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Original Article

Nephrin is an Important Component of the Barrier System in the Testis

Li Liu, Kunihiko Aya, Hiroyuki Tanaka*, Junya Shimizu, Shigeru Ito, and Yoshiki Seino

Department of Pediatrics, Okayama University Medical School, Okayama 700-8558, Japan

Nephrin, a gene product of the congenital nephrotic syndrome of the Finnish type (NPHS1), is a 1242-residue putative transmembrane protein of the immunoglobulin family of cell adhesion molecules. The expression of this gene is localized in rat and human glomerular epithelial cells. Here we report the expression of nephrin in various tissues other than the kidneys in mice. The expression of nephrin mRNA in various tissues of mice, including the kidneys, testes, spleen, thymus and brain, were first investigated by the RT-PCR method, and it was shown that a high level of nephrin mRNA could be detected in the testes of mice 1-6 weeks old. *In situ* hybridization revealed the expression of the nephrin gene in the Sertoli cells. Additionally, immunofluorescent staining studies indicated that nephrin was colocalized with anchoring protein ZO-1 in the mouse testis. From these results, it is inferred that nephrin is an important component of the barrier system in testes.

Key words: nephrin, Sertoli cells, glomerular epithelial cells, blood barrier

Congenital nephrotic syndrome of the Finnish type (CNF, NPHS1) is a distinct entity among nephrotic syndromes. The NPHS1 gene has recently been named nephrin [1]. The isolated nephrin message extends over 4.1 kb and contains a predicted open reading frame encoding a 1242 amino acid polypeptide with a predicted relative molecular mass of 135 kDa [2]. Recently, the expression of nephrin was examined in rats and humans. The expression is mainly localized at the glomerular podocytes as determined by *in situ* hybridization [1, 3], and the immunoreactivities were localized at the slit diaphragm [4]. These results suggest that nephrin may be important in the development, maintenance and function of the glomerular filtration barrier [5, 4]. However, the expression and function of nephrin in other organs have been poorly understood. It was assumed that nephrin may participate in a wide range of cellular functions including cell adhesion, spreading and migration [1]. We therefore investigated the distribution of nephrin in mouse tissues other than the kidneys.

Moreover, previous studies of kidneys suggested an association between nephrin and other major anchoring proteins such as ZO-1 [6]. The ZO-1 alpha-isoform is restricted to junctions of endothelial cells and highly specialized epithelial cells of both seminiferous tubules (Sertoli cells) and renal glomeruli (podocytes) [7]. Therefore, we also examined the colocalization of nephrin and ZO-1 in the testis.

Material and Methods

Animals. BALB/c mice were obtained from the mouse colony of Okayama University Medical School, Okayama, Japan. Zero-day-old and 1-, 2-, 3-, 4-, and 6-week-old male mice were killed by cervical dislocation. The brain, skin, and skull from 0-day-old male mice and the thymus, heart, lung, liver, spleen, kidney, testes and extremities from 3-week-old male mice were obtained.

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*Corresponding author. Phone: +81-86-235-7248; Fax: +81-86-221-4745
E-mail: hrtanaka@md.okayama-u.ac.jp (H. Tanaka)

The testes from 1–6 week-old male mice were also obtained immediately after the mice were killed.

RNA extraction. Total cellular RNA was isolated by means of acid-guanidium-phenol-chloroform extraction [8]. The isolated RNA was dissolved in diethylpyrocarbonate-treated water, and the RNA concentration was determined spectrophotometrically at 260 nm using a Gene Quant RNA/DNA Calculator (Pharmacia, Cambridge, UK).

Reverse transcription-polymerase chain reaction (RT-PCR). RT-PCR was performed in order to determine the amounts of nephrin mRNA and the internal control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. Five μg of the total RNA obtained from the mouse testis (the final volume of the reaction was 25 μl) was reverse-transcribed using an oligo (dT) primer with an RT-PCR kit (Stratagene, La Jolla, CA, USA).

Two point five μl of the first-strand cDNA reactions were used for each 50 μl PCR reaction. The PCR was performed using a Gene Amp DNA Amplification Reagent kit (Perkin-Elmer, Branchburg, NJ, USA). All reactions were performed with 1.5 mM $MgCl_2$ and 0.4 μM of each primer, and according to the manufacturer's instructions using a Programmable Thermal Controller (MJ Research, Watertown, MA, USA).

The sequences of the primers were as follows: sense, 5'-GTGTTTTTCTTCGGGTGTCA-3', (GenBank AF168466, position 1820–1839) and antisense, 5'-CCACTTTCGTCAGGGGAGTA-3' (position 2529–2548) for nephrin. The cycle times used were 94 °C for 4 min, 94 °C for 1 min, and 58 °C for 1 min, 72 °C for 1 min, for 35 cycles, followed by a single additional 8 min extension at 72 °C. The amplified product was a 729-bp fragment of nephrin cDNA. After 2% agarose gel electrophoresis, the gel stained with ethidium bromide was examined on a UV-transilluminator, which was equipped with a computerized CCD camera (ATTO densitograph, ATTO Co. Tokyo, Japan). The RT-PCR products were sequenced by the dideoxy method and confirmed as the published sequences. RT-PCR of GAPDH was performed according to a previously described method [9].

Preparation of riboprobes. For *in situ* hybridization, sense and antisense riboprobes were made according to a previously described manner [10]. The PCR product was subcloned into the vector pCRII (Invitrogen Carlsbad, CA, USA) to allow the generation of sense and antisense cRNAs as run-off transcripts with SP6 or T7 RNA polymerases (Boehringer Mannheim, GmbH, Germany) in the presence of digoxigenin-UTP (Boehringer Mannheim Biochemica, Mannheim, Germany). The antisense probe (T7) was used to detect nephrin mRNA and the sense probe (SP6) was used for detecting the nonspecific binding of RNA to the tissues.

In situ hybridization. Testes from 5-week-old mice were quickly dipped into freshly prepared 4% paraformaldehyde (PFA)-phosphate-buffered saline (PBS) for 2 h at 4 °C. After being washed with PBS, the testes were kept in 30% sucrose/PBS until they sank. Following preincubation with OCT compound (Miles, Elkhardt, IN, USA), the tissues were frozen with liquid nitrogen. Ten μm cryosections were collected on silane-coated slide glasses.

Sections were postfixed with 4% PFA-PBS for 15 min, digested with 1 μg/ml proteinase-K (37 °C; 30 min) in PBS and treated again with 4% PFA-PBS (10 min). After being washed with PBS, the sections were incubated with 0.2 N HCl (10 min). After being washed, the tissue sections were acetylated with 0.25% acetic anhydride in the presence of 1 M triethanolamine (10 min), dehydrated with increasing concentrations of ethanol and then dried. Hybridization was performed in a humidified chamber with 10 μg /ml DIG-labeled probe in a solution containing 50% formamide, 10% dextran sulfate, 1 $\times$ Denhardt's solution, 600 mM NaCl, 10 mM Tris-HCl pH 7.6, 1 mM EDTA, 0.25%SDS, and 200 μg/ml transfer RNA (57 °C; 16 h). After hybridization, the sections were briefly washed with 5 $\times$ SSC at 57 °C, 50 % formamide-2 $\times$ SSC (57 °C; 30 min), PBS (37 °C; 5 min), 1 μg/ml ribonuclease-A in PBS (37 °C; 30 min), PBS (37 °C; 5 min) and 2 $\times$ SSC (57 °C; 20 min), and twice with 0.2 $\times$ SSC (57 °C; 20 min). After this wash, the sections were immersed in a maleic acid buffer (0.1 M maleic acid pH 7.5 0.15 M NaCl) for 5 min, and incubated with 1% blocking reagent (DIG Nucleic acid detection kit, Boehringer Mannheim) in the maleic acid buffer for 60 min and then with 1.5 U/ml anti-digoxigenin Fab fragments labeled with alkaline phosphatase (Boehringer Mannheim) in the maleic acid buffer (30 min). Unbound antibodies were removed by washing twice with the maleic acid buffer (15 min $\times$ 2). The signal was detected by an AP Substrate kit including BCIP/NBT (Vector Laboratories, Burlingame, CA, USA) at room temperature overnight. Then the sections were counterstained by methylgreen.

Immunofluorescent microscopy. Dual immunofluorescent staining was performed according to the following method. Five μm cryostat sections were fixed with acetone for 5 min and incubated with the rabbit anti-rat nephrin and goat anti-mouse ZO-1 (Santa Cruz, CA, USA) for 1 h. The sections were then stained with FITC-conjugated anti-rabbit IgG and TRITC-conjugated anti-goat IgG (Chemicon International, Temecula, CA, USA) and observed under confocal microscopy (LSM-510, ZEISS, Germany).

Results

We first examined nephrin distribution by means of RT-PCR. We observed that a high level of nephrin mRNA is expressed in the kidney and testis, along with minor expression in the spleen, thymus and brain, but no expression was detected in the other organs such as the skull and extremities (Fig. 1A).

Because nephrin expression in the kidney has already been determined, our interest was in clarifying the expression of nephrin in the testis during postnatal development until sexual maturation. As shown in Fig. 1B, the expression of nephrin in the testis showed an age-dependent increase until 4 weeks of age following a stable expression period. This result suggests that nephrin in the testis may play a role in testicular maturation.

In order to define the cells which express the nephrin gene, we next performed *in situ* hybridization on the testis of 5-week-old mouse, in which the expression level of the nephrin reaches maximum. A DIG-labeled antisense RNA probe and a sense RNA probe were used. The use of antisense riboprobe tissue showed that Sertoli cells were stained deep purple and adhered to the wall of seminiferous tubules or near the walls (Fig. 2A). Moreover, we observed that spermatids and interstitial cells, which are typical epithelial cells in the testis, were not stained.

To analyze the colocalization of mouse nephrin and ZO-1 in the testis, we performed a dual-labeled staining. Similar to the *in situ* hybridization results, immunofluorescent staining indicated that nephrin was located along seminiferous tubular walls. Furthermore, the ZO-1 staining in a normal mouse testis showed an

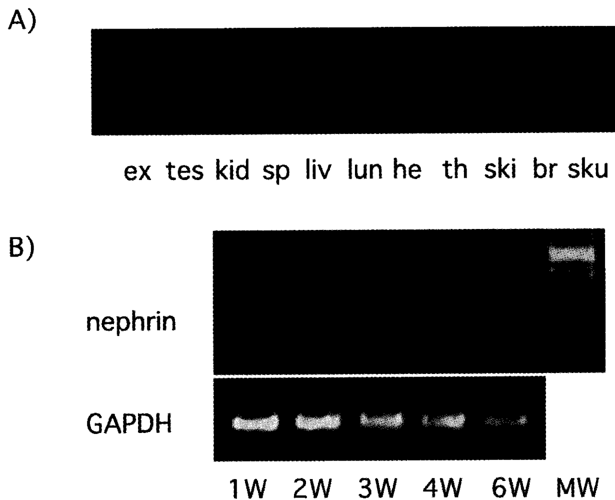

Fig. 1 By means of the RT-PCR method, we examined the amounts of nephrin in various tissues including the testis, kidney, spleen, liver, lung, heart, extremities, and thymus of 3-week-old mice and the skin, brain and skull of newborn mice. We observed that a high level of nephrin mRNA is expressed in the testis, kidney, spleen, thymus and brain (**A**). The nephrin mRNA and GAPDH mRNA expression in testes from the mice aged 1, 2, 3, 4, and 6 weeks were examined by RT-PCR. The experiment was repeated at least 3 times and the representative result is shown (**B**).

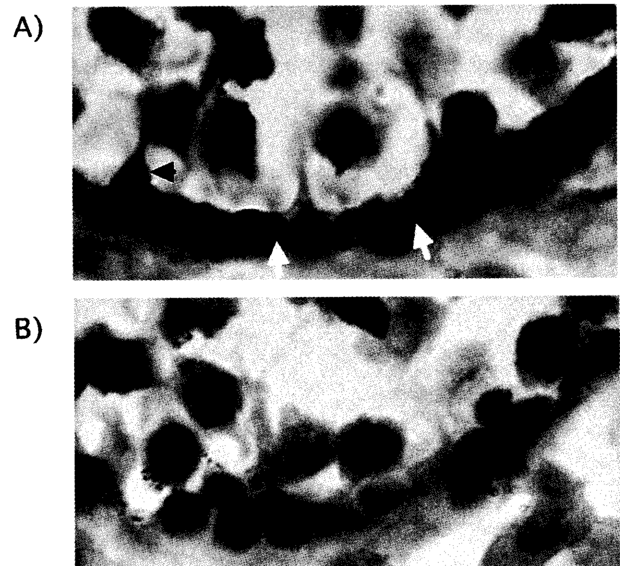

Fig. 2 Testis sections of 5-week-old mice were hybridized with a DIG-labeled antisense RNA probe (**A**) and with a sense probe (**B**). Nephrin mRNA was highly expressed in the Sertoli cells. Sertoli cells were stained deep purple and adhered to the wall of seminiferous tubules. The black arrows indicate a typical Sertoli cell and the white arrows indicate the wall of seminiferous tubules.

almost similar pattern. As illustrated in Fig. 3, the bright yellow colored stain, which shows colocalization of 2 signals, was observed in the Sertoli cells due to the dual staining.

Discussion

Our results indicated that nephrin mRNA is not only expressed in the kidney but is also expressed in extrarenal tissues such as the testis and brain. Thus, the presence of nephrin in a specific region of the testis and brain gave rise a question about the role of this protein in genital and neuronal development and function. Putaala *et al.* reported that about 10% of approximately 50 kidney-transplanted Finnish patients have congenital neurologic symptoms, but genital system symptoms have not been reported [11]. This may due to their short life without kidney transplantation [1]. Therefore, the role of nephrin in the testicular development of humans cannot be excluded. According to anatomical knowledge, the Sertoli cells constitute most of the seminiferous epithelium in the fetal testis. The Sertoli cells provide mechanical support, protection and probably nutrition for the developing germ cells [12] and constitute the blood-testis barrier [13].

Recent reports have shown that nephrin is produced in glomerular epithelial cells, and nephrin is a central component of the glomerular slit diaphragm, which is essential for a normal renal filtration process [11]. Previous studies also described the appearance of a belt-like tight junction on the glomerular epithelial region, which maintains a very high transepithelial potential. The protein of the tight junction has been named ZO-1 (zona occludens-1). It is a 210-225 kDa membrane protein and is tightly associated with the cytoplasmic surface of the tight junction [14, 15]. The ZO-1 protein possibly connects the slit diaphragm to the cytoskeleton directly or indirectly [4]. In the kidney, the tight junctions are known to function as a seal to restrict the passage of proteins, ions, and water through the intercellular spaces. They work as a fence to prevent mixing of apical and basolateral plasma membrane domains [16].

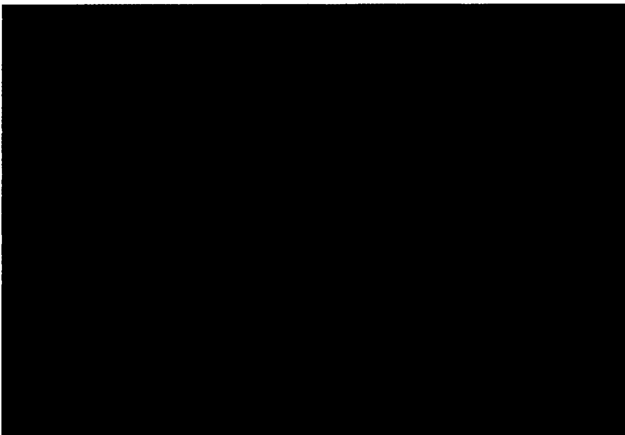

Fig. 3 Localization of nephrin and ZO-1 on Sertoli cells in mouse testis. The double immunofluorescent staining shows that the localization of nephrin (green staining) coincided with ZO-1 (red staining). The bright yellow color reveals that nephrin was colocalized with ZO-1.

Interestingly, a certain testicular structure is somewhat similar to a structure in the kidney. In testes, the arrangement of Sertoli cells is similar to that of podocytes in kidneys. The lateral processes of Sertoli cells in proximity to the basement membrane of seminiferous tubules form a parallel array of the tight junctions and constitute the blood-testis barrier [13], where the amount of proteins that pass through is limited. Balda *et al.* have reported that the ZO-1 isoform is restricted to the junction of endothelial cells and highly specialized epithelial cells of both seminiferous tubules (Sertoli cells) and renal glomeruli (podocytes)[7]. Our results indicated that nephrin was colocalized with ZO-1 in the mouse testis according to double immunofluorescence staining. This suggests that nephrin may play an important role in forming a testicular barrier with ZO-1.

Putaala *et al.* recently reported that nephrin mRNA was strongly expressed in the hindbrain and spinal cord of mouse embryos [11]. Physiological knowledge suggests that on brain tissues present a famous blood-brain barrier, where passage of protein is completely limited. Thus, nephrin may be a specific protein that is common to blood-barriers in the testis, kidney and brain. And in the testis, it is speculated that nephrin may play an essential role in the regulation of Sertoli cell function, creating the specialized micro-environment of the adluminal compartment of the seminiferous epithelium. The reports, which may discuss, in future, the fertility of male Finnish type congenital nephrosis might provide important information about the physiological roles of testicular nephrin. However, we have only limited data so far. The *in vitro* system, by which the function of nephrin could be evaluated, is essential to further clarification of its roles.

We observed that nephrin mRNA also existed in the

mouse spleen and thymus. Although we cannot explain the function of nephrin in these organs very clearly at this time, one might speculate that nephrin seems to be an element that participates in forming the blood barrier structure of these organs. One of the clinical symptoms of congenital nephrosis is widened cranial sutures and fontanels [17]. This symptom suggests that nephrin may play a role in skull maturation. However, we did not detect nephrin gene expression in the skull. Therefore, it is hard to believe that defective nephrin could be a direct pathogenetic factor of this symptom.

In conclusion, nephrin is an important component of the barrier system in the testis as well as in the kidney.

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