

The pathological study on membranous nephropathy in  
dogs with special reference to phospholipase A2 receptor  
in the podocytes

イヌの膜性腎症の病理学的研究：特に足細胞におけ  
る Phospholipase A2 receptor を中心として

2016年2月24日

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## **Abstract**

### **Introduction**

Membranous nephropathy (MN) is a major immune complex-mediated glomerulonephritis (ICGN) and can be grouped into idiopathic (primary) type and secondary type of MN in human.

Phospholipase A2 receptor (PLA<sub>2</sub>R) is expressed in the glomerular podocytes of human kidney. Anti-PLA<sub>2</sub>R autoantibodies were discovered in the majority of patients with idiopathic MN. Also, enhanced glomerular expression of PLA<sub>2</sub>R is reported in most patients of the disease. Therefore, PLA<sub>2</sub>R is suggested to be causative autoantigen of human idiopathic MN. On the other hands, PLA<sub>2</sub>R is expressed minimal or absent in murine and rat podocytes. Because a suitable animal model of PLA<sub>2</sub>R -associated glomerular disease has not been established, the function of this receptor in podocytes, and whether the circulating autoantibodies actually bind PLA<sub>2</sub>R on podocytes, remains unclear.

In dogs, MN is a major spontaneous glomerular disease and its histological and clinical characteristics are analogous to idiopathic MN in humans. However, there are no reports describing the expression of PLA<sub>2</sub>R in canine podocytes, and the pathogenesis of canine MN has not been investigated. In this study, the author

hypothesized that some case of canine MN might be induced by the autoimmune mechanism in common with human idiopathic MN.

## **Chapter 1**

Human MN can be grouped into idiopathic (primary) type and secondary type. In electron microscopy, the dense deposits are usually limited in subepithelial area in idiopathic MN, while these are observed also in subendothelial and mesangial area in addition to subepithelial area in secondary MN. In this chapter, the author investigated the localization of immune deposits in 8 cases of MN dogs by electron microscopy. In 4 cases, the deposits were limitedly observed in subepithelial area. In another 4 cases, subendothelial and mesangial deposition was also observed. In this study, the author demonstrated some cases of canine MN exhibiting restrictive subepithelial localization of dense deposits, which is consistent with characteristic finding in human idiopathic MN.

## **Chapter 2**

In this chapter the author performed gene cloning and motif analysis for canine PLA<sub>2</sub>R obtained from normal glomeruli and investigated the expression and detailed localization of PLA<sub>2</sub>R in normal glomeruli and a MN cases dogs by

immunofluorescence (IF). The author provided fundamental information that canine PLA<sub>2</sub>R obtained has high homology and common extracellular structures with human PLA<sub>2</sub>R. In addition to expression of PLA<sub>2</sub>R in normal glomeruli, the author demonstrated increased expression of PLA<sub>2</sub>R in some cases of MN dog showing idiopathic deposition pattern in chapter 1. Similar altered expression of PLA<sub>2</sub>R was reported in human idiopathic MN, in association with the presence of serum anti-PLA<sub>2</sub>R auto-antibody. The results in this chapter may support the author's hypothesis that some canine MN cases may be autoimmune mechanism associated with PLA<sub>2</sub>R as an autoantigen expressed in podocyte.

### **Chapter 3**

In the previous chapter, the author revealed glomerular expression of PLA<sub>2</sub>R in dogs. The object in this chapter is to establish the method of podocyte primary culture for dogs and to confirm the expression of PLA<sub>2</sub>R in the canine cultured podocyte. The author succeeded in effective isolation of glomeruli from canine kidney by modifying the method for rat. Canine cultured podocytes in the present study distinctly expressed PLA<sub>2</sub>R in addition to several podocyte-specific markers, including nephrin, podocin and synaptopodin. The podocyte expression of PLA<sub>2</sub>R was confirmed by reverse transcriptase polymerase chain reaction, western blotting and IF in dogs. The *in-vitro*

experiment provided from this method may contribute to progression of investigation for canine glomerular disease including MN.

## **Conclusion**

In this study, the author revealed new findings on ultrastructural feature of canine MN and PLA<sub>2</sub>R expression in glomerular podocytes of dogs as follows.

1. Canine MN showed two different deposition patterns of dense deposit in the glomeruli resembling human idiopathic or secondary MN. This may suggest the presence of different pathogenesis in canine MN as suspected in human MN.
2. Canine PLA<sub>2</sub>R obtained from the glomeruli has high homology and common extracellular structures with human PLA<sub>2</sub>R.
3. Canine PLA<sub>2</sub>R is expressed in podocyte and distributed on the foot process in tissue sections. This is important finding suggesting the possibility of dogs as an animal model of human idiopathic MN. Because PLA<sub>2</sub>R is not expressed in the podocytes of rodents, rabbits and no experimental or spontaneous animal models for idiopathic MN have been established, so far.

4. The increased expression of PLA<sub>2</sub>R, which is feature of human idiopathic MN associated with PLA<sub>2</sub>R, demonstrated in some of MN dog cases with idiopathic ultrastructural pattern of dense deposit. These results may support the author's hypothesis that some canine MN cases are autoimmune disease associated with PLA<sub>2</sub>R as an autoantigen expressed in podocyte.

5. The primary culture method for canine podocyte expressed several podocyte specific proteins was established. The *in vitro* experiment provided from this method may contribute to progression of investigation for canine glomerular disease including MN.

These results may support the author's hypothesis that some canine MN cases are autoimmune disease associated with PLA<sub>2</sub>R as an autoantigen expressed in podocyte. In addition, the author established the primary culture method for canine podocyte. The *in vitro* experiment provided from this method may contribute to progression of investigation for canine glomerular disease including MN.

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### Chapter 3

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## General Introduction

Membranous nephropathy (MN) is a major immune complex-mediated glomerulonephritis (ICGN) in both dogs and human, and characterized by the subepithelial formation of immune deposits containing immunoglobulin (Ig) G and complement components causing subsequent podocyte injury and glomerular basement membrane (GBM) changes in the glomeruli [8, 25].

In human medicine, MN can be grouped into idiopathic (primary) type and secondary type of MN by presence of underlying disease and histopathological feature for each type. In clinical strategy, differentiation of these types is very important, because the patients with secondary MN require detection and therapy of underlying disease, such as infection (hepatitis B), systemic autoimmune disease, medications or chemical exposures (NSAIDs, mercury), and certain malignancies [3, 33]. In pathogenesis, the different mechanisms of the subepithelial formation of immune deposits are assumed in each type of MN. The evidences in many recent reports suggested *in situ* immune complex (IC) formation in human idiopathic MN. A primary form of MN was reported in neonates born from mothers with defect of neutral endopeptidase (NEP) localized on podocytes cell membrane and having anti-NEP antibodies sensitized during previous pregnancies [3, 33]. Also, a well-known rat model of idiopathic MN, Heymann's



nephritis was first described in the rats injected with kidney extracts resulting in nephrotic syndrome in 1959 [19]. Thereafter, megalin was identified as a target protein in Heymann's nephritis which is expressed in both the brush border of proximal tubular cells and podocyte foot processes [13]. At the present day, Heymann's nephritis can be induced by immunization with rat proximal tubule brush borders fraction termed Fx1A (active Heymann's nephritis) or injection of anti- Fx1A antibody produced in other animals (passive Heymann's nephritis) [27]. Despite of these evidences, the podocyte antigens in most of idiopathic MN had not been identified. Recently, the expression of phospholipase A2 receptor (PLA<sub>2</sub>R) on glomerular podocytes in the human kidney was demonstrated and anti-PLA<sub>2</sub>R autoantibodies were discovered in the majority of patients with idiopathic MN [2, 18, 36, 39].

Phospholipase A2 (PLA<sub>2</sub>) is a family of lipolytic enzymes and hydrolyzes the sn-2 position of phospholipids to generate the corresponding fatty acid and lysophospholipid [11, 16]. Also, PLA<sub>2</sub> relate to generate various types of biologically active lipids, including prostaglandins hydroxy fatty acids, leukotrienes, thromboxanes, and platelet-activating factor [11, 16]. Secretory PLA<sub>2</sub> (sPLA<sub>2</sub>) are a group of low molecular weight (~14 kDa) and secreted from cells. To date, there are 10 type of mammalian sPLA<sub>2</sub> classified as groups IB, IIA, IIC, IID, IIE, IIF, III, V, X and XII. The sPLA<sub>2</sub>-IB is a

most major enzyme and provided the fundamental information on structure and mechanism. sPLA<sub>2</sub>-IB, as a ligands, induces various biological processes such as cell proliferation, cell migration, hormone release, and eicosanoid production in various cells and tissues [9, 15, 16] mediated by PLA<sub>2</sub>R, a type I transmembrane glycoprotein related to the C-type animal lectin family receptor. Studies using PLA<sub>2</sub>R-knockout mice have demonstrated a critical role of PLA<sub>2</sub>R in the regulation of the development of endotoxic shock [14].

Subsequent to the discovery of anti-PLA<sub>2</sub>R autoantibodies, PLA<sub>2</sub>R was highlighted as a causative antigen located on podocytes in human idiopathic MN and many clinical studies have been reported. For example, the prevalence of elevated anti-PLA<sub>2</sub>R autoantibody levels was reported in several countries [2, 18, 36, 39] and autoantibody titer was found to be strongly associated with disease activity and proteinuria in idiopathic MN patients [21]. In addition, the enhanced expression of PLA<sub>2</sub>R was histologically observed in the glomeruli of idiopathic MN patient and the immunostaining for PLA<sub>2</sub>R in biopsy samples is thought to be alternative method for diagnosis of idiopathic MN associating with anti- PLA<sub>2</sub>R antibody [23, 40, 43].

However, basic research to elucidate the pathogenesis of PLA<sub>2</sub>R associated MN has not been expanded, while these are many clinical studies as described above. In general,

the establishment of a suitable animal model and experimental reproduction of the disease is critical to understanding the pathogenesis of autoimmune diseases. However, the expression of PLA<sub>2</sub>R in the glomerular podocytes was found to be minimal or absent in mice and rats [4, 6, 37, 43] and to date, a suitable animal model of PLA<sub>2</sub>R - associated glomerular disease has not been established. Due to the lack of an animal model, the function of this receptor in podocytes, and whether the circulating autoantibodies actually bind PLA<sub>2</sub>R on podocytes, remains unclear.

In dogs, glomerular diseases are thought to be the common cause of chronic renal failure [32]. And it has been reported that canine glomerular diseases share many of the histological characteristics seen in their human counterparts [7, 8, 25, 31]. To develop a comprehensive understanding of canine glomerular disease, the World Small Animal Veterinary Association–Renal Standardization Study Group (WSAV-ARSSG) was conceived at Netherland’s Utrecht University in January 2005. They makes a diagnosis based on the World Health Organization classification system for human glomerular disease by routinely using standardized light microscopy (LM), immunofluorescence (IF), and transmission electron microscopy (TEM) methods [7, 8, 25, 31]. Canine glomerular diseases are divided into three broad categories including amyloidosis, ICGN, and non-ICGN. Canine MN is a representative ICGN as well as

membranoproliferative glomerulonephritis (MPGN) and accounts for about 30% of canine glomerular diseases [7, 8, 25, 31] and has following features; diffuse thickening of the glomerular walls without endocapillary hypercellularity (LM), granular deposition of Igs and compliments along the glomerular walls (IF), subepithelial depositions of dense deposits (TEM) and severe proteinuria (clinical feature).

As described above, because canine MN has high incidence in canine glomerular diseases and similarities to human MN, the elucidation of the pathogenesis canine MN could contribute not only to veterinary medicine but also to human medicine. However, there are no reports regarding the expression of PLA<sub>2</sub>R in canine podocytes, and the pathogenesis of canine MN has not been investigated.

In veterinary medicine, several researchers have investigated canine glomeruli using histologic sections. They showed normal expression of some podocyte specific markers and altered expression in various glomerular diseases in common with human and experimental animals [24, 30]. Podocyte cell culture derived from human, mice and rat glomeruli has been used in many studies aimed for elucidating their physiological and pathophysiological characteristics. However, there is no *in vitro* research using canine cultured glomerular podocytes because of the lack of culture methods for canine podocytes.

In this study, the author hypothesized that some cases of canine MN might be induced by the autoimmune pathogenesis in common with human idiopathic MN. Therefore, in chapter 1, the author demonstrated ultrastructural features in canine MN focusing on distribution of dense deposits in the glomeruli which suggested the mechanism of the subepithelial formation of immune deposits. In chapter 2, the author revealed fundamental information of canine PLA<sub>2</sub>R and shown normal expression of PLA<sub>2</sub>R in the glomeruli and also increased expression of that some canine MN cases exhibited ultrastructural feature of the glomerulus consistent with human idiopathic MN associating with PLA<sub>2</sub>R. In chapter 3, the author showed the method of primary cultured for canine podocytes and the distinct expression of PLA<sub>2</sub>R in canine cultured podocyte to confirm *in vivo* study on the expression of PLA<sub>2</sub>R in the normal and affected glomeruli.

## **Chapter 1**

Ultrastructural features of canine membranous nephropathy with special reference to distribution of dense deposits in the glomeruli

## **Introduction**

In dogs, MN is a major spontaneous glomerular disease and its histological and clinical characteristics are analogous to idiopathic MN in humans [7, 8, 25, 31].

In human medicine, MN can be grouped into idiopathic (primary) type and secondary type of MN. This distinction is thought to be important because clinical behavior and therapeutic strategy are different in both types. The most important point to confirm idiopathic MN is lack of features suggestive of underlying disease leading secondary MN, such as infection (hepatitis B), systemic autoimmune disease, medications or chemical exposures (NSAIDs, mercury), and certain malignancies and so on [3, 33]. However, the light microscopic feature of idiopathic and secondary MN is similar or even identical and the clinical distinction is difficult when the underlying disease is unclear. On the other hands, some pathological changes suggestive of idiopathic or secondary MN are often observed. For instance, IgG subclass containing in IC is mainly IgG4 in idiopathic MN [3, 33]. In ultrastructural feature, the dense deposits are usually limited in subepithelial area in idiopathic MN, while these are observed also in subendothelial and mesangial area in addition to subepithelial area in secondary MN [33].

On the other hands, the difference of deposition patterns associated with pathogenesis has not been described in canine MN. Therefore, in this chapter, the author investigated the localization of immune deposits in MN dogs.



## **Materials and Methods**

### *Renal Biopsies cases*

The clinical data and pathologic diagnosis of dogs employed in the present study is shown in Table 1. The glomerular diseases cases using in the study include eight MN (No. 1 to 8), two MPGN (No. 9 and 10) and two non-ICGN (No. 11 and 12) cases.

These biopsies were surgically carried out using 16 or 18-gauge Tru-cut-type needles by opening the abdominal cavity of the dogs. 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 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 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 LM. The World Health Organization's classifications of human glomerular diseases were consulted [33].

### Immunofluorescence (IF)

Immune deposition in glomeruli were evidenced by direct IF using antibodies of anti-dog IgG fluorescein isothiocyanate (FITC) conjugated (Cappel, Aurora, Ohio, 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.

### Ultrastructural analysis

To observe deposition of IC, a small piece from biopsy tissue was cut into 1-mm<sup>3</sup> cubes, fixed in 2.5% glutaraldehyde and post-fixed in 1% OsO<sub>4</sub> for 2 hr. 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. Two or more of glomerulus were observed in each case. The staging (stage I-IV) were according to human staging system for MN [33]. The deposition patterns of dense deposit were grouped by “restrictive subepithelial deposition” or “subepithelial deposition accompanied by subendothelial and/or mesangial depositions”

## **Results**

### *Light and fluorescence microscopic findings of membranous nephropathy*

Light microscopically, this disease is characterized by diffuse thickening of the glomerular walls without cellular proliferation. Consistent with this thickening, granular deposition of Igs and complement is detected by IF. And clinically, severe proteinuria was observed in most of dogs (Table 1).

### *Deposition pattern of immune complexes in Canine MN*

The deposition pattern of each case was shown in Table 1. In 4 cases, from No.1 to No. 4, the deposits were limitedly observed in subepithelial area (Fig. 1). In another 4 cases, from No.5 to No.8, subendothelial and mesangial deposition was also observed.

## Discussion

In this chapter, the author demonstrated some cases of canine MN exhibiting restrictive subepithelial localization of dense deposits, which is a characteristic finding in human idiopathic MN.

Similar deposition pattern was observed in human neonatal MN. This disease is reported in neonates born from mothers with defect of NEP localized on podocytes cell membrane and having anti-NEP antibodies sensitized during previous pregnancies [10]. Also, a representative experimental model of idiopathic MN, Heymann's nephritis was caused by binding of circling antibodies to an antigen, called as megalin, expressed on podocyte cell membrane [19, 27]. Recently, autoantibody against PLA<sub>2</sub>R, a protein on podocyte cell surface, was discovered in the majority of patients with idiopathic MN [2], thereafter PLA<sub>2</sub>R was highlighted as a candidate antigen composing IC deposited in the glomeruli of idiopathic MN.

In this manner, many evidences in previous reports suggested that this deposition pattern indicated autoimmune mechanisms against antigens on podocyte cell membrane causing *in situ* formation of IC, although the glomerular deposition of circulating antigen-antibody complex in serum is considered as mechanisms of IC deposition in the secondary MN glomeruli.

Therefore, canine MN cases showing idiopathic deposition pattern observed in this study may have autoimmune pathogenesis in common with human idiopathic MN associated with autoantigens on podocyte like PLA<sub>2</sub>R.

## Figures and Tables

Table 1. The clinical data, pathological diagnosis of dogs and the results of analysis in this study.

No.	Pthological diagnosis	Breeds	Sex/Age (years)	Urinary Protein	Immunocomplexes		Enhanced expression of
					Stage	Deposition pattern	
1		Miniature dachshund	Cas/6	9.3	I		NA
2		Boston terrier	Sp/10	4.6	III-IV	Subepithelial deposition	Yes
3		Miniature dachshund	F/10	2.8	I		
4	MN	Yorkshire terrier	F/8	1.4	II	Subepithelial +	NA
5		Papillon	Cas/12	2.2	II-III		
6		Lhasa apso	Sp/12	3+(strip)	I	Subendothelial and mesangial deposition	No
7		Yorkshire terrier	Sp/8	5.1	III-IV		
8		French bulldog	M/10	12.64	III	Subepithelial, Subendothelial and mesangial region	No
9	MPGN	Yorkshire terrier	F/5	12.84			
10		Toy poodle	F/5	3.5			
11	NIMGN	Italian greyhound	Sp/6	9.5		Non	No
12		Shetland Sheepdog	M/8	7.66			

M: Male, F: Female, Cas: Castrated, Sp: Spayed, Urinary Protein: Urinary protein/creatinin ratio or reagent strip, MN: membranous nephropathy, MPGN: membranoproliferative glomerulonehritis, NIMGN: Non-immune-mediated glomerulonehropathy, NA: not available

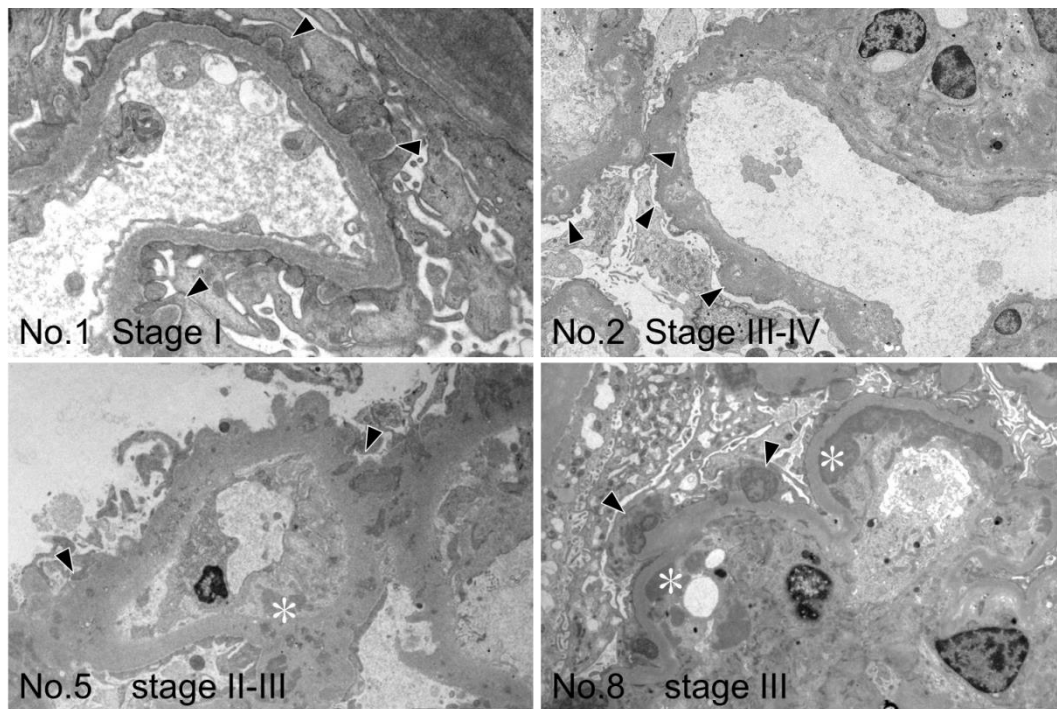


Fig. 1. Transmission electron microscopy of the glomerulus in canine MN.

The dense deposits were limitedly observed in subepithelial area along capillary walls in upper 2 cases (No. 1 and No. 2). The dense deposits were also observed in subendothelial and mesangial area in lower two. Arrowheads: dens deposit in subepithelial area, Asterisk: dens deposits in subendothelial area. TEM.

## **Chapter 2**

Expression of phospholipase A2 receptor in normal glomerulus  
and membranous nephropathy in dogs



## Introduction

In chapter 1, the author demonstrated that some canine MN cases have characteristic ultrastructural features seen in human idiopathic MN. Therefore, in this chapter the author focused on a new candidate autoantigen, PLA<sub>2</sub>R, probably associated with human idiopathic MN.

PLA<sub>2</sub>R is a type I transmembrane glycoprotein related to the C-type animal lectin family [16] and is reported to mediate various biological processes, such as cell proliferation, cell migration, hormone release, and eicosanoid production via s PLA<sub>2</sub> (ligand of PLA<sub>2</sub>R) stimulation in various cells and tissues [9, 15] .

Recently, the expression of PLA<sub>2</sub>R on glomerular podocytes in the human kidney was demonstrated and anti-PLA<sub>2</sub>R autoantibodies were discovered in the majority of patients with idiopathic MN [2]. Subsequent to this report, PLA<sub>2</sub>R was highlighted as a causative antigen located on podocytes in human idiopathic MN and many clinical studies have been reported. For example, the prevalence of elevated anti-PLA<sub>2</sub>R autoantibody levels was reported in several countries [2, 18, 34, 36, 39] and autoantibody titer was found to be strongly associated with disease activity and proteinuria in idiopathic MN patients [21]. In addition, the enhanced expression of PLA<sub>2</sub>R was histologically observed in glomeruli of idiopathic MN patient and the

immunostaining for PLA<sub>2</sub>R in biopsy samples is thought to be alternative method for diagnosis of idiopathic MN associated with anti- PLA<sub>2</sub>R antibody [23, 40, 43].

The expression of PLA<sub>2</sub>R on glomerular podocytes has been reported in human kidneys, while its expression was found to be minimal or absent in mice and rats [4, 6, 21, 37]. To date, a suitable animal model for human idiopathic MN associated with autoantibody against PLA<sub>2</sub>R has not been established. Due to the lack of an animal model, the function of this receptor in podocytes, and whether the circulating autoantibodies actually bind PLA<sub>2</sub>R on podocytes, remains unclear. Therefore, the identification of a suitable animal model is critical to understanding the pathogenesis of human idiopathic MN.

In dogs, there are no reports describing the expression of PLA<sub>2</sub>R in the normal glomeruli, and pathogenesis of MN has not been investigated despite its high incidence. Here, we describe the expression and detailed localization of PLA<sub>2</sub>R in normal canine glomeruli and altered expression of PLA<sub>2</sub>R in dog MN cases.

## **Materials and Methods**

### *Animals*

Various organs (heart, lung, liver, kidney, spleen, stomach, pancreas, small intestine, colon, uterus, ovary, testis, brain, lymph node, renal cortex, and renal medulla) were obtained from 12 month-old male and female beagle dogs for reverse transcriptase polymerase chain reaction (RT-PCR) to see the expression of PLA<sub>2</sub>R. All dogs were purchased from a laboratory animal breeding and supply company (Kitayama Labes Co, Ltd, Nagano, Japan) and confirmed to be healthy by physical examination. The animals were used for clinical education and euthanized in accordance with the guidelines approved by the Animal Research Committee of Azabu University (No. 100408-3)

### *Renal Biopsies cases*

Same renal Biopsies cases in this study except for three cases of MN (No. 1, 2 and 7) were used (summarized in Table 1 in chapter 1).

### *Immunofluorescence*

The primary and secondary antibodies using in this study were summarized in Table 2.

The renal tissues were embedded in OCT compound (Sakura Finetek, Tokyo, Japan) and snap-frozen. Cryosections (3  $\mu\text{m}$ ) were made and fixed in acetone for 20 min at -20  $^{\circ}\text{C}$  and washed with phosphate buffered saline (PBS; pH 7.2, 0.01 M). IF for PLA<sub>2</sub>R, cryosections were pretreated by citrate buffer (pH 6.0) at 90  $^{\circ}\text{C}$  for 10 min. Images were obtained using a FSX100 fluorescence microscope (OLYMPUS, Tokyo, Japan) or a Leica TCS SP5 confocal microscope (Leica Microsystems, Heidelberg, Germany).

#### *Isolation of glomeruli for Reverse Transcriptase Polymerase Chain Reaction*

The glomeruli were isolated from renal tissues using the sieving method as previously reported [42]. Briefly, renal cortical tissue was minced and sieved through 600, 250 and 125  $\mu\text{m}$  pore-size stainless-steel meshes with PBS. Finally, the glomeruli were collected on a 75  $\mu\text{m}$  mesh and placed into a low protein binding tube (SUMILON Proteosave; Sumitomo Bakelite, Tokyo, Japan).

#### *Reverse Transcriptase Polymerase Chain Reaction*

Total RNA from the isolated glomeruli and various organs was extracted using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, California). First-strand complementary DNA (cDNA) libraries were obtained from the total RNA using the Superscript III first-strand synthesis system (Invitrogen).

Table 3 summarizes the primer sequences used in this study. Amplification of the full length PLA<sub>2</sub>R sequence was accomplished using two forward primers and a reverse primer as follows: forward primer 1 (F1) is specific for the 5' un-translated region (UTR) of feline PLA<sub>2</sub>R, which has a high degree of homology among mammals, forward primer 2 (F2) is specific for the 5' terminal coding sequence (CDS) of canine PLA<sub>2</sub>R, and the reverse primer is specific for 3' terminal region of the predicted mRNA sequence of canine PLA<sub>2</sub>R.

PCR reactions using KOD FX Neo (Toyobo, Osaka, Japan) were performed as follows: denaturation at 94 °C for 2 min; 30 cycles (glyceraldehyde-3-phosphate dehydrogenase ; GAPDH) or 35 cycles (partial and full length PLA<sub>2</sub>R) of denaturation at 98 °C for 10s, annealing at 64 °C for 30s, and extension at 68 °C for 30s (partial PLA<sub>2</sub>R and GAPDH ) or 150s (full length PLA<sub>2</sub>R); and a final extension step at 68 °C for 30s.

### Cloning and sequencing for full-length canine PLA<sub>2</sub>R

The full-length canine PLA<sub>2</sub>R amplified from isolated glomeruli was inserted into the pTA2 vector according to the TArget Clone™ -Plus- protocol (Toyobo), and sequencing was performed at the Dragon Genomics Center (TAKARA BIO INC., Otsu, Japan). The forward primers utilized in sequencing PLA<sub>2</sub>R are summarized in Table 4. Alignment and motif analysis of the amino-acid sequence were performed using BLAST (Basic Local Alignment Search Tool) in NCBI.

### In situ hybridization

Small piece of the canine renal tissue were fixed in 10% neutral buffered formalin and embedded in paraffin wax. *In situ* hybridization (ISH) was performed on the paraffin sections (4 µm) using the QuantiGene® ViewRNA ISH Tissue Assay (Affymetrix, Santa Clara, CA, U.S.A.) according to the manufacturer's protocol. Gene-specific probe sets for canine PLA<sub>2</sub>R mRNA consisting of an average of 20 different probe pairs were custom designed based on the full length PLA<sub>2</sub>R sequence determined in this study, and synthesized by Affymetrix. The signal for PLA<sub>2</sub>R mRNA was visualized with fast red

and examined using differential interference contrast microscopy. An ISH reaction solution without the probes was used as a negative control, according to Affymetrix's recommendations.

## Results

### Alignment and motif analysis of the canine PLA<sub>2</sub>R amino-acid sequence

Two different forward primers were employed to amplify the full-length sequence of canine PLA<sub>2</sub>R using RT-PCR. A single band was obtained using the F1 primer, which is specific for the 5' UTR of feline PLA<sub>2</sub>R, but not when using the F2 primer, which is specific for the predicted 5' terminal CDS of canine PLA<sub>2</sub>R in Genbank (Accession Number: XM\_545489) (Fig. 2A). The full-length CDS of canine PLA<sub>2</sub>R was determined from the amplicon and the sequence, except for primer-binding sites, was registered with Genbank (Accession Number: LC049956). The amino acid sequence of canine PLA<sub>2</sub>R determined in this study had 1463 amino acid residues, and 34 amino acid residues in the N-terminal was completely different from the predicted PLA<sub>2</sub>R sequence in the Genbank database (Fig. 2B), whereas the subsequent amino-acid sequence were consistent with the predicted PLA<sub>2</sub>R sequence, except for amino acid 1301. This single nucleotide substitution (C to T) causing missense mutation (S to F) is documented as a genetic variation reported in Genbank. Motif analysis demonstrated that canine PLA<sub>2</sub>R contains extracellular structures with an N-terminal ricin-type beta-trefoil (RICIN), a fibronectin II-like (FN2) domain, and eight C-type lectin-like (CLECT) domains in common with human PLA<sub>2</sub>R (Fig. 2C).



Table 5 shows the sequence comparison of PLA<sub>2</sub>R amino acids between dog and different animals including human (Accession Number: NP\_031392), cat (XP\_006935367), mouse (NP\_032893) and rat (NP\_001094307). Full sequence of canine PLA<sub>2</sub>R exhibited a higher degree of homology with cat and human sequence compared with mouse and rat. Among all domains, CLECT domain 4 had high degree of homology with that of all animals.

#### *Expression of PLA<sub>2</sub>R protein and mRNA in canine renal glomeruli*

The expression of PLA<sub>2</sub>R protein and mRNA in canine renal glomeruli was demonstrated using IF and ISH. In the IF experiments, the labeling of PLA<sub>2</sub>R exhibited a podocyte pattern, that is, diffuse staining in a linear pattern along the glomerular capillary wall (Fig. 3A). Consistent with this result, the labeling of PLA<sub>2</sub>R mRNA in the ISH experiments was restricted to the glomeruli (Fig. 3C).

#### *Localization of PLA<sub>2</sub>R protein in the glomeruli*

Double IF was performed to reveal the detailed localization of PLA<sub>2</sub>R protein. PLA<sub>2</sub>R staining colocalized with podocin along the glomerular capillary wall (Figs. 4A to F).

On the other hand, the labeling of PLA<sub>2</sub>R was distinct from vimentin and type IV

collagen, and was localized between vimentin (Figs. 5G to I) and type IV collagen (Figs. 5J to L).

*Protein expression of PLA<sub>2</sub>R in the cases of glomerulonephropathy*

In 2 MN cases (No. 3, 4) showing idiopathic deposition pattern as shown in chapter 1, increased staining for PLA<sub>2</sub>R was observed along the glomerular capillary walls (Fig. 5). However, this increased staining was not observed in other 3 MN cases with non-idiopathic deposition pattern (No. 6 to 8) and other 4 glomerular diseases.

## Discussion

In this study, we provide fundamental information on canine PLA<sub>2</sub>R, including the high structural similarity to human PLA<sub>2</sub>R, mRNA tissue distribution, and detailed localization of the protein on glomerulus. Double IF demonstrated colocalization of PLA<sub>2</sub>R and podocin in the canine glomeruli. This finding suggested that canine PLA<sub>2</sub>R is distributed on the foot process and podocyte cell surface.

The N-terminal sequence of the PLA<sub>2</sub>R determined in this study differs considerably from the predicted sequence in the two major sequence databases (NCBI and Ensemble). Incidentally, we could not detect any specific bands by RT-PCR using several additional forward primers specific for the 5' terminal of the predicted sequence. These results indicate that the sequence obtained in this study, using forward primers specific for a highly homologous mammalian UTR, is the correct canine PLA<sub>2</sub>R sequence from glomeruli.

The amino acid sequence of canine PLA<sub>2</sub>R had high homology with human and also cat which is one of the most popular companion animals and could be candidate of animal model for MN as dogs. However, there are no reports about PLA<sub>2</sub>R in cat and its fundamental information is unknown, for example it is expressed in the podocyte. In point of function, CLECT domains 3 to 5 are essential recognition regions for sPLA<sub>2</sub>-IB (a subtype of sPLA<sub>2</sub>). Especially, the conserved sequence of CLECT domain 4,

observed in this study, has been reported and suggested to play an important role for various function induced sPLA<sub>2</sub>-IB [20].

In addition to expression of PLA<sub>2</sub>R in normal glomeruli, the author demonstrated increased expression of PLA<sub>2</sub>R in some of MN dog cases showing idiopathic deposition pattern in chapter 1. Similar altered expression was reported in human idiopathic MN, in strongly association with the presence of serum anti-PLA<sub>2</sub>R auto-antibody [23, 40, 43]. At the present time, immunostaining for PLA<sub>2</sub>R is considered as a useful method for diagnosis of human idiopathic MN and can be alternative method for autoantibodies detection. These results may support the author's hypothesis that some of canine MN cases are autoimmune disease associated with PLA<sub>2</sub>R as an autoantigen expressed in podocyte.

However, so far, there are no reports to detect auto-antibody against PLA<sub>2</sub>R or other antigens in dogs. In human idiopathic MN, auto-antibodies to PLA<sub>2</sub>R in serums from patients only recognize the protein in the non-reduced state (intact disulfide bonds), and cannot be detected in serums from several dozen percent of idiopathic MN and almost all of secondary MN. In companion animals, no regard is given to pathogenesis of MN and differentiation between idiopathic and secondary types may not be concerned. Therefore, it is important future issues for certifying the presence of anti-

PLA<sub>2</sub>R auto-antibodies in dogs to conduct a large scale research on a large number of MN cases and to establish detection techniques for PLA<sub>2</sub>R auto-antibodies.

## Figures and Tables

Table 2. Primary antibodies used in immunofluorescence staining

Primary antibody for IF	Clone	Dilution	Reference or Source	Secondary antibody	Fixation for immunocytology
Goat PLA <sub>2</sub> R	goat polyclonal	1:100	Abcam, Cambridge, United Kingdom	Alexa Fluor 568-conjugated donkey anti-goat IgG (Invitrogen, Tokyo, Japan)	Acetone
Mouse PLA <sub>2</sub> R	CL0474	1:100	Atlas Antibodies AB, Stockholm, Sweden	Alexa Fluor 568-conjugated goat anti-mouse IgG (Invitrogen)	Methanol
Vimentin	V9	1:100	Dako, Glostrup, Denmark	Alexa Fluor 488-conjugated goat anti-mouse IgG (Invitrogen)	-
Type IV collagen	rabbit polyclonal	1:100	a gift from Dr. K. Arai, Tokyo University of Agriculture and Technology, Tokyo, Japan	Alexa Fluor 488-conjugated goat anti-rabbit IgG (Invitrogen)	-
Podocin	rabbit polyclonal	1:100	Sigma, St Louis, Missouri	Alexa Fluor 488-conjugated goat anti-rabbit IgG (Invitrogen)	Acetone
Canine nephrin	rabbit polyclonal	1:50	Kobayashi <i>et al.</i> 2012	Alexa Fluor 488-conjugated goat anti-rabbit IgG (Invitrogen)	Acetone
von Willebrand factor (vWF)	rabbit polyclonal	Prediluted	Dako, Glostrup, Denmark	Alexa Fluor 488-conjugated goat anti-rabbit IgG (Invitrogen)	Methanol
Ionized calcium binding adaptor molecule 1 (Iba-1)	rabbit polyclonal	1:200	Wako Pure Chemical Industries Ltd., Osaka, Japan	Alexa Fluor 488-conjugated goat anti-rabbit IgG (Invitrogen)	Methanol
Synaptopodin	goat polyclonal	1:100	Santa Cruz Biotechnology, Santa Cruz, California, USA	Alexa Fluor 488-conjugated rabbit anti-goat IgG (Invitrogen)	Acetone

Table 3. Primers used for RT-PCR used in this study

	Primer sequence (5'-3')	Sequence accession	Location
Full length PLA <sub>2</sub> R	F1: CGAGTCCAGTGGTTAGGGATG	XM_006935305 (Predicted feline PLA <sub>2</sub> R)	153-173
	F2: ATGCGTGCCAGAGAGCCAGGAG R: GCATTCTCTGAGTTCATTGGTAT GG	XM_545489 (Predicted canine PLA <sub>2</sub> R)	1-21 4495-4519
Partial PLA <sub>2</sub> R	F: ACAGGAGAAAACGGAGCAGG R: ACTCCCAAGAGCCAGCATTC	LC049956	1938-1957 2223-2242
Podocin	F: CAAAGTGCGGGTGATCGCT R: GGCAACCAAAGGAAGTGCAG	XM_547443.2	984-1002 1353-1372
Nephrin	F: TCGGGTAGCAGGAACTTGG R: AAGCCACCTCCCAGAAGAGT	XM_541685	3781-3800 4043-4062
Synaptopodin	F: ACCCCATGGACTCAACGAAG R: GGCCCAACGCTGGTTTCTG	XM_536465.5	223-242 581-599
GAPDH	F: CTGAACGGGAAGCTCACTGG R: GTCGAAGGTGGAAGAGTGGG	AB038240	719-738 918-937

Table 4. Primers for seqncing canine PLA<sub>2</sub>R

	Primer sequence (5'-3')	Location
1	GTAATACGACTCACTATAGGGC	T7
2	CAGATGGTGGGAAGCATTGTGA	563-584
3	CATTTTGGCCAAGTGCCTGGA	1109-1129
4	CTGAGGGACGCTGGAAAGTT	1556-1575
5	ACAGGAGAAAACGGAGCAGG	2055-2074
6	GAGCGCTATCAAAGTATGGTGT	2699-2720
7	TTCCTCTCTGTGCTTTGCTATC	3299-3320
8	TGAGGCTGCTCATGAATTTTGC	3927-3948

Table 5. Comparison of amino acid sequences between canine PLA<sub>2</sub>R and several animals.

Animals	Human	Cat	Mouse	Rat
	%			
Full sequence	86	92	74	73
RICIN	85	89	78	70
FN2	94	92	73	78
CLECT1	86	92	75	75
CLECT2	85	91	73	77
CLECT3	88	92	78	78
CLECT4	98	98	89	89
CLECT5	80	87	67	68
CLECT6	90	96	82	79
CLECT7	89	99	76	77
CLECT8	88	93	77	73
Transmembrane region	75	90	62	58
Cytoplasmic domain	80	93	69	64

RICIN; ricin-type beta-trefoil; FN2, fibronectin type II domain; CLECT, C-type lectin like domains.

Accession Number of PLA<sub>2</sub>R protein:  
 NP\_031392 (Human), XP\_006935367(cat),  
 NP\_032893(mouse), NP\_001094307(rat)



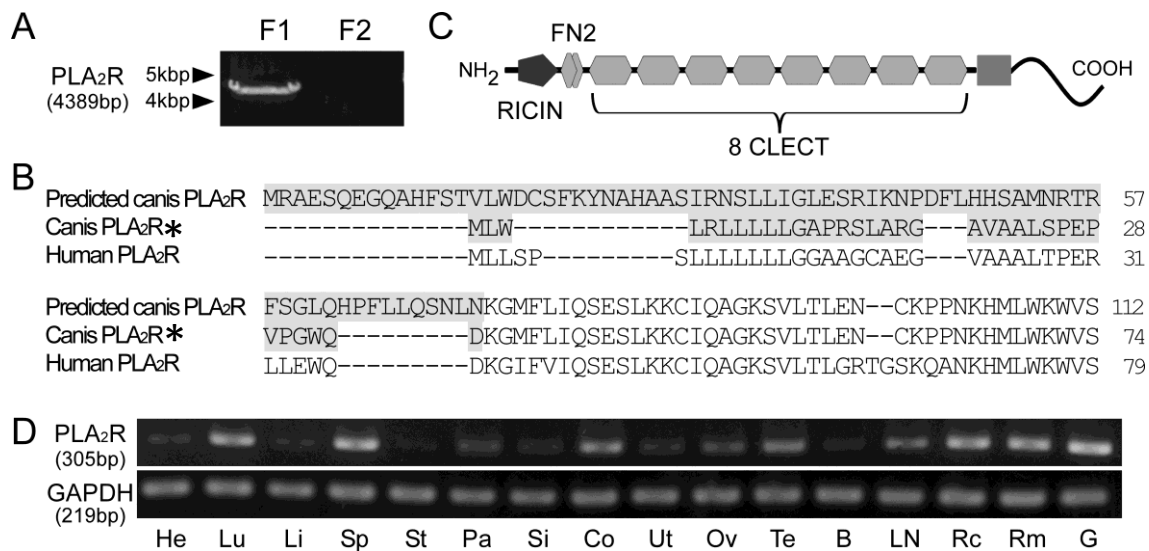


Fig. 2. Gene structure and tissue distribution of canine PLA<sub>2</sub>R. (A) RT-PCR for full length PLA<sub>2</sub>R using two different forward primers; F1 is specific for the 5' untranslated region of feline PLA<sub>2</sub>R, which has high homology among mammals; F2 is for the 5' terminal CDS of predicted canine PLA<sub>2</sub>R. (B) Comparison of the deduced amino acid sequence of canine PLA<sub>2</sub>R in the N-terminal region with the predicted canine PLA<sub>2</sub>R sequence in Genbank (Accession Number: XM\_545489) and those of human PLA<sub>2</sub>R (NP\_031392). In the shaded region, the sequence determined in this study was completely different from the predicted PLA<sub>2</sub>R amino acid sequence in Genbank. \*The sequence obtained in this study. (C) The predicted schema of the PLA<sub>2</sub>R protein. RICIN; ricin-type beta-trefoil; FN2, fibronectin type II domain; CLECT, C-type lectin like domains. (D) RT-PCR for the partial canine PLA<sub>2</sub>R sequence in various organs. cDNA was prepared from heart (He), lung (Lu), liver (Li), spleen (Sp), stomach (St),

pancreas (Pa), small intestine (Si), colon (Co), uterus (Ut), ovary (Ov), testis (Te), brain

(B), lymph node (LN), renal cortex (Rc), renal medulla (Rm), and isolated glomeruli

(G). GAPDH cDNA was amplified as an internal control.

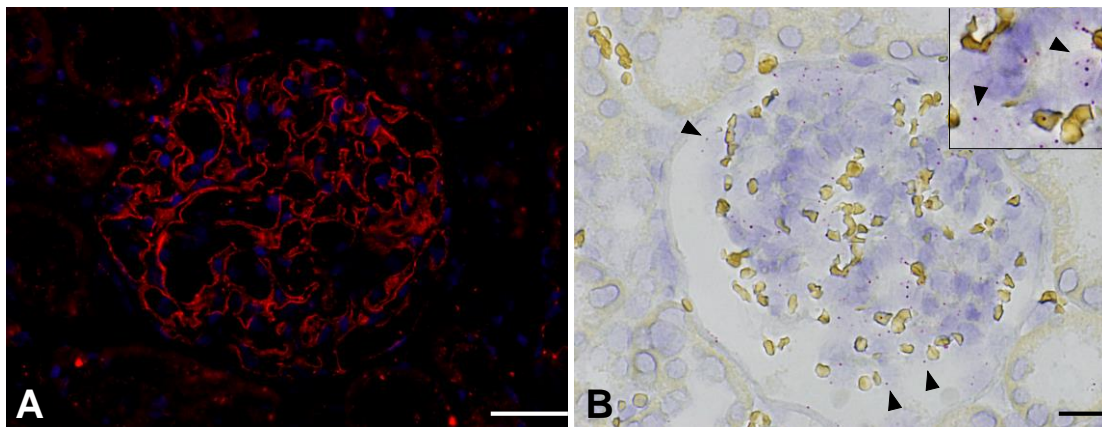


Fig. 3. Expression of PLA<sub>2</sub>R protein and mRNA in canine renal tissue.

Immunofluorescence staining (A) and *in situ* hybridization (B). The expression of PLA<sub>2</sub>R protein (red) was observed along the capillary walls in the glomeruli (A). The expression of PLA<sub>2</sub>R mRNA (red dots; arrow heads) was observed in glomerulus (B).

Bar, 30  $\mu$ m.

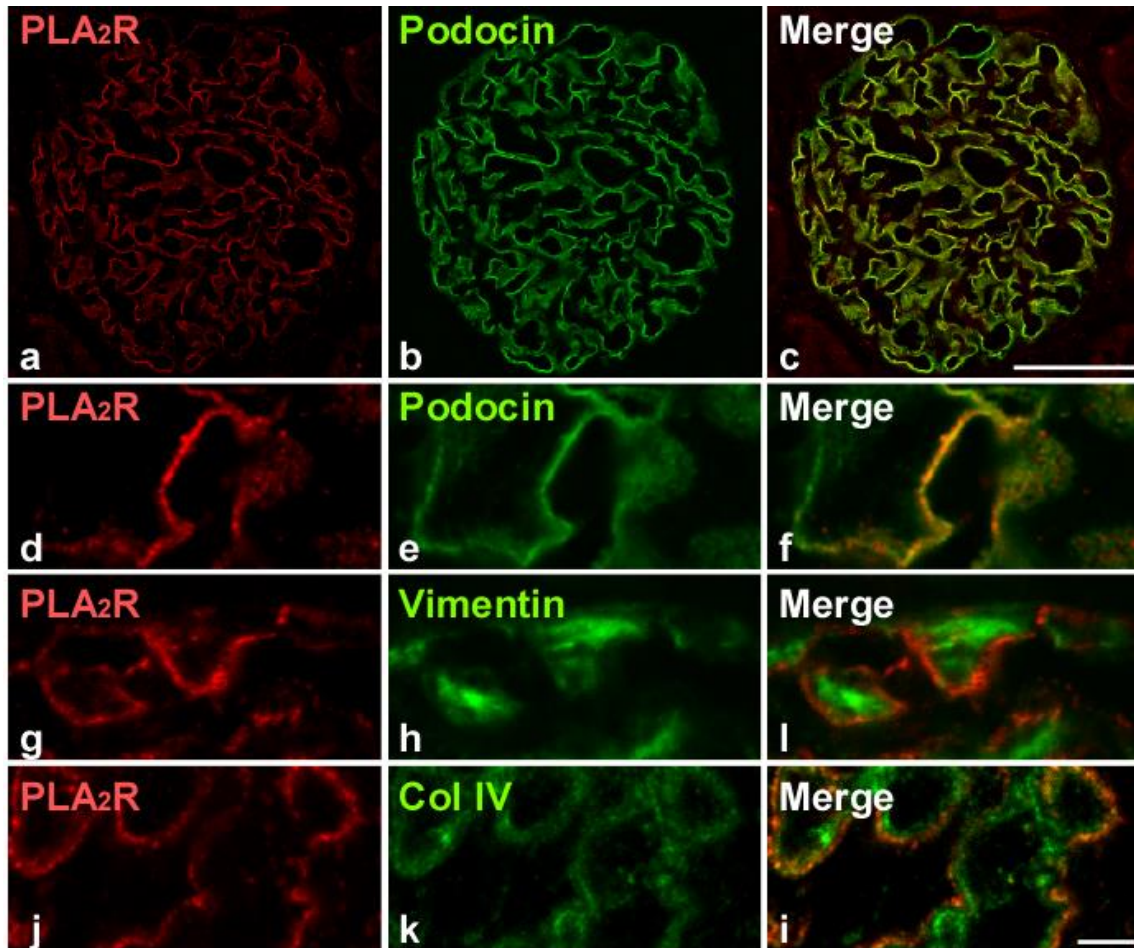


Fig. 4. Localization of PLA<sub>2</sub>R protein in glomeruli. Confocal images of PLA<sub>2</sub>R (A, D, G and J), podocin (B and E), vimentin (H), type IV collagen (K), and merged images of PLA<sub>2</sub>R and podocin (C and F), vimentin (I) or type IV collagen (L). PLA<sub>2</sub>R colocalized with podocin along the glomerular capillary wall (A to F), but not with vimentin and type IV collagen, and was localized between vimentin (G to I) and type IV collagen (J to L). Bar, 50 μm (C) and 4 μm (L).

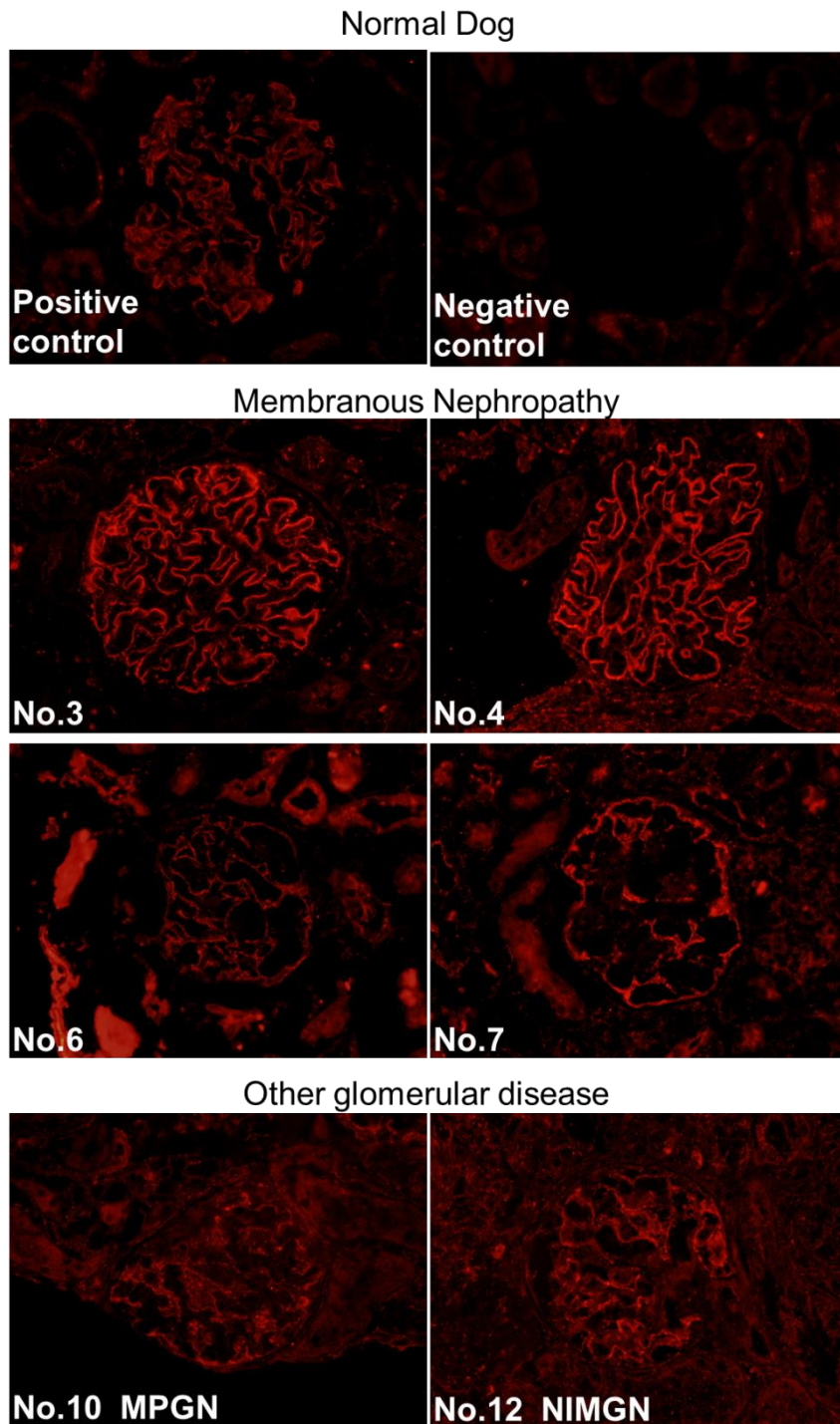


Fig. 5. Immunofluorescence for PLA<sub>2</sub>R in cases of glomerular disease.

In two MN cases (No.3 and No.4), increased staining for PLA<sub>2</sub>R along the glomerular capillary walls was identified but not in other three cases (No.6-8). In other glomerular disease such as membranoproliferative glomerular nephritis and non-immune-mediated glomerular nephropathy, increased expression of PLA<sub>2</sub>R was not observed.

## **Chapter 3**

Establishment of the primary culture method for canine podocyte  
and detection of expression of PLA<sub>2</sub>R in primary cultured  
podocytes

## Introduction

Podocytes are the highly differentiated epithelial cells covering with GBM and form particular ultrastructure, foot process and slit diaphragm. These particular structures have a central role of glomerular selective filtration and loss of these specific structures in various pathological conditions profoundly associates with protein leakage from glomerular capillary walls [38].

In veterinary medicine, several researchers investigated canine glomeruli using tissue samples and showed normal expression of some podocyte specific markers and altered expression in various pathological conditions in common with human and experimental animals [24, 31]. However, there is no *in vitro* research report using canine cultured glomerular podocytes because of the lack of culture methods for canine podocytes.

Podocyte cell culture derived from human, mice and rat glomeruli has been used in many studies aimed for elucidating their physiological and pathophysiological characteristics. The methods for primary podocyte culture are relatively convenient and well-established methods in rodents [29, 41]. Also, primary cultured podocytes growing out from isolated glomeruli are assumed to retain their *in vivo* phenotype.

In the previous chapter, the author revealed glomerular expression of PLA<sub>2</sub>R. However, strictly speaking, the expression in podocyte is still unproven. Considering the

mechanism of *in situ* IC formation in idiopathic MN, it is important to prove exactly podocyte expression of PLA<sub>2</sub>R.

The object in this chapter is to establish the method of podocyte primary culture of dogs and to reveal the expression of PLA<sub>2</sub>R in the cultured podocyte.



## **Materials and Methods**

### *Animals*

Renal tissues were obtained from three 12 month-old male and beagles. The detailed information of the animals was described in chapter 2. All dogs were purchased from a laboratory animal breeding and supply company (Kitayama Labes Co, Ltd, Nagano, Japan) and confirmed to be healthy by physical examination. The animals were used for clinical education and euthanized in accordance with the guidelines approved by the Animal Research Committee of Azabu University (No. 100408-3)

### *Primary culture of podocytes*

The method used for the primary culture of podocytes was based on a method for rat kidney [29]. Canine kidneys were enucleated with the renal artery, veins, and ureter. A 21-gauge butterfly needle was inserted into the renal artery and the kidney was perfused with 20 ml PBS (pH 7.2, 0.01M) and 30 ml PBS containing 36 mg of iron powder ( $\phi 6 \mu\text{m}$ ) (Wako). The renal cortical tissue was then cut into 1-2 mm cubes in Hanks' balanced salt solution (HBSS; Invitrogen). The tissues were digested in HBSS containing 1 mg/ml collagenase A (Roche Diagnostics GmbH, Mannheim, Germany)

and 0.2 mg/ml deoxyribonuclease I (Roche Diagnostics GmbH) at 37 °C for 60 min with gentle agitation. The collagenase-digested tissues were gently pressed through a 250 µm tissue strainer (Thermo Fisher Scientific, Basingstoke, United Kingdom) using a flattened pestle. Glomeruli containing iron powder in the tissue suspension were gathered using a magnetic particle concentrator (Dynal AS, Oslo, Norway) and washed at least four times with PBS. Finally, collected glomeruli were suspended in a suitable amount (approximately 12 ml) of PBS and the suspension was poured onto a 70 µm cell strainer (BD Biosciences, Stockholm, Sweden) and glomeruli remaining on the cell strainer were carefully collected. During the procedure, kidney tissues were maintained at 4 °C, except during the collagenase digestion.

Isolated glomeruli were seeded on type I collagen-coated culture dishes or glass coverslips (Asahi techno glass, Tokyo, Japan) in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (D-MEM:F-12, 1:1; Invitrogen) containing 5% fetal bovine serum (FBS; Hana-nesco Bio., Tokyo, Japan) supplemented with 0.5% Insulin-Transferrin-Selenium-A (ITS-A) liquid media supplement (Invitrogen) and 1% Antibiotic-Antimycotic liquid (Invitrogen). IF was performed after incubation at 37°C in a humidified incubator with 5% CO<sub>2</sub> for three days. For subculture of primary cultured podocytes, the cellular outgrowths obtained following three days of incubation

were detached using a trypsin ethylenediaminetetraacetic acid (EDTA) solution containing 2 g Difco™ Trypsin 250 (BD Biosciences) and 0.4 mg EDTA, disodium salt, dihydrate in 200 ml of 0.01 M PBS, pH 7.2, passed through a 40- $\mu$ m sieve (BD Biosciences) to remove the remaining glomerular cores, and the filtered cells were gathered by centrifugation at 1000  $\times$  g. The obtained cells were then cultured on collagen I-coated dishes for 1 day and processed for RT-PCR or western blotting (WB) preparation.

### Immunofluorescence

To confirm that the cultured cells express PLA<sub>2</sub>R and podocyte specific markers (podocin, nephrin and synaptopodin), immunocytochemical experiment was performed. For immunocytochemical experiment, cultured podocytes were fixed in acetone for 10 min at -20 °C (goat PLA<sub>2</sub>R, podocin, nephrin and synaptopodin) or methanol for 5 min at 4 °C (mouse PLA<sub>2</sub>R, vWF, and Iba-1). The staining procedure and equipment were described in chapter 2.

### Reverse Transcriptase Polymerase Chain Reaction using cultured podocytes

To confirm that the cultured cells express PLA<sub>2</sub>R and podocyte specific markers (podocin, nephrin and synaptopodin) RT-PCR was performed. Total RNA was extracted from cultured podocytes using the RNeasy Plus Micro Kit (Qiagen, Hilden, Germany). First-strand cDNA was obtained from the total RNA using a Sensiscript RT kit (Qiagen) with random primers (Invitrogen), in the presence of RNasin ribonuclease inhibitor (Promega, Madison, Wisconsin). Table 2 summarizes the primer sequences used in this study. The primer sequences were designed based on the predicted mRNA sequences of *Canis lupus familiaris* nephrosis 1, congenital, Finnish type (nephrin) (NCBI database, accession number: XM\_541685), *Canis lupus familiaris* nephrosis 2, idiopathic, steroid-resistant (podocin) (XM\_547443.2), *Canis lupus familiaris* synaptopodin (XM\_536465.5). PCR reactions were performed as follows: denaturation at 94°C for 2 min; 40 cycles of denaturation at 98°C for 10 s, annealing at 64°C for 30 s, and extension at 68°C for 30 s; and a final extension step at 68°C for 30 s. The other information for PCR was described in chapter 2.

#### *Isolation of glomeruli for western blotting*

Isolation of glomeruli for WB was performed according to the above description in chapter 2.

### Western blotting

To confirm that the cultured cells express PLA<sub>2</sub>R and podocin, WB was performed. The proteins from isolated glomeruli and cultured podocytes were extracted and lysed in RIPA buffer (consisting of 0.1% sodium dodecyl sulphate [SDS], 1% NP-40, 150 mM NaCl and 0.5% sodium deoxycholate in 50 mM Tris-HCl, pH 7.4) with Protease Inhibitor Cocktail Set I (Wako, Osaka, Japan) and protein concentrations were assayed using 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 containing 10% beta-mercaptoethanol (5% final concentration) 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 10% skimmed milk in PBS with 0.1% Tween 20 for 1 h at room temperature and incubated overnight at 4 °C with the mouse anti-PLA<sub>2</sub>R (1 in 700 dilution) or the rabbit anti-podocin (1 in 1000) antibodies diluted in Immuno-Enhancer Reagent A (Wako). After washing, the membranes were incubated with a horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody (Dako Cytomation, Glostrup, Denmark; 1 in 2000 dilution) or HRP-conjugated goat anti-rabbit IgG antibody (Dako Cytomation; 1 in

2000 dilution) diluted in Immuno-Enhancer Reagent B (Wako) for 1 hr at room temperature. Immunoreactivity was visualized using Luminata<sup>™</sup> Forte Western HRP Substrate (Millipore, Temicula, California, USA) and detected using an LAS4000 (GE Healthcare Japan Co., Ltd., Tokyo, Japan).

## Results

### Podocytes outgrowth from isolated glomeruli and expression of podocyte-specific proteins in cultured podocytes

A number of isolated glomeruli were collected and all of the isolated glomeruli were decapsulated. However, a low rate of blood vessel and tubular contamination was observed ( $3.3 \pm 1.5$  %; n = 3). Cellular growth of podocytes from isolated glomeruli was first detected within 2 days and became prominent by 3-4 days in culture (Fig. 6).

The podocyte morphology was irregular, often exhibiting arborization with long cytoplasmic processes, and multinucleation. Their morphology was identical to that of primary cultured rat podocytes described in previous reports [29, 45]. Also a small number of fibroblast and tubular epithelial cells proliferation from contaminated blood vessel and tubules were sometimes observed.

These cultured cells also showed distinct expression of the podocyte specific proteins (nephrin, podocin, and synaptopodin) by IF (Fig. 7A). Synaptopodin exhibited most characteristic staining pattern, stress fiber-like appearance and cross striation. The cultured cells expressed nephrin and podocin in the cytoplasm and marginal region. Gene expression of these proteins in the primary cultured podocytes was also confirmed by RT-PCR (Fig. 7B). In WB, the specific band for podocin with an extra band was

observed. None of the outgrowing cells showed positive staining for von Willebrand factor (an endothelial marker) or ionized calcium binding adaptor molecule 1 (a macrophage markers) (data not shown).

#### Expression of PLA<sub>2</sub>R in cultured podocytes

The cultured cells also showed distinct positive staining for PLA<sub>2</sub>R by IF using different antibodies. The positive staining was observed to be prominent in the cytoplasm and marginal region (Fig. 8A). Expression of PLA<sub>2</sub>R in the primary cultured podocytes was also confirmed by RT-PCR and WB (Fig. 8B and C). A distinct single band was observed in WB for cultured podocytes.



## Discussion

The author succeeded in effective isolation of glomeruli from canine kidney by modifying an isolation method established for rat in previous report [29, 41]. In previous reports, it is considered as a good preparation when 95% of the cellular constituents in isolated tissue is glomeruli [41] and the samples obtained in the present study was assumed to be good preparation according to this criteria.

Cultured podocytes in the present study expressed several podocyte-specific markers, including nephrin, podocin and synaptopodin. Consistent with previous reports, nephrin and podocin was localized in the cytoplasm and cell surface in cultured podocytes in this study [12, 26, 29]. The cytoplasmic expression pattern of nephrin and podocin in cultured podocytes is inconsistent with the expression pattern observed in glomeruli in the tissue section, and might related to the morphological features of cultured podocytes, which lack foot processes and slit-diaphragms, or unusual intracellular transport of these proteins in the *in vitro* environment.

Considering the mechanism of *in situ* IC formation in idiopathic MN, the localization of the target antigen on podocyte cell membrane is important. The marginal localization of PLA<sub>2</sub>R in canine cultured podocytes consistent with that of foot process-associated proteins (nephrin and podocin) is also an important finding supporting this possibility,

because the binding of circulating antibodies against PLA<sub>2</sub>R on the basal surface of the podocyte foot processes is assumed to be the mechanism for subepithelial deposition of IC which is a characteristic feature in idiopathic MN, as suggested by various reports using experimental rat models of MN [19, 27] and human neonatal MN [10].

The cultured podocytes have been used in previous research for idiopathic MN. Meyer-Schwesinger *et al.* established a new mouse model of MN in which sheep anti serum against murine cultured podocytes caused subepithelial IC formation in the glomeruli of injected mice and suggested some candidate auto-antigens in podocytes in the models [34]. Moreover, commercial detection kit (Euroimmun AG) for anti- PLA<sub>2</sub>R autoantibodies in patient serum of human idiopathic MN is an IF test using cultured HEK293 cells transfected with the PLA<sub>2</sub>R [40]. Yangbin *et al.* reported that the increased levels of serum sPLA<sub>2</sub>-IB (a subtype of sPLA<sub>2</sub>) in MN patients enhanced glomerular expression of PLA<sub>2</sub>R [37]. Furthermore, Pan *et al.* demonstrated using immortalized human cultured podocytes that the interaction between sPLA<sub>2</sub>-IB and PLA<sub>2</sub>R stimulate podocyte apoptosis through activating ERK1/2 and cytosolic PLA<sub>2</sub>α, as well as through increasing podocyte arachidonic acid content. However, given the high degree of specialization and complex cytoarchitecture of podocytes, it is more significant to explain the involvement of PLA<sub>2</sub>R in podocyte dysfunction by

investigating the expression of podocyte specific proteins or the reconstruction of actin cytoskeleton network closely linked to podocyte functions, rather than detection of apoptosis [1, 26, 35, 44]. For instance, Yasuno *et al.* revealed podocyte injury induced by angiotensin II by evaluating reorganization of F-actin fiber in subcultured podocyte derived from Osborn Mendel rats, showing progressive spontaneous glomerulonephropathy [46]. Therefore, the primary culture of canine podocytes may be useful for evaluation the effect of sPLA<sub>2</sub> and PLA<sub>2</sub>R on podocyte or detection of autoantibodies in serums in affected dogs. In addition, several questions about the direct effect of binding of autoantibodies against PLA<sub>2</sub>R, such as whether the binding of anti-PLA<sub>2</sub>R autoantibodies itself can cause podocyte injury or enhanced expression of PLA<sub>2</sub>R in podocytes, could be evaluated by using canine podocytes. Therefore, *in-vitro* method demonstrated in this study may contribute to advance research about podocytes or glomerular diseases in dogs.

In this chapter, the author demonstrated the method of canine podocyte primary culture. The primary cultured cells expressed PLA<sub>2</sub>R in addition to several podocyte-specific proteins. This is the first study of primary cultured footed mammalian podocytes expressing PLA<sub>2</sub>R. The result in this chapter did prove the expression of PLA<sub>2</sub>R in podocyte suggested by the results of tissue section in previous chapter. The *in-vitro*

experiment provided from this method may contribute to progression of investigation  
for canine glomerular disease including MN.

## Figures and Tables

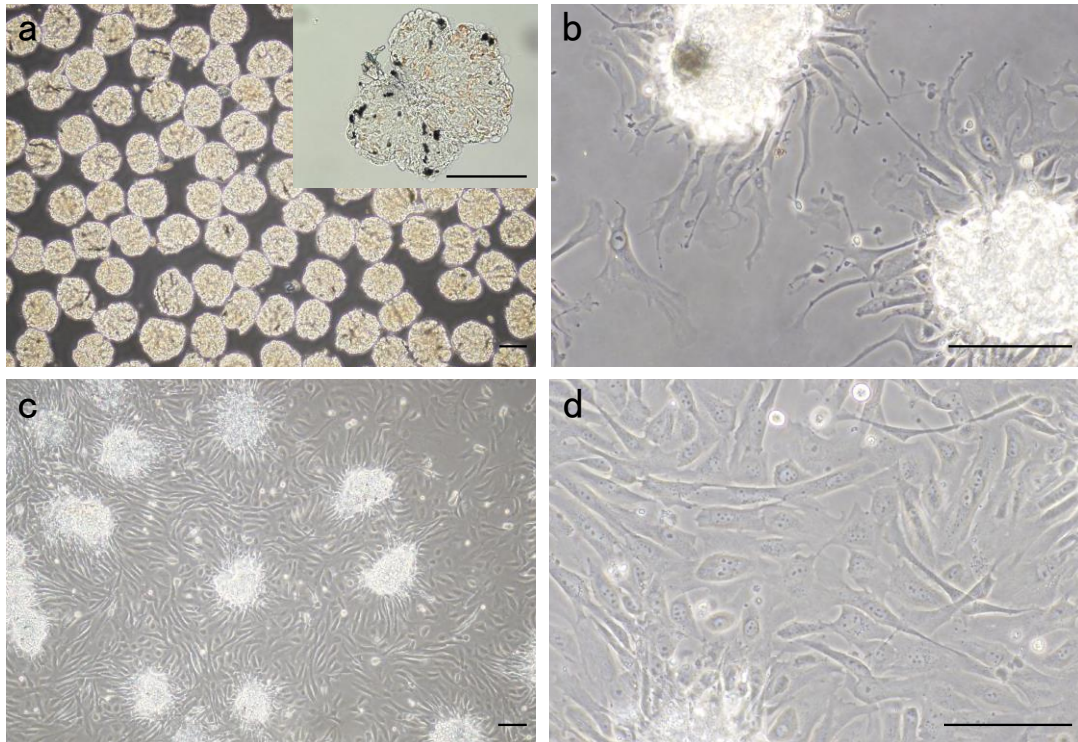


Fig. 6. Phase-contrast microscopy of isolated canine glomeruli and cellular growth of podocytes. Phase-contrast microscopy of isolated glomeruli from canine renal tissue (A) and their cellular outgrowths after 2 days (B) and 3 days (C and D) in culture. \*Isolated glomerulus, Bar = 100  $\mu\text{m}$ .

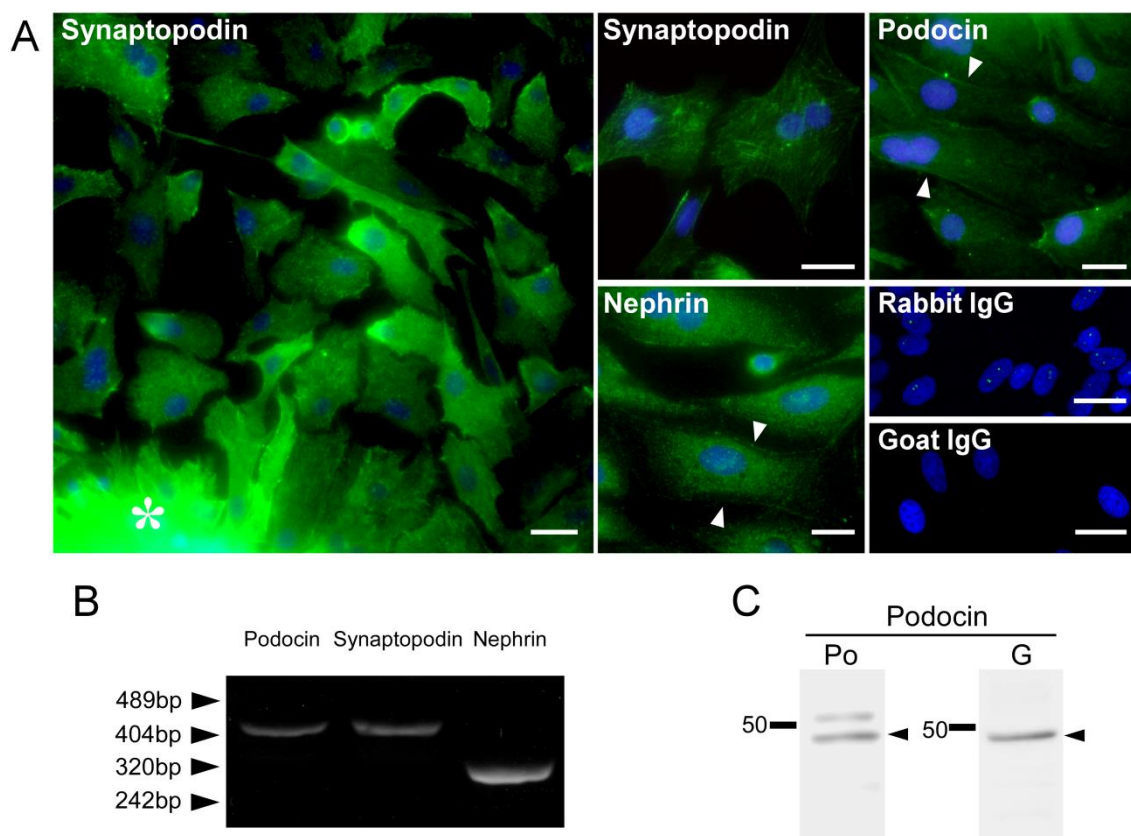


Fig. 7. Expression of podocyte markers in primary cultured canine podocytes.

Immunofluorescence for podocyte-specific proteins (synaptopodin, podocin and nephrin) (A), reverse transcriptase polymerase chain reaction (RT-PCR) for podocyte-specific markers (podocin, synaptopodin and nephrin) (B), and western blotting for podocin protein (C) in canine primary cultured podocytes. Rabbit and goat IgG: negative control. Arrowheads: expression on marginal region. Asterisk: Isolated glomerulus. Bar = 25 μm.

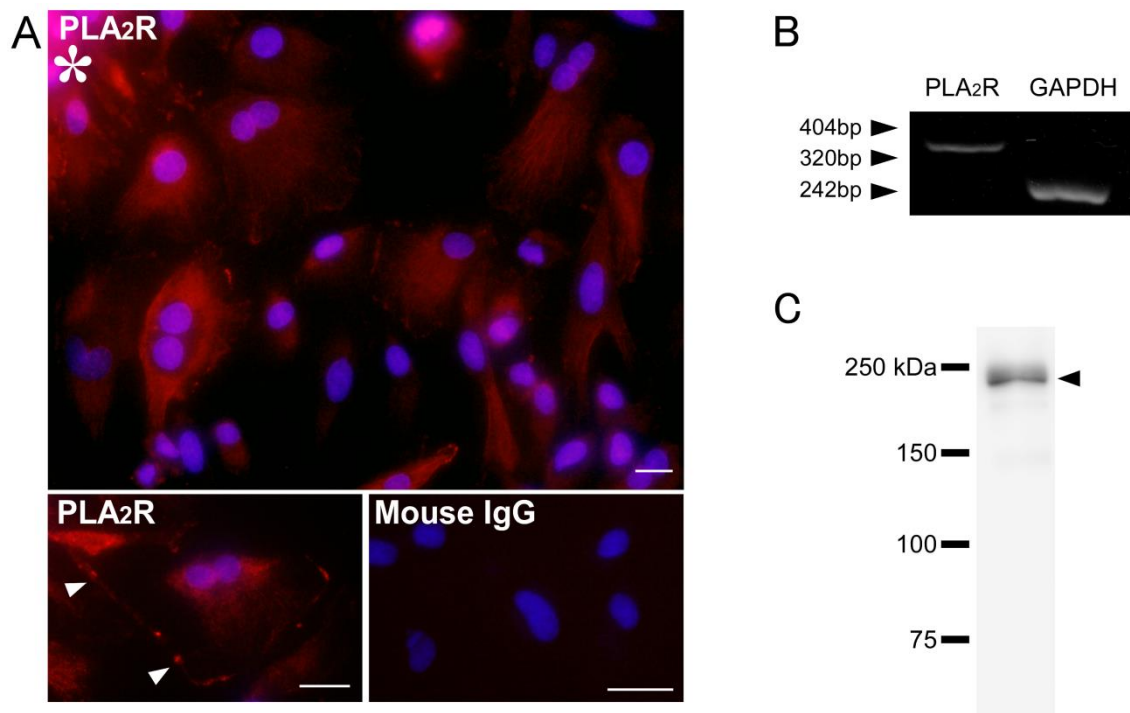


Fig. 8. Expression of PLA<sub>2</sub>R in primary cultured canine podocytes. (A)

Immunofluorescence for PLA<sub>2</sub>R staining (A), reverse transcriptase polymerase chain reaction (RT-PCR) for *PLA2R1* and *GAPDH* (B), and Western blotting for PLA<sub>2</sub>R protein (C) in primary cultured canine podocytes. Mouse IgG: negative control.

Arrowheads: expression on marginal region. Asterisk: Isolated glomerulus. Bar = 25

μm.

## Conclusion

The author revealed the expression of PLA<sub>2</sub>R in canine podocytes and, the ultrastructural features observed in some MN dogs were shared with human idiopathic MN associated with PLA<sub>2</sub>R. Conclusion is summarized as follows.

1. Canine MN showed two different deposition patterns of dense deposit in the glomeruli resembling human idiopathic or secondary MN. This may suggest the presence of different pathogenesis in canine MN as suspected in human MN.
2. Canine PLA<sub>2</sub>R obtained from the glomeruli has high homology and common extracellular structures with human PLA<sub>2</sub>R.
3. PLA<sub>2</sub>R is expressed in canine podocyte and distributed on the foot process in tissue sections. This is important finding suggesting the possibility of dogs as an animal model of human idiopathic MN. Because PLA<sub>2</sub>R is not expressed in the podocytes of rodents, rabbits and no experimental or spontaneous animal models for idiopathic MN have been established, so far.
4. The increased expression of PLA<sub>2</sub>R, which is feature of human idiopathic MN associated with PLA<sub>2</sub>R, demonstrated in some of MN dog cases with idiopathic ultrastructural pattern of dense deposit. These results may support the author's



hypothesis that some canine MN cases are autoimmune disease associated with PLA<sub>2</sub>R as an autoantigen expressed in podocyte.

5. The primary culture method for canine podocyte expressed several podocyte specific proteins was established. The *in vitro* experiment provided from this method may contribute to progression of investigation for canine glomerular disease including MN.

In this study, the author suggested that some canine MN might be associated with PLA<sub>2</sub>R, but it was not proven. As future subject to confirm the author's hypothesis, detection of anti-PLA<sub>2</sub>R autoantibodies in the serum of affected dogs is most important.

In human medicine, technologies applied to detect anti-PLA<sub>2</sub>R autoantibody such as WB using isolated glomeruli [2, 17, 36, 39], IF test using cultured HEK293 cells transfected with the PLA<sub>2</sub>R [5, 21-23] and ELISA using recombinant PLA<sub>2</sub>R obtained from the same cells [5, 21, 28], were established. The establishment of these examination tools for dogs and suitable and routine storage of the serum from dogs given renal biopsy could contribute to the detection of autoantibodies in the serum from dogs with MN. Also, the establishment of an animal model caused by the immunization with PLA<sub>2</sub>R or injection of anti-PLA<sub>2</sub>R antibodies made from other animals may prove

that the circulating anti-PLA<sub>2</sub>R antibodies can cause MN. As identified in this study, dogs may be a candidate animal for the useful model of human idiopathic MN.

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## **Acknowledgment**

The author wishes to express his sincere appreciation and gratitude to Dr. Kinji Shiota, 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 Dr. 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 and Dr. Yoko Fujii and Dr. Keisuke Sugimoto, Laboratory of Surgery I, Azabu University.

Thanks are also due to all graduate and undergraduate students of the author's laboratory, especially Dr. Takayuki Mineshige, 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.

A part of this thesis was published as a following article.

1. Sugahara, G., Kamiie, J., Kobayashi, R., Mineshige, T. and Shirota, K. 2016.

Expression of phospholipase A2 receptor in primary cultured podocytes derived from dog kidneys. *J. Vet. Med. Sci. (in press)*