Note (Pathology)

Expression of neuronal nitric oxide synthase and renin in dysplastic kidneys of young dogs

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Running Head: DYSPLASTIC KIDNEYS OF YOUNG DOGS

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Abstract

Renin and neuronal nitric oxide synthase in the kidney control the renin-angiotensin and tubuloglomerular feedback systems. The present study investigated the expression of renin and neuronal nitric oxide synthase in the dysplastic kidneys of three young dogs. Renin-immunoreactivity, which occurs in the juxtaglomerular and tubular cells of dysplastic kidneys, did not differ from that in the normal kidneys of young dogs. Macula densa cells in the normal kidneys showed neuronal nitric oxide synthase -immunoreactivity, but those in the dysplastic kidneys showed no apparent signals. This observation may be correlated with the pathological mechanisms of renal failure in young dogs.

Key words: chronic kidney disease, dysplastic kidney, immunohistochemistry, neuronal nitric oxide synthase, renin
Renal failure occasionally occurs in young dogs. In such cases, histological examination frequently demonstrates dysplastic kidney lesions. However, the pathophysiological mechanisms that occur in the dysplastic kidneys of dogs remain unclear. Recently, we demonstrated the attenuation of cyclooxygenase (COX)-2 in chronic kidney diseases in young dogs with dysplastic kidneys using immunohistochemical analysis [19].

COX-2 is an inducible inflammatory enzyme which is essential for normal kidney development in the fetal and neonatal stages [12, 15]. After birth, COX-2 is constitutively expressed in the kidney and is concentrated in the restricted area of the macula densa (MD) of the thick ascending limbs [1, 4, 22]. The MD is a specialized region adjacent to the vascular pole of the glomerulus. It controls renin secretion and the tubuloglomerular feedback (TGF) system. COX-2 expressed in MD cells regulates these mechanisms [6, 8]. In addition to COX-2, neuronal nitric oxide synthase (nNOS), which is also expressed in MD cells, is known to be the principal regulator of the TGF system [3, 18]. These two molecules, which are co-expressed in MD, are known to interact with each other [2, 5, 9]. In the present study, we investigated the expression of renin and nNOS in dysplastic kidneys by immunohistochemistry to determine whether the attenuation of COX-2 in dysplastic kidneys induces the disruption of the renin-angiotensin system and TGF system in young dogs.

The formalin-fixed paraffin blocks of dysplastic kidneys from young dogs (n=3), normal kidneys from young dogs (n=3), and normal kidneys from adult dogs (n=4) were the same as those used in our previous study, in which the clinical history and tissue preparation have been previously described [19]. The study was approved by the Committee for Animal Experimentation of Kagoshima University, Japan (VM15020). Sections were prepared with a 3-µm thickness, and immunohistochemical analysis for renin and nNOS was performed according to the following protocols: (1) deparaffinization; (2) antigen retrieval by microwave heating in 10 mM of citrate buffer (pH 6.0) for nNOS; (3) incubation with 3% H2O2 for 30 min; (4) blocking with 3% bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) for renin or 0.25% casein (Sigma-Aldrich) for nNOS, diluted in phosphate-buffered saline, for 30 min; (5) incubation overnight at 4 °C with a 1:3,000 dilution of rabbit anti-recombinant renin polyclonal antiserum (supplied by Dr. Murakami, University of Tsukuba, Japan) or a 1:3,000 dilution of rabbit anti-nNOS polyclonal antiserum (Cayman Chemical, Ann Arbor, MI, USA), in a blocking solution; (6)
incubation for 30 min at room temperature with a peroxidase-polymer-conjugated universal antibody (anti-mouse immunoglobulin (Ig) G and anti-rabbit IgG; simple stain MAX-PO [MULTI]; Nichirei Biosciences, Tokyo, Japan); and (7) immunosignal detection using the 3,3′-diaminobenzidine system (3,3′-diaminobenzidine buffer tablet; Merck, Darmstadt, Germany). For the negative control sections, normal rabbit IgG (Lab Vision, Fremont, CA, USA) was used in place of the primary antibody.

Randomized morphometric analysis for renin immunoreactivity was performed according to a previously described method [20]. Briefly, the renin index was defined as the ratio of the total number of renin-positive arterioles to the total number of glomeruli and expressed per 100 glomeruli. Differences between the groups were analyzed using Bonferroni’s multiple comparison method with SPSS version 24 (IBM SPSS Statistics, Armonk, NY, USA).

Histopathological diagnosis was performed according to the microscopic criteria of canine renal dysplasia by Picut and Lewis [14]. The histology sections of all three cases presented asynchronous differentiation, which indicates the presence of lesions with fetal/immature glomeruli. Secondary changes of variable severity such as tubular damage, interstitial fibrosis, and sclerotic glomeruli were observed within the mature renal tissues.

In the dysplastic kidneys, renin-immunoreactivity signals were observed in the juxtaglomerular cells (JGCs) (Fig. 1A), and tubular cells were located in the distal nephrons (Fig. 1B). In some glomeruli, a few cells showed renin-positive signals near the vascular poles (Fig. 1A). In the young control kidneys, renin signals were also detected in both JGCs and distal tubules, including the MD cells. In the adult control kidneys, renin-positive signals were restricted to the JGCs. Statistically, the number of renin-positive areas in the dysplastic kidneys showed no significant differences from those in young and adult kidneys.

Stimulation of renin production is known to play a role in COX-2 signaling, and previous studies have demonstrated that plasma renin activity and concentration were decreased after administration of the COX-2 inhibitor or in the knockout of the COX-2 gene [10, 11]. As the dysplastic kidneys examined in the present study showed no expression of renal COX-2 [19], a reduction in the immunosignals of renin in JGCs was initially suspected as a possible physiological abnormality in the dysplastic kidneys. However, the present immunohistochemical analysis could not confirm this hypothesis, and no apparent
reduction of renin was suggested in JGCs. Although a physiological role of the tubular renin has not been clarified previously, renal handling of the sodium might be a possible role of the tubular renin, since the enhancement of the renin expression was induced in the distal nephron after low-salt diet feeding in rats [16], and the dysplastic kidneys of dogs examined in the present study might have maintained this tubular function.

COX-2 is also important in the regulation of the TGF system, which is important for maintaining the glomerular filtration rate. A study in rats using the micropuncture method demonstrated the suppression of the TGF response after treatment with a COX-2 inhibitor [2]. TGF response in dysplastic kidneys might be attenuated due to the lack of renal COX-2 expression. However, no relevant findings have been previously reported. In the kidney, nNOS is expressed in the MD cells and helps to regulate the TGF system [18].

In the present nNOS analysis, no apparent signals were detected in the MD regions of all cases (Fig. 2). This reduction of nNOS was considered as etiopathogenic event in the dysplastic kidneys rather than an acquired one after the renal failure of the cases. Actually, our previous study using OLETF (Otsuka Long-Evans Tokushima Fatty) rats, as a model of type 2 diabetes, demonstrated that nNOS was overexpressed in the MD and its around tubular cells in the later stage of diabetic nephropathy [21]. In regulation of the TGF system, the function of nNOS is similar in and interacts with that of COX-2. Some studies have suggested that nNOS is positioned upstream of the signaling pathway and stimulates the expression of COX-2 [5, 9]. On the other hand, other studies have suggested the opposite that COX-2 is positioned upstream of then NOS [2, 13]. Regardless of the difference in the signaling pathway, lack of nNOS-immunosignals in the MD cells may trigger the downregulation of renal COX-2 expression in dysplastic kidneys.

A few tubules showed positive signals for nNOS in two cases. In the remaining case, many dilated and flattened tubules, probably in the distal nephron, displayed clear positive signals (Fig. 3). In this case, with prominent nNOS-positive tubules, the development of the renal tissues was the most immature. In the young and adult normal kidneys, nNOS-positive signals were observed in the thick ascending limbs, especially in the MD cells (Fig. 4). Distribution of the nNOS changes during the
developmental course of the kidney. In developing kidneys of pigs, nNOS distributes widely and observed in portions of the thick ascending limb leading to the macula densa [17]. In rats, the presence of nNOS in the developing distal tubule is encountered already in the S-shaped body from the earliest stages of nephrogenesis [7]. Although the distribution of the nNOS in developing kidneys of dogs has not been investigated previously, there is a possibility that the prominent nNOS signals in tubules might be a testament to the highly immature tubules in the dysplastic kidney rather than the damaged mature tubules.

In conclusion, renin and nNOS expression in the dysplastic kidneys of three young dogs were immunohistochemically investigated in the current study. This demonstrated the deterioration of nNOS in the MD cells with constant renin expression. The reduction of nNOS expression in the MD cells could attenuate the TGF system of the kidneys, and this may be one of the pathological mechanisms underlying renal failure in young dogs with dysplastic kidneys.

**POTENTIAL CONFLICTS OF INTEREST:** The authors have nothing to disclose.

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FIGURE LEGENDS

Fig. 1. Immunohistochemistry for renin in a dysplastic canine kidney. A: Renin-positive signals were observed in the juxtaglomerular cells near the vascular poles and some glomerular cells (arrows). Bar: 50 μm. B: A different region of the section shown in panel A. Tubules in the unidentified nephron segment show renin-immunoreactivity. Bar: 50 μm

Fig. 2. Immunohistochemistry for neuronal nitric oxide synthase in a dysplastic kidney. The tubular cell macula densa region (arrow) shows no apparent positive immunoreactivity. Bar: 50 μm

Fig. 3. Immunohistochemistry for neuronal nitric oxide synthase in a dysplastic kidney. In this case, many dilated and flattened tubules show clear positive signals. Bar: 100 μm

Fig. 4. Immunohistochemistry for neuronal nitric oxide synthase in a normal kidney from a young dog. The neuronal nitric oxide synthase-positive signals were observed in the macula densa regions (arrows) of the thick ascending limbs. Bar: 50 μm
