Retinoids Regulate the Repairing Process of the Podocytes in Puromycin Aminonucleoside-induced Nephrotic Rats

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Abstract. The foot processes forming the slit diaphragm are disrupted in diseases associated with proteinuria. Although they are often repairable, regulators for the repairing process remain unknown. By extrapolating from the fact that vitamin A is essential for the nephrogenesis, this study examined whether or not injured podocytes in the middle of the repairing process require retinaldehyde dehydrogenase type 2 (RALDH2), one of the key enzymes to produce all-trans-retinoic acid (ATRA). RALDH2 was dramatically upregulated in podocytes of puromycin aminonucleoside-induced nephrosis (PAN nephrosis) rats. On day 5 of PAN nephrosis, RALDH2 showed a remarkable induction, whereas glomerular expression levels of nephrin and midkine, one of the ATRA target genes, were downregulated. Daily administration of ATRA ameliorated proteinuria, which was accompanied by the improvement in the effacement of the foot processes and by the induction of nephrin and midkine. In contrast, recovery from PAN nephrosis was delayed in rats fed with a vitamin A-deficient diet. Consistently, the promoter region of human nephrin gene (NPHS1) contained three putative retinoic acid response elements (RARE) and showed the enhancer activity in response to ATRA in a dose-dependent manner. This transcriptional activation was regulated through the receptors for retinoids because BMS-189453, an antagonist to the retinoid receptors, counteracted it in a dose-dependent manner. In conclusion, active metabolites of vitamin A, especially ATRA produced by RALDH2 play relevant roles during the repairing process of injured podocytes. The results obtained from PAN nephrosis rats might be applicable to human renal diseases.

All-trans-retinoic acid (ATRA), an active metabolite of vitamin A, exerts a wide variety of biologic effects such as cell proliferation, apoptosis, differentiation, reproduction, maintenance of normal tissues, especially of epithelial cells (1). ATRA is generated from vitamin A through a series of oxidation mediated by both the alcohol dehydrogenase family and the aldehyde dehydrogenase family, including retinaldehyde dehydrogenase type 2 (RALDH2), the key enzyme producing ATRA (2). ATRA induces the expression of several target genes responsible for the diverse biologic effects via the cognate receptors for retinoids, which are referred to as retinoic acid receptors (RAR) and retinoid X receptors (RXR). The fact that different genes are activated by different concentration of retinoic acid receptors (RAR) and retinoid X receptors (RXR). The fact that different genes are activated by different concentration of retinoic acid response elements (RARE). The RARE can be divided into three classes. The most potent response element comprises the direct repeat of the consensus sequence, AGGTCA, which is separated by one, two, three, four, or five nucleotides. The palindrome structure made of two consensus sequences is less responsive to retinoic acid than the direct repeat, and it requires overexpression of RAR to be active. Finally, the complex type, which is composed of sequences that are highly degenerated from the consensus and that scatter randomly along the promoter region, is only weakly responsive (5).

Vitamin A plays a crucial role in the nephrogenesis as well as the embryogenesis. In the 1940s, Wilson et al. (6) demonstrated that pregnant rats fed with a diet lacking in vitamin A showed diverse renal abnormalities such as hypoplastic or ectopic ureters, horseshoe kidney, and renal hypoplasia. Even mild vitamin A deficiency induces a reduction of the nephron number, while other organs develop in a normal way (7). Recently, it is reported that a role of vitamin A in the nephrogenesis is to regulate epithelial/mesenchymal interactions through the expression of c-ret, a receptor for glial cell line-derived neurotrophic factor (GDNF), and to induce branching of the ureteric bud (8–10). However, these facts are not sufficient to account for the various renal abnormalities observed in vitamin A-deficient animals.

The glomerular epithelial cell, also called the podocyte, arises from the metanephric blastema that also produces other nephron segments such as the proximal and distal tubules. Despite highly specialized features of the podocyte, factors regulating its differentiation process and maintaining its struc-
tural integrity after the terminal differentiation remains unknown. Effacement of foot processes, which is associated with proteinuria in many renal diseases, is reparable when therapeutic approaches are successfully employed. However, regulators for this regeneration process are also unknown.

In this study, we adopted rat puromycin aminonucleoside-induced nephrosis that represents a self-limiting injury of the podocytes and examined the significance of vitamin A and its active metabolites in maintaining the integrity of the podocyte.

Materials and Methods

Reagent and Chemicals

All trans-retinoic acid (ATRA) and puromycin aminonucleoside (PAN) were purchased from Sigma (St. Louis, MO). BMS-189453, a compound that antagonizes all classes of RAR, was supplied by Bristol-Myers-Squibb (Princeton, NJ). Vitamin A-deficient diet modified from AIN-93G (11), which contained less than 0.01 mg retinol/100 g, was purchased from Oriental Yeast (Osaka, Japan). Mouse monoclonal antibody (clone 5-1-6) that recognizes the extracellular domain of rat nephrin and rabbit polyclonal antibody that recognizes the intracellular domain of rat nephrin were prepared as described previously (12). Polyclonal anti-RARα antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Animals

Male Sprague-Dawley (SD) rats weighing 200 to 250 g were purchased from Japan SLC (Hamamatsu, Japan). PAN nephrosis was induced by a single intravenous injection of PAN (10 mg/100 g body wt). To rats treated with ATRA, 10 or 20 mg/kg body wt of ATRA was administered subcutaneously once a day from the day of the disease induction through the day of the sacrifice. Equal volume of DMSO was administered subcutaneously to the control rats. Where indicated, vitamin A-deficient diet was started to rats 3 wk before the administration of PAN and was continued throughout the experiments. All procedures were approved by the animal committee of Osaka University School of Medicine.

Tissue Preparation

At the time indicated, rats were anesthetized and sacrificed by intraperitoneal administration of pentobarbital (50 mg/kg body wt). After systemic perfusion with phosphate-buffered saline (PBS), kidneys were removed and were sieved to obtain glomeruli. Alternatively, kidneys were thoroughly perfused with PBS followed by 4% paraformaldehyde (PFA)/PBS and were further fixed by immersion in 4% PFA/PBS for 6 h at 4°C. The tissues were then embedded in OCT compound (Sakura Finetechnical Co., Ltd, Tokyo, Japan) or in paraffin.

Measurement of the Urinary Protein/Creatinine Ratio

Spontaneously voided urine was collected between 2 p.m. and 5 p.m. every 2 d. Concentration of protein and creatinine was measured by using Micro TP Test Wako and Creatinine Test Wako, respectively, according to the instructions (Wako, Osaka, Japan).

Northern Blot Analyses

RNA was extracted with Trizol (Life Technologies, Rockville, MD) according to the manufacturer’s instruction, and was subjected to Northern blot analyses (13). Briefly, 10 μg of RNA per lane were separated in 1.2% agarose/formaldehyde gel and were transferred to nylon membrane filters (Hybond N+, Amersham-Pharmacia Biotech, Little Chalfont, UK). The filter hybridized with 1 x 10⁶ cpm/ml of P32-labeled cDNA probes at 42°C for 12 h were washed twice in 2 x SSC/0.1% SDS at room temperature for 15 min, and then twice in 0.2 x SSC/0.1% SDS at 65°C for 15 min. After the hybridized probes were stripped off, the filters were rehybridized with a probe for rat glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) as the control.

Reverse Transcription-PCR (RT-PCR)

Semiquantitative RT-PCR was performed as described previously (14). Briefly, 0.4 μg of RNA was converted to single-strand DNA with random primers (Invitrogen, Carlsbad, CA) and SuperScript II (Invitrogen). The DNA was diluted to find a linear range for the following PCR reaction. The DNA was diluted at 1:25 for nephrin, 1:25 for midkine, 1:10 for RARα, 1:10 for RARβ, 1:10 for RXRα, and 1:1 for RXRβ. Each diluted DNA was mixed with 2 μl of 10 x PCR buffer (Takara, Tokyo, Japan), 1 μl of dNTP mixture (Takara), 1 μM of forward and reverse primers, and 0.1 μl of Takara Taq. Synthetic oligonucleotides described below were used as the primers: for nephrin, 5’-AGCCTCTTGACCACCTGCTA-3’ and 5’-CCCAAG TCACCTGAAGGTA-3’; for midkine, 5’-CCGCGTGCCCT- CAAAAAGAAAG-3’ and 5’T-CTGTGGTCTTGGAGGTCC-3’; for RARα, 5’-CAGCCCAAGCCACCTCAATC CCAT-3’ and 5’-GC- CACGAGAGAGCCTGCTTCATCG-3’; for RXRα, 5’-AAAGGG GCAGAGTTTGAGTGATCC-3’ and 5’-AGACGGGTCTTGAT CACTCGGA-3’; for RXRα, 5’-GTACACAGG AACAGGCCGCTCA- CATGG-3’ and 5’-TTAGAAAGAAAGACTGAGGTTCC AGGC-3’; for RARβ, 5’-ATTCGCCAGCCACCAAGACTCCTGC-AT-3’ and 5’-GCA- CAAAGTCGTGGGCCAGCGCAG-3’; for GAPDH, 5’-CTTCA CACGCGCAAGTCTCACA-3’ and 5’-GGATGCACCTTGGCCACACGC-3’. Twenty-eight cycles of PCR were performed with GeneAmp PCR System 9700 (Applied Biosystems). Annealing temperature was 56°C for nephrin, 54°C for midkine, 55°C for RARα, RARβ, and RXRα, 57°C for RXRβ, and 56°C for GAPDH. The PCR products were separated in 2% agarose gel and visualized with ethidium bromide.

In Situ Hybridization Analysis

Seven-micrometer-thick sections were deparaffinized, rehydrated, and further fixed in 4% PFA/PBS for 20 min at room temperature. The sections were sequentially incubated in 50 mM Tris·HCl (pH 7.4) containing 5 μg/ml of proteinase K and 5 mM of EDTA for 15 min at room temperature, in 0.2 N HCl for 10 min at room temperature, and in 0.25% acetic anhydride/0.1 M Triethanolamine for 10 min at room temperature. The sections were prehybridized with the hybridization buffer (4 x SSC/50% formamide/10% dextran sulfate/1 x Denhardt solution/2 mM EDTA/denatured salmon sperm DNA [500 ng/ml]) for 1 h at 37°C. Hybridization was performed with 2.5 μg/ml of digoxigenin-labeled cRNA probes in the hybridization buffer at 37°C for 17 h. Once hybridization was completed, the sections were washed in 2 x SSC at 50°C for 15 min and were incubated at 37°C for 10 min in 10 mM Tris·HCl (pH 7.5)/1 mM EDTA/0.5 M NaCl/ribonuclease A (20 μg/ml). After being washed twice in 2 x SSC at 50°C for 15 min and twice in 0.2 x SSC at 50°C for 15 min, digoxigenin was immunologically detected by using DIG Nucleic Acid Detection kit (Boehringer Mannheim) according to the manufacturer’s instruction. The sections were counterstained with Vector Methylgreen (Vector, Burlingame, CA).

Immunohistochemistry

For nephrin, 4-μm cryostat sections were incubated with 3% normal horse serum/PBS for 30 min and then with anti-nephrin mono-
clonal antibody 5-1-6 for 2 h. The sections were washed in PBS and then incubated with Texas red-conjugated anti-mouse IgG antibody (Vector) for 1 h. For RARα, 4-μm paraffin sections were incubated with 3% normal goat serum/PBS for 30 min and then with anti-RARα (C-20) antibody (Santa Cruz Biotechnology). After the sections were washed in PBS, they were incubated with biotinylated anti-rabbit IgG antibody. Finally, the sections were washed again and then incubated with VECTASTAIN Elite ABC-horseradish peroxidase Reagents for 30 min. To visualize the signals, the sections were incubated in DAB solution (0.05% of 3,3′-diaminobenzidin tetrahydrochloride, 0.01% of H₂O₂, pH 7.2). These paraffin sections were counterstained with Vector Methylgreen. All images were obtained with Nikon ECLIPSE E600 (Nikon, Tokyo, Japan) connected to a Macintosh computer.

**Western Blot Analyses**

Isolated glomeruli were lysed in the cell lysis buffer (Cell Signaling Technology, Beverly, MA) using a glass/Teflon homogenizer. The protein concentration of each sample was measured by the BCA Protein Assay Kit (Pierce, Rockford, IL) with the use of bovine serum albumin as the standard. The lysate was mixed with Laemmli sample buffer and was boiled for 10 min and cooled on ice. Twenty micrograms of protein were subjected to SDS-polyacrylamide gel electrophoresis and electroblotted onto a polyvinylidene difluoride membrane (Amersham Pharmacia). The membrane was incubated in 5% skim milk/20 mM Tris · HCl (pH 7.6)-buffered saline (TBS)/0.1% Tween 20, and then in anti-nephrin rabbit polyclonal antibody (1:500) or anti-RARα mouse monoclonal antibody (Santa Cruz) (1:1000). To visualize the signals, the horseradish peroxidase-conjugated anti-rabbit or mouse IgG antibody (Dako) was used at 1: 10000 in combination with the SuperSignal West Pico Chemiluminescent Substrate (Pierce).

**Transmission Electron Microscopy**

Kidneys were dissected after systemic perfusion of PBS. Immediately, the tissues were fixed in 4% glutaraldehyde for 4 h, postfixed in 1% osmium tetroxide, dehydrated in graded acetones, and embedded in Epon-Araldite. Ultra-thin sections, cut into 0.08-μm thickness and stained with uranyl acetate and lead citrate, were examined with Hitachi H-7100 (Hitachi, Tokyo, Japan).

**Construction of Luciferase Reporter Vectors**

Human genomic DNA was extracted with DNAzol BD (Life Technologies) from peripheral blood obtained from an informed healthy volunteer. Two kb of the 5′-genomic sequence flanking the transcription start site of human nephrin gene was obtained by PCR with the following primers: 5′-TGAGGCTCGAGAATTCGGACCT-3′ and 5′-TTGATGATCCGAGATCC-3′ and 5′-TTCCAGATCTTCTCCGTGATATC-3′. The PCR product was sequenced and inserted into XhoI/BglII-cut pGL2-promoter vector that has SV40 promoter positioned on the upstream of firefly luciferase gene (Promega, Madison, WI). A 325-bp fragment covering the region from #1-1060 through #735 was subcloned into XhoI/BglII-cut pGL2-promoter vector by using PCR with the following primers: 5′-GGAGGGCTCAACGAGATGGGAT-3′ and 5′-AGCTGGGCCC CCAGATCTCTTT-3′.

**Cell Culture and Luciferase Assay**

HeLa cells were cultured in DMEM supplemented with 10% fetal calf serum (FCS), 100 unit/ml penicillin, and 100 μg/ml streptomycin. The cells were seeded on 24-well plates at 1 × 10⁴ cells per well 48 h before the transfection. Plasmid DNA was introduced with TransFast transfection reagent (Promega) according to the manufacturer’s instruction. Briefly, the cells cultured in 24-well plates were washed with Opti-MEM (Invitrogen). The cells were transfected with the pGL2-promoter vector harboring the 2-kb promoter region of human nephrin (0.75 μg/well) or the 325-bp region (0.75 μg/well), pRL-TK vector (5 ng/well; Promega), which has the herpes simplex virus thymidine kinase promoter positioned on the upstream of Renilla luciferase gene, was transfected simultaneously to correct the transfection efficiency. The culture medium was switched to DMEM supplemented with 0.5% FCS 4 h after the transfection. After the following 20 h, the cells were stimulated with 10⁻⁸, 10⁻⁷, 10⁻⁶, or 10⁻⁵ M of ATRA in DMEM supplemented with 0.2% FCS. To inhibit the activity of the RAR family, the cells were stimulated with 10⁻⁵ M of ATRA in the presence of 10, 1, or 0.1 μM of BMS-189453. Twenty-four hours after the addition of ATRA, the cells were lysed in the Passive Lysis Buffer (Promega) according to the manufacturer’s instruction and were subjected to freeze-thaw twice. Firefly luciferase activity and Renilla luciferase activity were measured with Dual-Luciferase reporter assay system (Promega) and Lumat LB9507 (Berthold Technologies, Wildbad, Germany) according to the manufacturer’s instruction. The results were shown as the ratio of the Firefly luciferase activity to the Renilla luciferase activity.

**Statistical Analyses**

Statistical significance between experimental values was evaluated by a non-paired t test. Significance was defined as P < 0.05.

**Results**

**Induction of RALDH2 in the Podocytes of PAN Nephrosis Rats**

To examine whether vitamin A and/or its active metabolites are involved in the regeneration of the foot processes, PAN nephrosis rats were made and the expression level of RALDH2 in the glomeruli was measured by Northern blot analyses. As shown in Figure 1A, RALDH2 was dramatically upregulated in response to the administration of PAN. On day 5, the expression level was ten times higher than that on day 0 and returned to the basal level on day 20 (Figure 1B). In situ hybridization, which was applied to the tissue on day 5 of PAN nephrosis rats, revealed that RALDH2 was expressed exclusively in the podocytes (Figure 1C).

**Expression of Retinoid Receptors in Glomeruli**

ATRA regulates transcription of its target genes through nuclear receptors for retinoids such as RARα, RARβ, RXRα, and RXRβ. Semiquantitative RT-PCR revealed that transcripts of all the receptors were expressed in isolated glomeruli of PAN nephrosis rats, and that RARα was the dominant isoform (Figure 2A). RARα mRNA and its protein product were induced slightly on day 5 of PAN nephrosis, while the expression levels of other receptors remained stable over time (Figure 2, A and B). Both in situ hybridization and immunohistochemistry demonstrated that RARα was localized mainly to the podocytes (Figure 2C).

**Effects of Exogenous ATRA and Vitamin A–Deficient Diet on PAN Nephrosis**

According to our results described above, it was expected that exogenous ATRA or lack of vitamin A (an upstream...
Figure 1. Glomeruli were obtained from puromycin aminonucleoside-induced (PAN) nephrosis rats on days 0, 5, 10, and 20. The expression level of retinaldehyde dehydrogenase type 2 (RALDH2) mRNA was examined by Northern blot analyses. (A) RNA transferred to a filter was probed with the \(^{32}\)P-labeled cDNA probes, and the filter was exposed to a film: a 365-bp Rsal-Styl fragment of rat RALDH2 cDNA (upper panel) and a rat GAPDH (lower panel). (B) The autoradiogram was subjected to the densitometry using ImageQuant (Molecular Dynamics). The signal ratio of RALDH2 to GAPDH was shown as the fold increases against the value on day 0 (mean ± SD, n = 3). (C) The localization of RALDH2 mRNA was detected by in situ hybridization. A 365-bp Rsal-Styl fragment of rat RALDH2 was subcloned into pST-Blue1. Antisense and sense digoxigenin (DIG)-labeled cRNA probes were made by using Riboprobe Combination System (Promega). (a) the antisense probe for RALDH2 mRNA (×400). (b and c) the cells pointed by the arrows are shown in higher magnification (×1000). The sense probe for RALDH2 did not show any signals (data not shown).
Figure 2. (A) Expression levels of retinoic acid receptor-α (RARα), RARβ, retinoid X receptor-α (RXRα), and RXRβ in isolated glomeruli were examined by RT-PCR. DNA ladder markers are shown in the left lane. (B) Expression level of RARα was examined by Western blot analysis. Twenty micrograms of protein was applied to each lane. (C) Localization of RARα mRNA (a and b) and its protein product (c and d) was examined by in situ hybridization and immunohistochemistry, respectively. (a and b) a 202-bp (#1596–#1798) of RARα fragment was subcloned into pSTblue-1. Antisense and sense digoxigenin (DIG)-labeled cRNA probes were made by using Riboprobe Combination System (Promega). The cell pointed by the arrow in (a) (×400) is shown at a higher magnification in (b) (×1000). The sense probe for RARα did not show any signals (data not shown). (c and d) the protein product is also localized to the podocytes (c, ×400; d, ×1000).
substrate of RALDH2) would attenuate the phenotype of PAN nephrosis. From the first day of PAN nephrosis, 10 or 20 mg/kg of ATRA was administered subcutaneously once a day to PAN nephrosis rats. Daily administration of 10 mg/kg of ATRA reduced the urinary protein/creatinine ratio, which was statistically significant on day 15 and thereafter (Figure 3A). When 20 mg/kg of ATRA was used, this beneficial effect was more remarkable and was obtained at an earlier time point (Figure 3A). Consistently, the effacement of the foot processes was less serious in rats treated with ATRA than in untreated rats (Figure 4). A reciprocal phenotype was obtained in vitamin A−deficient rats. When rats had been fed with a vitamin A−deficient diet from 3 wk before the induction of PAN nephrosis, the urinary protein/creatinine ratio remained high without the spontaneous remission that is normally expected in PAN nephrosis (Figure 3B). Importantly, rats fed with a vitamin A−deficient diet did not develop nephrosis in the absence of the administration of PAN (Figure 3B). These results clearly indicate that the demand for vitamin A and/or its active metabolites such as ATRA increases in response to PAN and that these molecules are indispensable for the repair of the injured podocytes in PAN nephrosis rats.

Expression Levels of Nephrin and Midkine in Glomeruli of PAN Nephrosis Rats

Perturbation of nephrin results in massive proteinuria with the effacement of the foot processes (15,16). The expression level of nephrin decreases in PAN nephrosis rats (17,18). Therefore, we assumed that the administration of ATRA would affect the expression of nephrin in glomeruli of PAN nephrosis rats. Midkine, one of the known target genes of ATRA (19,20) was also examined as a reference for the local concentration of ATRA. Semiquantitative RT-PCR applied on day 0, 3, 5, and 7 of PAN nephrosis revealed that the amount of nephrin mRNA was well maintained in rats treated with ATRA, while it decreased in rats without treatment (Figure 5, top panel). This effect was obvious on day 5 but not on days 3 and 7. Because the response of midkine to ATRA was almost identical to that of nephrin (Figure 5, middle panel), it is likely that ATRA regulates both genes through a common or similar mechanism. The effect of ATRA on nephrin protein product was also confirmed by immunohistochemistry and Western blot analysis. The protein product of nephrin was clearly stained and was localized linearly along the capillary wall of normal glomeruli, which was not affected by the administration of ATRA (Figure 6A, a and b). In the glomeruli of PAN nephrosis rats on day 5, however, only a trace of nephrin was seen (data not shown). Intriguingly, the response of PAN nephrosis rats to ATRA was more pronounced than that of normal rats (Figure 4).

Figure 3. (A) The effect of exogenous ATRA on the urinary protein/creatinine ratio is shown. Broken line with open circles, normal rats; broken line with closed circles, PAN nephrosis rats treated with vehicle alone; solid line with open squares, rats treated with 10 mg/kg of ATRA; solid line with closed squares, rats treated with 20 mg/kg of ATRA. (B) The effect of a vitamin A−deficient diet on the urinary protein/creatinine ratio is shown. Broken line with open circles, normal rats treated with vitamin A−deficient diet; solid line with closed squares, PAN nephrosis rats fed with the normal diet; solid line with open squares, PAN nephrosis rats fed with a vitamin A−deficient diet. The sample number at each time point is three. The values are expressed as mean ± SD. Statistical significance was obtained by comparison with the basal value: * P < 0.002; ** P < 0.002; *** P < 0.016; **** P < 0.05.

Figure 4. Ultrathin sections of the glomeruli obtained from day 5 of PAN nephrosis were observed by electron microscopy (×15000). (A) rats treated with 20 mg/kg of ATRA; (B) rats treated with vehicle alone. The effacement of the foot processes is less serious in rats treated with ATRA than that in untreated rats. The representative images are shown.
nephrosis rats to ATRA presented a striking contrast to that of normal rats. PAN nephrosis rats partially recovered the normal expression pattern of nephrin even though the expression level was still lower than the normal (Figure 6A, c and d). Western blot analyses confirmed that nephrin protein was dramatically reduced in PAN nephrosis rats and that the reduction was mild when rats were treated with ATRA (Figure 6B). These data indicate that ATRA regulates the expression of nephrin mRNA and its protein product.

**RARE in the Promoter Region of Human Nephrin Gene**

To examine whether ATRA and presumably other active metabolites of vitamin A directly affect the transcription of nephrin gene, we retrieved the sequence of human nephrin gene (NPHS1) from the existing database and analyzed it. As shown in Figure 7, there were at least three putative retinoic acid response elements (RARE) within 2-kb of the promoter region. We made a plasmid construct harboring the 2-kb of the promoter region or the 325-bp fragment including the two putative RARE: (#-774~#-757) and (#-1041~#-1021), and we measured the enhancer activity in HeLa cells. HeLa cells were chosen as the host cell because the cells express several known retinoic acid response elements (RARE) within 2-kb of the promoter region. We made a plasmid construct harboring the 2-kb of the promoter region or the 325-bp fragment including the two putative RARE: (#-774~#-757) and (#-1041~#-1021), and we measured the enhancer activity in HeLa cells. HeLa cells were chosen as the host cell because the cells express several known types of receptors for retinoids (21). Transcriptional activity of the 2-kb promoter region was clearly augmented by ATRA in a dose-dependent manner and increased by fourfold in the presence of 10^{-5} M of ATRA (Figure 8A). Apparently, this activation was achieved through RAR bound to ATRA because BMS-189453, an antagonist for all types of RAR (22), canceled the ATRA-dependent transactivation in a dose-dependent manner (Figure 8B). Putative nonspecific cytotoxicity that might be brought by BMS-189453 did not account for the antagonization because the toxicity, if any, was detected as the decline in the activity of Renilla luciferase that was driven independent of ATRA. To further specify the element responding to ATRA, we performed a similar assay using the 325-bp region. As shown in Figure 8A, this region exerted much stronger transcriptional activity than the longer 2-kb region in the presence of ATRA. The maximum activation was achieved at the concentration of 10^{-6} M of ATRA, and the activity did not show any further increase at 10^{-5} M. This result indicates that the 325-bp region contains the RARE as the enhancer.

**Discussion**

Usefulness of ATRA and other derivatives of vitamin A (retinoids) is confirmed or proposed in a wide range of disease entities such as dermatitis, leukemia, and stenotic arterial diseases (23–27). In proliferating renal diseases, exogenous administration of ATRA, 9-cis-retinoic acid, or 13-cis-retinoic acid suppresses the proliferation of mesangial cells (26,27). However, even though anti-proteinuric effects are observed in these studies (26,27), the precise modes of action exerted by retinoids and the molecular targets of retinoids remain unknown. In this study, we demonstrated that injured podocytes require ATRA or other related retinoids to repair themselves. In addition, we have revealed a novel molecular mechanism that the podocytes rely on.

The effacement of the foot processes reportedly reached the maximum on day 5 in PAN nephrosis rats, and the amount of proteinuria reached the maximum on day 5 (28). The temporal expression pattern of RALDH2 closely correlated to the perturbation of the normal glomerular functions and structures (Figure 1) (28). Therefore, it is most likely that injured podocytes elevated the intracellular concentration of ATRA using the catalytic activity of RALDH2 that is upregulated in their own cell bodies. We also found out that RALDH2 was expressed in the developing renal epithelium such as the comma-shaped body, the S-shaped body, and weakly in the capillary loop stage epithelium (unpublished data), which suggested RALDH2 might be essential for podocyte development. The localization of RARα supports the significance of ATRA in the podocytes (Figure 2). Now, what is the role of ATRA in the podocytes?

The normal structure of the slit diaphragm is essential for preventing serum protein from leaking out of glomerular capillaries. Disruption of the slit diaphragm is histologically recognized as the effacement of the foot processes projected by the podocytes, which is typically observed in human nephrotic syndrome such as minimal change nephrotic syndrome (MCNS). In response to appropriate therapy including the administration of glucocorticoid, proteinuria in MCNS ameliorates along with the recovery of the slit diaphragm. PAN nephrosis, which is accompanied by the foot process effacement and focal detachment of podocytes from the glomerular basement membrane (GBM) (29,30), mimics human MCNS, at least in a sense that PAN nephrosis shows temporal massive proteinuria and subsequent complete remission without any scar in the kidney. Many investigators have studied the molecular mechanisms of PAN-induced proteinuria (31–35) and
demonstrated that nephrin is one of the most responsible genes (12,17). Nephrin, discovered as the gene whose mutation causes Finnish type of congenital nephrotic syndrome (36), is localized to the slit diaphragm.

We demonstrated here that ATRA directly enhance the transcription of nephrin gene (Figure 8). However, there are several mechanisms how ATRA and other retinoids regulate the target genes. The first mechanism is through a change in the rate of gene transcription (37). The second one involves a change in the stability or half-life of a particular mRNA in response to retinoids (38). In addition, the transcriptional regulation by ATRA can be divided into two types, the direct one and the indirect one. The direct regulation by ATRA is generally rapid and mediated through direct binding of RAR or RAR-RXR complexes to the RARE (37). On the contrary, the indirect regulation appears later, and no direct binding of RAR or RAR-RXR to the RARE is observed (37). Whereas ATRA enhanced the transcription of nephrin through the direct regulation, we have no data to know whether ATRA changes the stability or half-life of nephrin mRNA. The 2-kb promoter region of human nephrin gene has three feasible RARE, one direct repeat, one palindrome, and one complex type (Figure 7). Each element matches the consensus sequence with minor variation, which suggests that each of the regions has a weak

Figure 6. (A) Immunofluorescence image of nephrin. Kidney sections prepared from normal rats, normal rats treated with ATRA, PAN nephrotic rats on day 5, and PAN nephrotic rats treated with ATRA on day 5 were stained with anti-nephrin antibody in combination with Texas Red-conjugated secondary antibody. (a) Normal rats treated with vehicle alone (×400). (b) Normal rats treated with 20 mg/kg of ATRA (×400). (c) PAN nephrotic rats treated with vehicle alone (×400). (d) PAN nephrotic rats treated with 20 mg/kg of ATRA (×400). (B) Western blot analyses of nephrin. Glomeruli were isolated from the four groups of rats. Twenty milligrams of protein was subjected to each lane. Lane a, normal rats treated with vehicle alone; lane b, normal rats treated with 20 mg/kg of ATRA; lane c, PAN nephrotic rats treated with vehicle alone; lane d, PAN nephrotic rats treated with 20 mg/kg of ATRA.
response to ATRA. According to our results obtained from HeLa cells, at least the region #−1041 through #−1021, the region #−774 through #−757, or both are the functional RARE (Figure 8). If cells expressing transacting factors essential for nephrin were used instead of HeLa cells, the response to ATRA would be more remarkable.

Although the demand for ATRA increases during the repair process of the injured podocytes, those might result from temporally increased consumption of retinoids within the cells. Given the case, one possible explanation is that oxidative stress in glomeruli of PAN nephrosis rats (39) could lead to acute degradation of retinoids and to lack of retinoids because the oxidation step through CYP26 is one of the natural degradation mechanisms for ATRA (40). This is an intriguing hypothesis that might account for the perturbation of the slit diaphragm in PAN nephrosis or other podocyte injuries. However, our result that normal rats fed with a vitamin A−deficient diet did not spontaneously develop nephrosis implies that the low concentration of ATRA in the podocytes is not a direct cause of the perturbed slit diaphragm. Because it is difficult to measure the intracellular concentration of ATRA and/or other retinoids in the podocytes, different strategies will be required to address this question. It is reported that the distribution of RALDH2 provides the most accurate guide to the localization of ATRA (41–44). Therefore, studying the transcriptional regulation of RALDH2 gene might be informative to further understand the temporal change of intracellular concentration of ATRA.

We propose a mechanism that the injured podocytes take on to repair the disturbed slit diaphragm. First, the core system to produce active metabolites of vitamin A is upregulated. At this point, sufficient amount of the substrates such as vitamin A and β-carotene are required as the substrate. It can be expected that the significant lack of vitamin A retard the repair. Second, active metabolites including ATRA bind to retinoid receptors that are constitutively expressed in the podocytes and then transcribe their target genes. Similar mechanisms might work in human diseases because the expression of nephrin also decreases in human acquired nephrotic syndrome (45–47). In addition, ATRA might play a direct role to organize the structural components for the slit diaphragm. Our results also suggest that the disturbance of the retinoids signaling in the podocytes could be one of causes leading to progressing renal diseases.

Figure 7. The 2-kb promoter region of the human nephrin gene (NPHS1) was retrieved from the existing database and analyzed. Three putative retinoic acid response elements (RARE) are found. The numbers indicate the position of the nucleotide counted from the transcriptional start site that is reported by Wong et al. (48).

Figure 8. The enhancer activity of the 2-kb and the 325-bp promoter regions (#−1060 through #−735) was examined as described in Materials and Methods. (A) HeLa cells transfected with the Firefly luciferase reporter plasmid harboring the 2-kb region (closed circles) or the 325-bp region (open circles) was stimulated with various concentration of ATRA. The pGL-2 promoter vector was transfected to the cells as a control (open squares). (B) Luciferase assay was performed in the presence of various concentration of BMS-189453 by using the plasmid harboring the 2-kb promoter region. Transfection efficiency was adjusted by the activity of the cotransfected Renilla luciferase. The results are shown as the ratio of Firefly luciferase activity to Renilla luciferase activity (mean ± SD; n = 3 at each points). Each values was compared with the basal value, and the statistical significance was indicated as follows: * P < 0.04; ** P < 0.04; *** P < 0.02; * P < 0.05; ## P < 0.05.
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