podocytes, a cells with limited potential for proliferation, is attributed to apoptosis or detachment from capillary wall in glomerular diseases.(Mundel and Shankland, 2002) The loss of podocyte leads to irreversible damage and sclerosis in glomeruli. Our data indicated that podocyte loss was deeply associated with molecular depression and progression of glomerular injury in canine glomerular diseases. On the other hand, WT1

expression was prominently decreased in some podocyte. WT1 gene/protein has important roles in renal development and function of matured podocyte as transcription factor, and mutations of WT1 gene induce several nephrotic diseases in humans.(Morrison *et al.*, 2008) In human cultured podocyte and transgenic mice, TGF-β1 that was increased expression in various glomerular disease reduce WT1 expression.(Sakairi *et al.*, 2011) Our results suggested that injured podocyte was reduced WT1 expression in dogs as well as humans. In canine glomerular diseases, WT1 expression and function in matured podocyte have not been investigated in details. We speculated that WT1 have a key role not only development of kidney but maintenance of matured podocyte in dogs.

In the correlation tests, nephrin and podocin score was significantly negatively-correlated with UPC. In humans, urinary podocyte(Hara *et al.*, 1998) and excreted podocyte-associated molecules such as nephrin, podocin and podocalyxin(Kanno *et al.*, 2003; Sato *et al.*, 2009) have been thought to be useful biomarker of glomerular injury in urine. In addition, podocyturia might be more sensitive to assess glomerular damage than proteinuria.(Yu *et al.*, 2005) Our statistical results suggested that podocyte-associated molecules, especially nephrin and podocin might reflect glomerular damage in canine glomerular disease. In the previous report, we revealed that nephrin and podocin expression was limited to glomeruli in the kidney of dogs.(Kobayashi *et al.*, 2011) In addition, it has been revealed that nephrin mRNA was detectable in urine of dogs.(Ichii *et al.*, 2011) For these reasons, nephrin and podocin in urine could be functional and specific markers of glomerular injury in dogs.

The nephrin gene expression was increased in glomerular disease of our cases compared with normal controls, although protein expression was decreased. It has been reported that gene expression of nephrin was either increased(Koop *et al.*, 2003; Schmid *et al.*, 2003) or decreased(Furness *et al.*, 1999) in human acquired glomerular disease. We thought that the inconsistency was attributed to compensatory reaction to the damage on the podocyte, and analysis of protein expression might be adapted as the functional evaluation in canine glomerular disease.

In our results, podocyte injury was more severe in NIMGD than IMGD. In IMGD, podocyte injury presumably induced by glomerular damage which was related to other causes such as immune deposition to the glomerular tuft and proliferation of the cells in glomeruli. By contrast, NIMGD group included a case with MGA and 3 cases with FSGS. In humans, MCD and FSGS are common cause of nephrotic syndrome, in which morphological changes were mainly observed in podocyte and extensive effacement of FPs and SDs are noted. Although the pathogenesis of MCD and FSGS has been remains

unclear, the many studies provide evidence that primary podocyte injury intimately involves in the diseases.(Mathieson, 2007) For instance, mutations of podocyte-associated gene such as α-actinin-4 and CD2-associated protein are lead to FSGS.(Gigante *et al.*, 2009; Kaplan *et al.*, 2000) Polymorphisms and mutations of the genes, such as podocin(Franceschini *et al.*, 2006) and WT1,(Orloff *et al.*, 2005) might be predisposing factors of FSGS in human. In addition, puromycin aminonucleoside injection which injures podocyte into the rat induced histology resemble to MCD or FSGS.(Caulfield *et al.*, 1976; Diamond and Karnovsky, 1986) It has been evidenced that podocyte and its specific molecules play critical role in MCD and FSGS. In dogs, 2 case of MCD,(Sum *et al.*, 2010; Vilafranca *et al.*, 1993) and one case of FSGS(Aresu *et al.*, 2010) have only been reported but molecular analyses were not done in the studies. Our results accentuate significance of assessing podocyte-associated molecules for diagnosis and analysis of MCD and FSGS in dogs. We thought that analyses of MCD and FSGS in dogs might contribute to disclosing pathogenesis of podocyte injury.

The results in the present study suggested that podocyte injury more or less contribute to various canine glomerular diseases. In the view point, podocyte would be promising therapeutic target for the glomerular diseases in dogs. In humans and experimental models, the direct benefits for podocyte of various drugs have been evidenced, including renin inhibitor,(Sakoda *et al.*, 2010) angiotensin-converting enzyme inhibitors,(Macconi *et al.*, 2009) angiotensin receptor blocker,(Matsusaka *et al.*, 2010) statin,(Shibata *et al.*, 2006) rituximab(Fornoni *et al.*, 2011) and immunosuppressive agents such as cyclosporine A.(Faul *et al.*, 2008) These pharmacological agents are expected to protect podocyte and prevent loss of renal function in dogs.

In conclusion, we revealed that podocyte injury and altered expression and localization of nephrin, podocin, α-actinin-4, α3-integrin and WT1 were induced in various canine glomerular diseases. All glomerular diseased in the study showed morphological and molecular changes in podocyte with glomerular injury, although the further investigation would be needed to reveal whether podocyte is injured in other types of glomerular diseases in dogs.

Figures and Tables

Table 1. The clinical data and pathologic diagnosis of the 21 dogs

	Case number	Breed	Sex/Age (years)	Urinary Protein	BUN	S-Cre	Alb	Pathologic diagnosis
Immune mediated glomerular disease	1	Jack Russell terrier	Cas/6	9.8	114.4	2.8	2	MPGN
	2	Jack Russell terrier	Sp/6	3.5	21.8	1	1.8	MPGN
	3	Labrador retriever	Sp/6	5.1	9	0.7	2.1	MePGN
	4	Yorkshire terrier	Cas/10	1.9	NT	NT	NT	MePGN
	5	Lhasa Apso	Sp/12	3+	11	0.9	3.8	MN stage I
	6	Miniature Dachshund	F/10	2.8	8	NT	2	MN stage I
	7	Yorkshire terrier	F/6	2.3	23.3	0.7	4.4	MN stage I
	8	Yorkshire terrier	F/8	1.4	22.5	0.5	4.3	MN stage II
	9	Papillon	Cas/7	6.5	35	1.8	1.4	MN stage II-III
	10	Papillon	Cas/12	2.2	46.2	1.1	3	MN stage II-III
	11	Boston terrier	Sp/10	4.6	28.5	0.6	2	MN stage III-IV
Non-Immune mediated glomerular disease	12	Beagle	F/unkown	NT	NT	NT	NT	GA
	13	Miniature Dachshund	Cas/6	9.3	16.4	0.3	2.3	MGA
	14	Welsh Corgi	M/12	2+	58	2.9	1.9	FSGS
	15	Welsh Corgi	M/7	5	9.4	0.5	2.9	FSGS
	16	Miniature Schnauzer	M/8	9.3	37.9	1.9	2.9	FSGS
Non-glomerular diseases	17	Welsh Corgi	F/6	0.1	6	1.2	2.6	tubular degeneration
	18	Australian Labradoodle	Cas/2	0.1	30.9	1.4	3.7	renal congenital anomaly
	19	Chihuahua	F/0.8	0.1	37.6	0.7	3.5	renal congenital anomaly
	20	Bernese Mountain dog	M/1	0.3	25	1.4	3.7	no significant changes
	21	Chihuahua	Sp/2	3.2	4.5	0.2	2.5	no significant changes

M: Male, F: Female, Cas: Castrated, Sp: Spayed, Urinary Protein: Urinary protein/creatinin ration or reagent strip, BUN: Blood urea nitrogen (mg/dl), S-Cre: Serum creatinin (mg/dl), Alb: Serum albumin (g/dl), NT: not tested, MPGN: membranoproliferative glomerulonehritis, MePGN: mesangial proliferative glomerulonephritis, MN: membranous nephropathy, GA: glomerular amyloidosis, MGA: minor glomerular abnormality, FSGS: focal segmental glomerulosclerosis

Table 2. Correlations between WT1 index and molecular expression score

	nephrin***	podocin***	α-actinin-4**	α3-integrin*
P value	< 0.0001	< 0.0001	0.003	0.013
r	0.728	0.761	0.565	0.492

*, **, ***Statistically significant in Spearman's correlation test (*P<0.05, **P<0.01, ***P<0.001).

Table 3. Correlations between UPC and molecular expression score or index

	nephrin**	podocin**	α-actinin-4	α3-integrin*
P value	0.003	0.001	0.05	0.02
r	-0.655	-0.702	-0.468	-0.544

*, **Statistically significant in Spearman's correlation test (*P<0.05, **P<0.01).

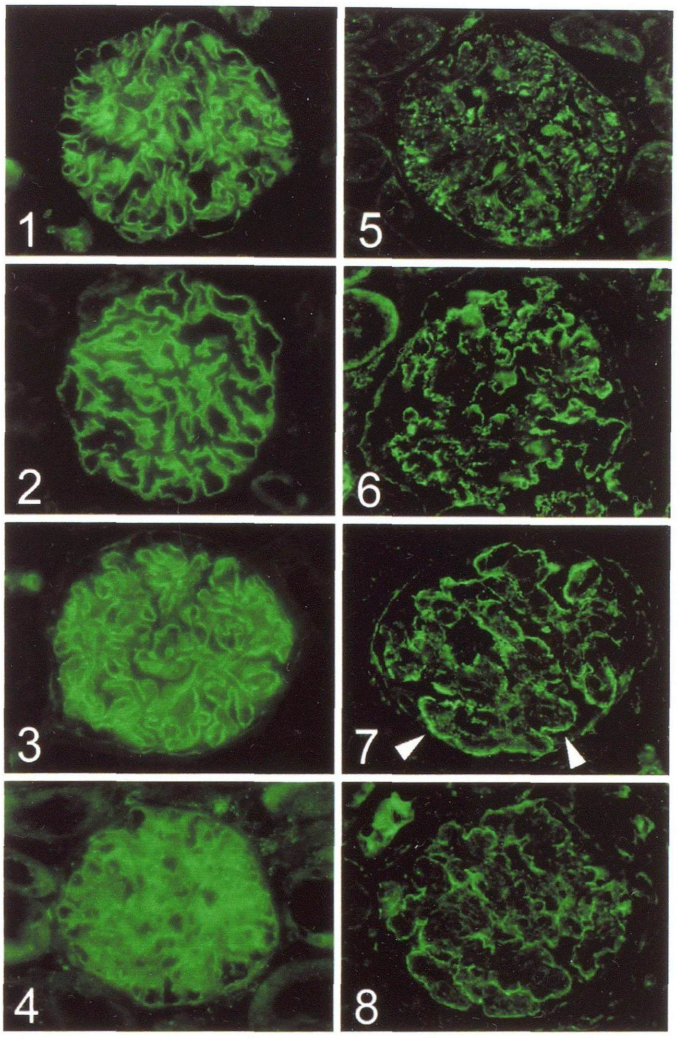

Figure 1. Glomerulus; normal dog. The linear pattern of nephrin covered with glomerular tufts. IF. **Figure 2.** Glomerulus; normal dog. The sharp and linear staining pattern of podocin. IF. **Figure 3.** Glomerulus; normal dog. α-actinin-4 localized in the glomerular tufts and the mesangium area. IF. **Figure 4.** Glomerulus; normal dog. The expression of α3-integrin in the glomerular tufts and mesangium areas. IF. **Figure 5.** Glomerulus; dog, case No. 7. The expression of nephrin is severely depressed and the staining pattern is shifted to coarse granular pattern. IF. **Figure 6.** Glomerulus; dog, case No. 9. Although the staining pattern of podocin is not changed, expression is decreased. IF. **Figure 7.** Glomerulus; dog, case No. 1. α-actinin-4 are depressed and aggregated in a portion of the tufts (arrow heads). IF. **Figure 8.** Glomerulus; dog, case No.1. The expression of α3-integrin in glomerular tufts and mesangium are globally decreased. IF.

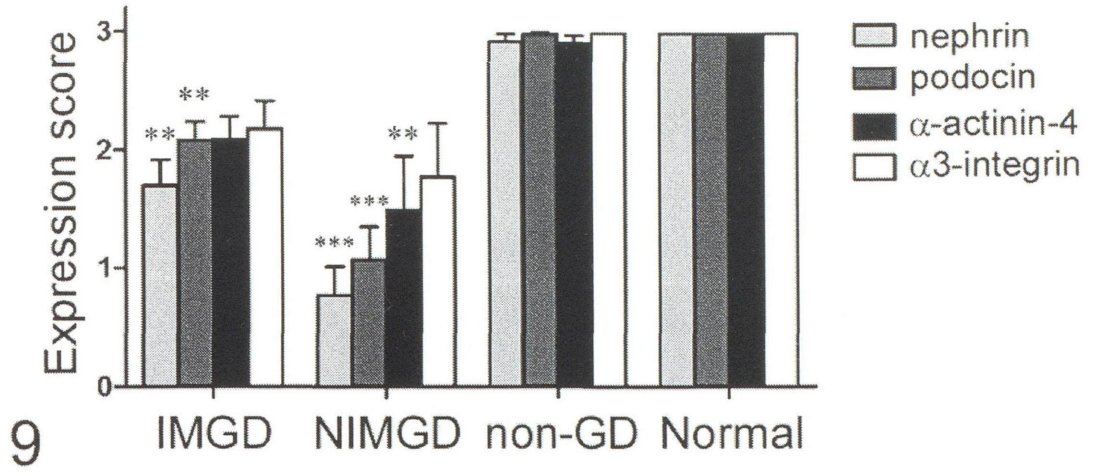

Figure 9. Expression score of nephrin, podocin, α-actinin-4 and α3-integrin in IMGD, NIMGD, non-GD and normal dogs (Normal). In IMGD and NIMGD, nephrin score is most reduced. Degrees of decreasing expression are more severe in NIMGD than in IMGD as a whole. $^{**}P < 0.01$ verses normal dogs, $^{***}P < 0.001$ verses normal dogs.

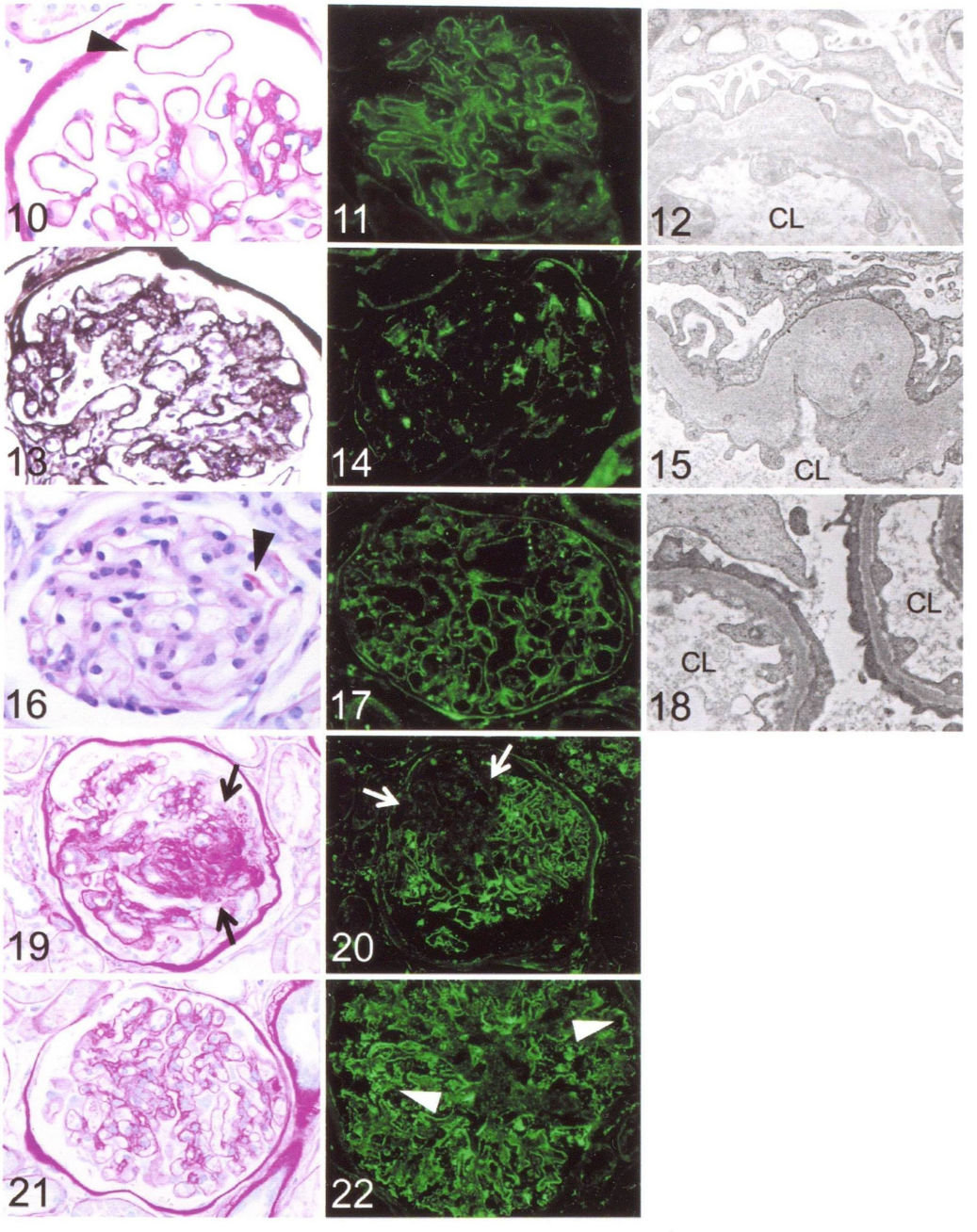

Figure 10. Glomerulus; dog, case No. 7. The change of glomerular basement membranes is scant in the glomerulus (arrow head). PAS. **Figure 11.** case No. 7. Nephrin expression is scarcely changed and keep linear pattern. IF. **Figure 12.** case No. 7. There is irregular thickening of glomerular basement membrane but the structures of FPs are maintained. CL, capillary rumen. TEM. **Figure 13.** case No. 11. Global and severe thickening of the glomerular basement membranes show bubble-like appearance. PAM. **Figure 14.** case No. 11. Nephrin expression is almost entirely depressed. IF. **Figure 15.** case No. 11. FPs are fused with advanced glomerular basement membranes

thickening. CL, capillary rumen. TEM. **Figure 16.** case No. 13. On light microscopy, the morphological changes are minimal. The hyaline droplets are observed in a podocyte (arrow head). PAS. **Figure. 17.** case No. 13. The expression of nephrin is globally decreased and the staining pattern shift to pseudolinear or finely granular. IF. **Figure. 18.** case No. 14. On Electron microscopy, FPs are globally flattened but other lesion is not observed. TEM. **Figure. 19.** case No. 16. Segmental sclerosis (arrows) and adhesion between glomerular tuft and Bowman's capsule. Hyaline droplet degeneration are remarkable in podocytes. PAS. **Figure 20.** case No. 16. Nephrin expression is disappeared in a sclerotic portion (arrows). **Figure 21.** case No. 16. Non-sclerotic glomerulus in FSGS. **Figure 22.** case No. 16. In non-sclerotic glomerulus, expression of nephrin is decreased and show finely granular pattern (arrow heads).

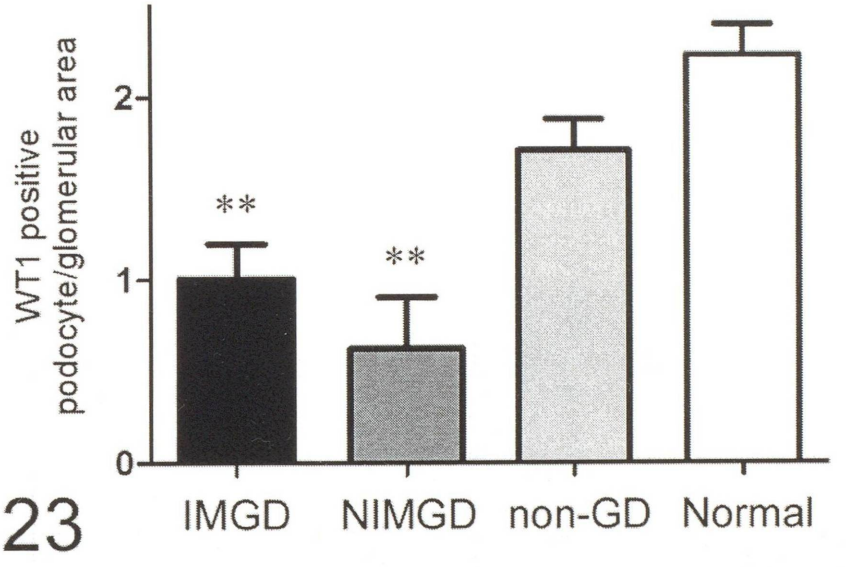

Figure 23. WT1 positive podocyte/glomerular area in IMGD, NIMGD, non-GD and normal dogs (Normal). $^{**}P < 0.01$ verses normal dogs.

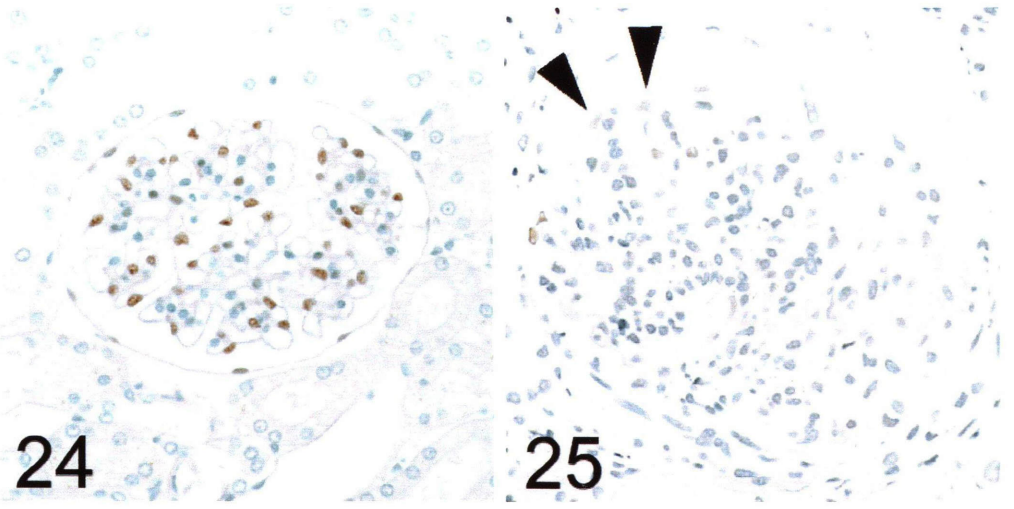

Figure 24. Glomerulus; normal dog. The nuclei of podocytes were specifically and intensely labeled by WT1. IHC. **Figure 25.** case No. 14. WT1 expression was decreased in some podocytes (arrow heads). IHC.

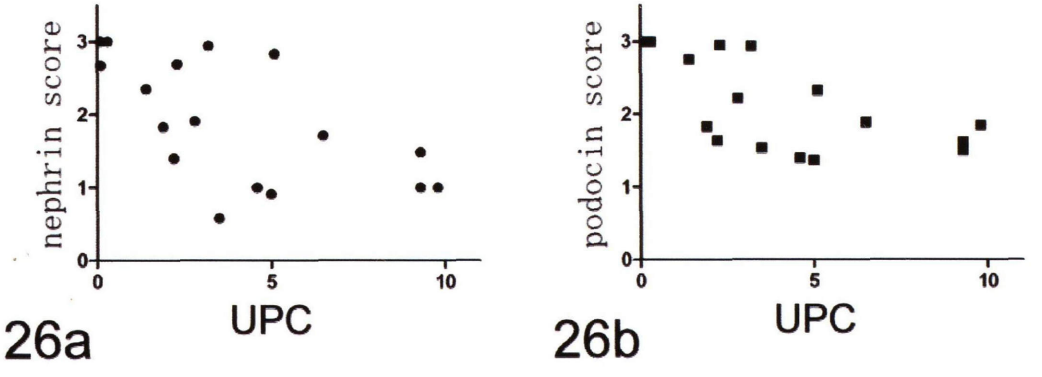

Figure 26. Correlation plots. The value of UPC is negatively-correlated with nephrin score (a) and podocin score (b) (P = 0.003 and 0.001, r = –0.655 and –0.702, respectively).

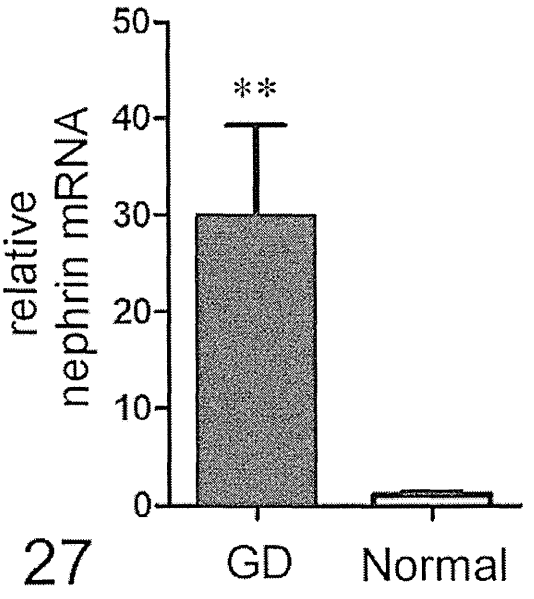

Figure 27. The results of real-time PCR using microdissected glomeruli. In glomerular disease (GD), elevation of nephrin gene expression is a 26.2-fold higher as compared to normal glomeruli. $^{**}P < 0.01$ verses normal dogs.

Supplementary Data

Table 3. Detail data of molecular expression score, localization changes, WT1 index and relative nephrin mRNA in 21 dogs

Case number	Pathologic dianosis	Urinary Protein	Expression score				Localization change		WT1 index	relative nephrin mRNA
			neprhin	podocin	α-actinin-4	α3-integrin	nephrin	α-actinin-4		
1	MPGN	9.8	1	1.85	1.37	1.27	○	○	2.532	14.41
2	MPGN	3.5	0.58	1.54	2	2.67	○	–	0.246	NT
3	MePGN	5.1	2.83	2.33	2.8	2.17	–	–	1.414	NT
4	MePGN	1.9	1.83	1.83	2.62	2.91	–	–	0.702	35.47
5	MN stage I	3+	1.43	2.5	2.25	2.54	○	○	0.794	12.65
6	MN stage I	2.8	1.91	2.22	2.32	2.06	–	○	0.782	64.93
7	MN stage I	2.3	2.69	2.95	2.74	3	–	–	0.828	NT
8	MN stage II	1.4	2.35	2.76	2.68	2.89	–	–	1.158	NT
9	MN stage II-III	6.5	1.71	1.89	1.71	2.5	○	○	1.541	NT
10	MN stage II-III	2.2	1.4	1.64	1.68	1.26	○	–	0.717	4.12
11	MN stage III-IV	4.6	1	1.4	0.8	0.67	○	○	0.362	NT
12	GA	NT	0.1	0.15	0.7	0.25	–	–	0.195	NT
13	MGA	9.3	1.48	1.62	2.07	2	–	–	1.744	62.34
14	FSGS	2+	0.37	0.73	0.6	1.5	○	○	0.515	NT
15	FSGS	5	0.91	1.37	1.1	2.12	○	○	0.33	15.65
16	FSGS	9.3	1	1.5	3	3	○	–	0.334	NT
17	tubular degeneration	0.1	3	3	3	3	–	–	1.518	NT
18	renal congenital anomaly	0.1	2.67	3	2.67	3	–	–	1.948	NT
19	renal congenital anomaly	0.1	3	3	3	3	–	–	1.874	NT
20	no significant changes	0.3	3	3	3	3	–	–	1.14	NT
21	no significant changes	3.2	2.94	2.94	2.9	3	–	–	2.084	NT

Urinary Protein: Urinary protein/creatinin ration or reagent strip, NT: not tested, MPGN: membranoproliferative glomerulonehritis, MePGN: mesangial proliferative glomerulonephritis, MN: membranous nephropathy, GA: glomerular amyloidosis, MGA: minor glomerular abnormality, FSGS: focal segmental glomerulosclerosis

Conclusion

The study revealed that the altered expression and localization of nephrin, podocin, α-actinin-4, α3-integrin and WT1 with podocyte injury in canine glomerular diseases. Our conclusion is summarized as follows.

1. Nephrin, podocin, α-actinin-4 and α3-integrin express in normal canine glomeruli. The normal staining patterns of these molecules are linear covered with glomerular capillary walls, and expression of nephrin and podocin are limited to glomeruli in the kidney.
2. In various canine glomerular diseases, podocyte injury accompanied by decreased expression of nephrin, podocin, α-actinin-4 and α3-integrin is induced. Especially nephrin might be useful marker to evaluate podocyte and glomerular injury in specimens.
3. Localization of nephrin and α-actinin-4 are characteristically shifted to granular and aggregated pattern, respectively. The alteration may reflect on the pathogenesis of podocyte.
4. Molecular changes reflect to disease severity or features in MN, MGA, FSGS of the dogs.
5. WT1 positive podocyte is reduced in diseased glomeruli and WT1 index significantly correlated with molecular expression scores. Thus, podocyte loss might deeply associate with progression of glomerular injury in dogs. Also, expression of WT1 is reduced in injured podocyte.
6. Expression score of nephrin and podocin significantly negatively-correlated with UPC. The molecules might be urinary marker for glomerular injury.
7. In canine glomerular diseases, nephrin mRNA expression is increased.

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Japanese Abstract

足細胞は腎糸球体の表面を覆う上皮細胞で、基底膜、内皮細胞と共に糸球体血管係蹄における選択的濾過機能に重要な役割を果たしている。特に高分子蛋白質に対するバリアとして機能しており、糸球体疾患の臨床徴候であるタンパク尿の発現には足細胞傷害が密接に関連している。近年、ヒトでは先天性ネフローゼ症候群の原因分子として同定された nephrin をはじめとし、足細胞に発現する様々な分子（足細胞関連分子）がその機能ならびに足突起、スリット膜などの特殊な形態を維持していることが明らかになってきた。すなわち、正常な足細胞の機能と構造はスリット膜を中心とした足細胞関連分子の相互作用により維持されていることになるが、何らかの要因により分子発現が変化すると形態学的な足突起のアクチン細胞骨格の改変や糸球体基底膜との接着能の低下などにつながり、蛋白が漏出する。このため現在医学領域では、足細胞を標的とした治療や、尿中に排泄された足細胞を糸球体傷害マーカーとして用いるための研究も進められている。

獣医学領域では、タンパク尿に伴ない足細胞傷害が電顕的に観察されることはわかっていたが、分子の変化についてはほとんど研究されていない。イヌの腎疾患では糸球体疾患が多く、ヒトと同様に足細胞および糸球体傷害の早期診断、治療が臨床的に重要であると考えられる。また、ヒトの糸球体疾患とイヌのそれには類似点が多く、比較医学的に興味深い。本研究の目的はイヌの糸球体疾患において足細胞の傷害とその関連分子の発現変化を解析し、病態との関連を明らかにすることである。

第一章　正常イヌ糸球体における足細胞関連分子の発現と局在

従来、イヌにおける足細胞傷害の評価には超微形態学的観察が行われてきたが、機能的評価には足細胞関連分子の発現解析が重要である。本章ではその基盤として、これまでに明らかでなかった正常なイヌ糸球体における足細胞関連分子の発現と局在を調べた。【材料と方法】正常なビーグル犬 5 頭から採材した腎皮質組織と、皮質組織よりシービング法により単離した単離糸球体を用いた。ウエスタンブロット法（WB）と蛍光抗体法（IF）にてタンパク発現と局在を、RT-PCR にて遺伝子発現を検索し、透過型電顕で足細胞の構造の観察とスリット膜の長さの計測をおこなった。検索した分子はスリット膜に発現する nephrin、スリット膜基部細胞内に局在する podocin、足突起のアクチン細胞骨格に関連するα-actinin-4、足突起と糸球体基底膜との接着に関与するα3-integrin である。抗体は作製した抗イヌ nephrin ポリクローナル抗体及び市販抗体を用い、プライマーは各分子の予測配列より設計した。【結果と考察】IF において nephrin と podocin は糸球体表面を覆うび漫性線状の足細胞パターンを示し、α-actinin-4 及びα3-integrin は足細胞に加えメサンギウム細胞にも発現していた。また、WB, RT-PCR により、予測された分子量のバンドが得られた。これらの結果よりイヌにおける 4 分子の発現と局在がヒト、ラット、マウスと同様であることが明らかになっ

た。また、電顕観察によりイヌのスリット膜の長さは約 379 ± 24.4 Å で、マウスとほぼ同等であった。(Kobayashi, R., *et al. J. Comp. Pathol.* 145:220-225. 2011.)

第二章　イヌの糸球体疾患における足細胞関連分子の発現および局在変化

第一章において明らかにした正常イヌ腎糸球体における足細胞関連分子の発現と局在を基に、イヌの糸球体疾患における足細胞傷害と関連分子の発現変化を明らかにし、タンパク尿、糸球体傷害との関連を明らかにするため、腎生検症例を用いた解析をおこなった。【材料と方法】持続的タンパク尿を伴う糸球体疾患（16 例）および非糸球体疾患（5 例）、計 21 例の Tru-cut 腎生検ならびに 4 頭の正常ビーグル犬腎組織を材料として用いた。糸球体疾患は膜性増殖性糸球体腎炎（MPGN）、メサンギウム増殖性糸球体腎炎（MePGN）、膜性腎症（MN）、糸球体アミロイドーシス（GA）、微小糸球体病変（MGA）、巣状分節性糸球体硬化症（FSGS）を含み、これらをさらに免疫介在性糸球体疾患（IMGD, n=11）、非免疫介在性糸球体疾患（NIMGD, n=5）に分け、非糸球体疾患（non-GD, n=5）と正常ビーグル犬（Normal, n=4）を対照群として解析をおこなった。また透過型電顕を用い足細胞の観察をおこなった。IF では nephrin, podocin, α-actinin-4, α3-integrin の発現・局在変化を観察し、半定量的にスコア化し、グループ間での比較とタンパク尿（Urinary protein/creatinine ratio）との相関関係を解析した。また、足細胞の核に特異的に発現する WT1 の免疫染色により、各症例の糸球体における WT1 陽性足細胞数を計測した。さらに、蛋白レベルで最も発現低下の著しかった nephrin の遺伝子発現を定量的に解析するため、laser microdissection（LMD）法にて凍結切片より糸球体を各症例 100 個切り抜き、real-time RT-PCR により解析した。【結果】電顕観察では、検索した糸球体疾患全例において様々な程度の足細胞傷害（細胞体の腫大、足突起の扁平化、スリット膜の消失、細胞表面微絨毛の増加）が確認された。一方、非糸球体疾患では足細胞はほぼ正常であった。IF においては、足細胞傷害に伴う nephrin の顆粒状化、α-actinin-4 の染色パターンの変化がみられた。また、スコアリングによる比較では糸球体疾患のいずれにおいても nephrin が最も高度に発現が低下していた。NIMGD では IMGD と比較し 4 分子ともに発現低下が高度であり、NIMGD と IMGD における nephrin、podocin の発現は non-GD, Normal と比較し有意に低下していた。タンパク尿と発現スコアの相関解析では、特に nephrin, podocin で高い負の相関を示した。また、疾患ごとの差を見てみると、最も症例数の多い MN の比較では形態学的変化の重篤なものほど分子の発現低下は著しく、MGA と FSGS ではそれぞれの病態を反映した局在の変化を示していた。WT1 陽性足細胞数は NIMGD で最も高度に減少し、IMGD と共に Normal に比較して有意に減少していた。また、一部の糸球体疾患では傷害足細胞において WT1 発現の低下がみられた。nephrin mRNA の定量的解析では、疾患糸球体では正常糸球体の約 26.2 倍の発現上昇がみられた。【考察】本章の研究により、イヌの糸球体疾患では形態学的に足細胞傷害が明らかで、同時に足細胞関連分子の発現低下、足細胞数の減少が起こることが明らかになった。ヒト、実験動

物において nephrin は podocin と共にスリット膜の機能に密接に関わり、発現低下により係蹄からの蛋白漏出が誘発されることが証明されている。本研究において、様々なイヌの蛋白漏出性糸球体疾患においてスリット膜の消失と同時に nephrin の顕著な発現低下が観察されたことから、nephrin がイヌの糸球体疾患の病態に深く関与すること、足細胞傷害の感受性の高いマーカーになることが示唆された。WT1 陽性足細胞数の計測では、各糸球体疾患において足細胞数の減少が明らかになった。さらに WT1 index は各分子の発現スコアと高い相関を示した。足細胞は増殖能に乏しく、数の減少は不可逆的な糸球体硬化に至る重要な要因であることから、イヌにおいても糸球体疾患の進行に足細胞の脱落・減数が関与していることが示された。また、近年ラットなどの実験動物で傷害足細胞における WT1 の発現低下が報告されており、WT1 は腎発生期だけでなく転写調節因子として正常な足細胞の機能の維持にも役割を担っていると考えられている。イヌの足細胞における WT1 の役割は不明であるが、正常糸球体では成熟足細胞に限局して強発現している WT1 が、傷害により低下することが明らかとなった。タンパク尿と関連分子の発現スコアの相関解析の結果からは、nephrin と podocin が係蹄からの蛋白漏出に重要な役割を果たすこと、さらに尿中の早期診断マーカーとして有用である可能性が示唆された。傷害糸球体における nephrin 遺伝子発現は顕著に上昇していた。ヒトの臨床検体を用いた研究では、糸球体傷害に伴う nephrin mRNA 発現上昇、低下いずれの報告もあるが、本研究における発現上昇の意義は不明であった。NIMGD における足細胞傷害は、いずれの解析においても IMGD より高度であった。NIMGD に含まれる MCD および FSGS は、ヒトにおいては一次的な足細胞傷害が病態に深く関わるとされる疾患である。イヌにおける MCD, FSGS の報告は非常に少なく、病態などの詳細は未だ不明であるが、本研究においてヒトと同様に高度な足細胞傷害が示された。

以上、イヌの足細胞関連分子に関する基礎的研究と、主に生検材料を用いた臨床例の研究により、イヌの糸球体疾患においてその治療や慢性腎不全への進行阻止には足細胞を標的とした戦略が重要であり、足細胞及び関連分子の解析は生検組織における病態把握に有用であることが示された。

Acknowledgements

The author wishes to express his sincere appreciation and gratitude to Dr. Kinji Shirota, Professor of Veterinary Pathology, Azabu University, for his valuable guidance and encouragement for 4 years, as well as critical review of this thesis, as a supervisor.

The author thanks Dr. Junichi Kamiie, Veterinary Pathology, Azabu University, for his valuable advices and technical teaching in all experiments. The author also sincerely appreciates Dr. Kikumi Ogihara, Laboratory of Environmental Pathology, Azabu University, for her valuable teaching on manipulation and techniques about transmission electron microscopy. The author sincerely expresses her thanks to Dr. Yumi Une, Professor of Veterinary Pathology and his thanks to Hiroo Madarame, Professor of Veterinary Teaching Hospital, Azabu University, for their valuable guidance as veterinary pathologist.

This study was supported by many veterinary clinicians and the author especially thankful to Dr. Toshifumi Watanabe and Dr. Mika Mishina, Department of Nephrology and Urology, Teaching Animal Hospital, Azabu University, Dr. Yoko Fujii, Department of Surgery 1, School of Veterinary Medicine, Azabu University, Dr. Masami Uechi and Dr. Shigeki Yamano, Veterinary Cardiovascular Medicine and Surgery Unit, Department of Veterinary Medicine, College of Bioresource Sciences, Nihon University.

Thanks are also due to all graduate and undergraduate students of the author's laboratory, especially Dr. Kyohei Yasuno, for his correct advice and kind support. I would like to express gratitude to my colleague in graduate school for their friendly support. Finally, the author expresses special thanks to my parents for their careful support in daily life.