Protein Gene Product 9.5 Is Selectively Localized in Parietal Epithelial Cells of Bowman’s Capsule in the Rat Kidney

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Abstract. Parietal epithelial cells (PEC) of Bowman’s capsules cover the inner aspect of Bowman’s capsules and are believed to contribute to extracapillary lesions of glomerulonephritis such as crescent formation. In glomerular research including cell culture experiments and pathology, differentiation between PEC and podocytes has frequently been a major problem. Immunohistochemistry of the adult rat kidney for protein gene product 9.5 (PGP 9.5), a neuron-specific ubiquitin C-terminal hydrolase, demonstrated selective localization of the immunoreactivity in PEC. At the urinary pole of the glomerulus, immunoreactive PEC were clearly differentiated from proximal tubular cells that were negative for PGP 9.5. In the subcapsular nephrogenic zone of newborn rat kidney, immunoreactivity was observed in almost all cells in the comma-shaped body and early S-shaped body and selectively in PEC in the late S-shaped body and capillary-stage glomerulus. In rat glomerular disease models (Masugi-nephritis and puromycin aminonucleoside nephrosis), cells that consisted of cellular crescents or adhered to glomerular tufts were positive for PGP 9.5. The selective localization of PGP 9.5 in PEC in rat kidney provides a new cytochemical marker for identifying the cells. Developmental expression of the protein suggests that PGP 9.5 is involved in the processes of nephrogenesis of rat kidney.

Parietal epithelial cells (PEC) of Bowman’s capsules are flat cells that cover the inner aspect of Bowman’s capsules. Flat PEC undergo a sudden transition to taller proximal epithelial cells at the urinary pole and are continuous with the visceral epithelial cells (podocytes) at the vascular pole. PEC and podocytes develop from the same primodium, but the proliferative capacity of these cells is different. The PEC exhibits an active proliferation even in normal conditions (1), whereas the podocyte is a terminally differentiated cell (2). The proliferation of PEC in response to pathologic stimuli seems to contribute to extracapillary lesions of glomerulonephritis. However, podocytes have recently been reported to dedifferentiate and proliferate in specific conditions such as HIV-associated and idiopathic-collapsing glomerulopathy (3). Accordingly, the differentiation between parietal and visceral epithelial cells has become a major problem in pathology.

Protein gene product 9.5 (PGP 9.5) is a neuron- and neuroendocrine cell-specific ubiquitin C-terminal hydrolase isoenzyme L1 that generates free active monomeric ubiquitin (4,5). Immunohistochemical studies revealed the localization of PGP 9.5 in all central and peripheral neurons as well as in cells of the ovary, testis, and synovial membrane (6–8). The present study reports the immunohistochemical localization of PGP 9.5 in the normal rat kidney and rat glomerular disease models. Immunostaining using a monoclonal antibody against PGP 9.5 demonstrated the selective localization of PGP 9.5 in PEC, which has not been reported previously.

Materials and Methods

Animals

All animal experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Adult male Sprague-Dawley (SD) and Wister-Kyoto (WKY) rats (weighing approximately 200 g) were obtained from Charles River Japan (Kanagawa, Japan). Newborn SD rats (4 d after birth) were obtained from the same company immediately before the experiments.

Induction of Glomerular Disease Models

Masugi-nephritis was induced in WKY rats (n = 4) by a single intravenous injection of rabbit anti-rat glomerular basement membrane serum (50 μl/100 g body wt). Puromycin aminonucleoside (PAN) nephrosis was induced in SD rats (n = 4) by a single intraperitoneal injection of PAN (15 mg/100 g body wt, dissolved in 0.9% saline). Development of both diseases was confirmed by the appearance of proteinuria at day 4.

Immunohistochemistry

Adult rat kidneys of normal SD rat (n = 4), day-7 Masugi-nephritis WKY rat, and day-28 PAN nephrosis SD rat were perfusion fixed. After the rats were anesthetized with Nembutal (100 mg/kg body wt),
the kidneys were retrogradely perfused via the abdominal aorta for 5 min with 4% paraformaldehyde in 0.1 mol/L phosphate buffer (PB), followed for 5 min with 20% sucrose in 0.01 mol/L phosphate-buffered saline (PBS). Then kidneys were removed and dipped in 20% sucrose solution at 4°C overnight. Finally, kidneys were cut into several pieces and shock-frozen in isopentane cooled with liquid nitrogen. Kidneys from newborn rats (n = 4) were immersed in 4% paraformaldehyde in 0.1 mol/L PB for 2 h at 4°C and processed as for adult rat kidneys.

Frozen sections (5 μm thick) were immunostained by indirect immunofluorescence. As the primary antibody (Ab), a mouse monoclonal anti-human PGP 9.5 monoclonal antibody (Clone 13C4, Ultraclone, Wellow Isle of Wight, England) was used. The secondary Ab was FITC-conjugated goat anti-mouse IgG antiserum (Cappel, ICN

Figure 1. Immunostaining of an adult rat kidney for protein gene product 9.5 (PGP 9.5). (a) Low-magnification view of cortex demonstrates localization of PGP 9.5 in nerve fibers around a small artery (A) and along Bowman’s capsules of glomeruli (G). (b) In a section of glomerulus containing the urinary pole, parietal epithelial cells (PEC) are sharply separated from proximal epithelial cells by immunoreactivity (arrows). (c) A section of glomerulus containing the vascular pole. The expression of PGP 9.5 in PEC exhibits a continuous fluorescence over all of the PEC. The transition from PEC to podocytes is clearly demonstrated (arrows). Nerve fibers (arrowheads) distributed around a vascular pole are visualized. A nerve bundle (small arrow) and nerve fibers (double arrowheads) are also observed around a small artery (A). Magnifications: approximately ×110 in a; approximately ×370 in b and c.
Pharmaceuticals, Aurora, OH). After the sections were blocked with PBS containing 2% bovine serum albumin, 2% fetal calf serum, and 0.2% fish gelatin for 15 min, they were incubated with primary Ab diluted 1:100 in blocking solution at room temperature for 60 min. The sections were then incubated with secondary Ab diluted 1:100 in blocking solution at room temperature for 30 min.

**Immunoprecipitation**

Isolated glomeruli and brain from normal adult SD rats \((n = 2)\) were examined. Glomeruli were isolated by the differential sieving method (9). Samples were lysed in 0.5% NP-40 buffer (0.5% NP-40, 150 mM NaCl, 50 mM Tris-HCl [pH 7.5], 0.05% sodium dodecyl sulfate [SDS], 1 mM ethylenediaminetetraacetate) containing proteinase inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mM pepstatin A, 1 mM E-64C) on ice. Insoluble tissue lysates were discarded by centrifugation at 15,000 \(\times\) g for 5 min and precleared with protein G-PLUS-Agarose (Santa Cruz Biotechnology Inc., Santa Cruz, CA) at 4°C for 1 h under constant rotation. The precleared lysates were then subjected to immunoprecipitation with a mouse monoclonal Ab (13C4). The immunoprecipitates were collected on protein G-PLUS-Agarose, washed with NP-40 buffer, and extracted from the beads by boiling in SDS sample buffer. They were analyzed by SDS-polyacrylamide gel electrophoresis (12% polyacrylamide gel) with 2-mercaptoethanol and electrophoretically transferred to a polyvinylidine fluoride membrane (Nihon Millipore, Yamagata, Japan). Detection of the association was finally determined by mouse monoclonal Ab (13C4) and subsequent secondary Ab (horseradish peroxidase–conjugated anti-mouse IgG antiserum, Cappel) in enhanced chemiluminescence reagent (Amersham Pharmacia Biotech, Piscataway, NJ).

**Reverse Transcription-PCR**

Total RNA of isolated glomeruli and brain from adult SD rats \((n = 2)\) were extracted by the acid guanidium thiocyanate-phenol-chloroform method. One \(\mu\)g of total RNA was reverse-transcribed using oligo(dT) primers (Life Technologies, Rockville, MD) and reverse transcriptase (Superscript II, Life Technologies). The single-strand cDNA product was denatured and amplified in GeneAmp PCR system 9600 (Perkin-Elmer, Norwalk, CT) with two primers under the fol-

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**Figure 2.** Immunostaining of the newborn rat kidney for PGP 9.5. (a) In the subcapsular nephrogenic zone, structures of various stages of nephrogenesis are positive for PGP 9.5. In the left half, where nephrogenesis is almost complete, positive immunostaining for PGP 9.5 is observed only in PEC of maturing stage glomeruli (*). (b) In comma-shaped bodies, cells are diffusely positive for PGP 9.5. (c) In an S-shaped body, presumptive PEC are clearly positive for PGP 9.5. In other cells, including presumptive podocytes, immunoreactivity is weak. Magnifications: approximately \(\times 110\) in a; approximately \(\times 370\) in b and c.

**Figure 3.** Immunostaining of Masugi-nephritis and puromycin aminonucleoside (PAN) nephrosis rat kidney for PGP 9.5. (a) In a glomerulus on day-7 Masugi-nephritis with cellular crescents, most epithelial cells filling Bowman’s space are positive for PGP 9.5. (b) In a glomerulus on day-28 PAN nephrosis with tuft adhesion, PEC arising from Bowman’s capsule and covering the head of the adhering tuft are positive for PGP 9.5. Magnification: \(\times 370\).
following conditions: 1 min at 94°C, 1 min at 55 to 60°C, and 2 min at 72°C for 28 cycles (for PGP 9.5 of rat brain and for glyceraldehyde phosphate dehydrogenase) or for 35 cycles (for PGP 9.5 of isolated glomeruli). Amplification primer pairs for PGP 9.5 were as follows: forward 5’-GGAGATTAACCCCGAGATGC-3’ and reverse 5’-GAGCCACTGCGGAGAAGCGG-3’. The primer pairs of GAPDH (forward 5’-ACCACCATGGAGAAGGCTGG-3’ and reverse 5’-GGTTTCTTACTCCTTGGAGG-3’) were used as control for reverse transcription-PCR (RT-PCR). The amplified samples were electrophoresed with 2% agarose gel, stained with ethidium bromide, and photographed under an ultraviolet lamp.

Results

Immunohistochemistry

Adult Rat Kidney. In the cortex of the normal adult rat kidney, PEC of Bowman’s capsules and nerve fibers running along small arteries and arterioles showed a positive reaction for PGP 9.5. The expression in PEC displayed a continuous fluorescence over all of the PEC, but the intensity of the immunoreactivity was somewhat variable. At the urinary pole, the transition from PEC to proximal tubular cells was clearly visualized by positive PEC and negative proximal tubular cells. At the vascular pole, PEC were clearly separated from podocytes by the immunoreactivity. Arterioles that entered and exited the glomerular tuft were negative, whereas nerve fibers that ran along them were intensely stained just before the entrance of the glomerulus. Other components of the cortex, including glomerular tufts, tubuli, and interstitial cells, did not react with PGP 9.5 (Figure 1).

Newborn Rat Kidney. Various stages of nephron formation were detected in the subcapsular nephrogenic zone of newborn rat kidney. Immunofluorescence microscopy of newborn rat kidney revealed that the expression of PGP 9.5 was clearly related to the developmental stages of the nephron. Cells that formed a comma-shaped body exhibited a diffuse cytoplasmic immunoreactivity for PGP 9.5. Predominant expression of PGP 9.5 was initially detected in presumptive PEC in S-shaped body and early capillary-stage glomeruli. At these stages, presumptive podocytes gradually lost their immunoreactivity. Thereafter, at the capillary and maturing stage, the positive reaction for PGP 9.5 disappeared from most parts of the nephron and was maintained only in PEC. Tubular elements were positive in their cytoplasm for PGP 9.5 at early stages of nephrogenesis (comma-shaped and S-shaped bodies), then lost their immunoreactivities during the subsequent stages of nephrogenesis (Figure 2).

Masugi Nephritis and PAN Nephrosis Rat Kidney. Pathologic lesions of Masugi-nephritis induced in WKY rats were characterized by prominent crescent formation as well as intraglomerular hypercellularity (10). At day 7, crescents observed in most of the glomeruli were largely composed of proliferating epithelial cells. In immunohistochemistry, these cells were positive for PGP 9.5, indicating that PEC are major components of cellular crescents in the early phase of this model.

In PAN nephrosis at day 28, glomeruli were almost recovered from PAN-induced podocyte damage, but glomerular tuft adhesion was occasionally seen. PEC that consisted of adhesive lesions were clearly positive for PGP 9.5, confirming the contribution of PEC for the formation of adhesion as reported previously (11) (Figure 3).

Immunoprecipitation and RT-PCR of Isolated Glomeruli from Adult Rat Kidney

Immunoprecipitation demonstrated a single immunoreactive band, at approximately 27 kD, in the extract of brain and isolated glomeruli from normal adult SD rats (Figure 4).

Using RT-PCR, a single band of 637 bp for PGP 9.5 was detected in isolated glomeruli from normal adult SD rat kidney as well as in the brain (Figure 5).

Discussion

Localization of the immunoreactivity for PGP 9.5 in PEC of Bowman’s capsule in the rat kidney is unusual from the general viewpoint of selective localization of PGP 9.5 in neurons and some functionally related cells (4). Although we did not check the specificity of the immunoreactivity by a preincubation test with a corresponding antigen, PEC and nerve fibers in the same section showed positive immunostaining for the protein. Immunoprecipitation revealed an identical molecular weight for the immunoreactive substance between isolated glomeruli and the brain from adult rat. Moreover, RT-PCR demonstrated the expression of mRNA for PGP 9.5 in isolated glomeruli. Because isolated glomeruli contains no nerve fibers, it is reasonable to consider that glomerular PGP 9.5 immunoreactivity and mRNA are derived exclusively from PEC. Thus, our findings
strongly suggest that PGP 9.5 itself is contained in PEC of rat kidney.

Functionally, PGP 9.5 belongs to the ubiquitin C-terminal hydrolase (UCH) family (5), which regulates ubiquitin-mediated proteolysis by cleavage of amide bonds at the C-terminus of ubiquitin (12). It is becoming increasingly clear that these enzymes are a part of the cellular proteolytic pathway that regulates many processes that are essential for various cellular functions, including cellular differentiation (13,14) and cell cycle regulation (15). This multifunctional role of the UCH family in cellular functions suggests that PGP 9.5 expressed in newborn and adult rat kidney contributes to nephrogenesis and the cellular function of PEC.

In the present study, the expression of PGP 9.5 was clearly related to the developmental stages of the nephron. There was abundant expression in early stages of the nephron precursor, and this expression continuously decreased thereafter, disappearing from most parts of the nephron and finally remained only in PEC. These changes point to essential involvement in the developmental process. In the early stages of nephrogenesis, cells proliferate and begin a differentiation to produce the epithelial cells of glomeruli and tubules. On the basis of recent findings that the UCH family, including PGP 9.5, contributes to cellular differentiation and cell cycle progression as described above, it is reasonable to assume that PGP 9.5 expressed in early stages of nephron precursor is one of the important molecules for nephrogenesis. Cytoplasmic localization of PGP 9.5 in nephron precursor cells supports this assumption, because the same localization of the protein was reported in neuronal cells under differentiation and maturation (16,17).

The continuing strong expression of PGP 9.5 in PEC of the adult rat kidney suggests that some specific function of PEC might be regulated by the ubiquitin-mediated proteolysis system. Background data permitting speculation about specific functions of the protein are lacking, but it is noteworthy that the expression is not uniform among PEC. The same difference is also recognized more prominently in the expression of cytokeratins (18,19). As demonstrated in other cultured epithelial cells (20), there may be differences in the cellular condition or function among PEC, resulting in the heterogeneous expression of the proteins.

Even if the specific function of PGP 9.5 for PEC is not determined by the data presented in this study, the selective expression of the protein in PEC is significant in cell culture research and in pathology of rats. In cell culture experiments on podocytes, it has always been a problem to distinguish whether the outgrowing cells from isolated glomeruli are of parietal or visceral origin (21,22). On the basis of our findings, PGP 9.5 should contribute to a separation of cultured cells into podocytes and PEC. Moreover, discrimination between parietal and visceral epithelial cells has also been a major problem in pathology of experimental and human glomerulonephritis (3,23,24). The possibility of discrimination between these two cell types using PGP 9.5 should reopen the problem of how crescents develop under various experimental conditions in rats.

In summary, our results suggest that PGP 9.5 is a good marker for PEC to analyze the contribution of PEC to various extracapillary lesions in rat glomerular disease models. Furthermore, it is suggested that the ubiquitin-mediated proteolysis system plays an important role in nephron development and the function of PEC of rat kidney.

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References


