Apical Cell Membranes Are Shed into Urine from Injured Podocytes: A Novel Phenomenon of Podocyte Injury

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Previously it was shown that urine from patients with nephritis contains podocytes and their fragments (podocalyxin [PCX]-positive granular structures [PPGS]), reflecting the degree of podocyte injury. The present study was designed to trace PPGS to their origin. Urine samples and renal biopsy specimens from 53 children with nephrotic syndrome and nephritis were examined immunohistochemically. Immunofluorescence studies of kidney sections using an anti-PCX antibody demonstrated that PPGS originated from the glomerulus and flowed into the tubular lumen. Electron microscopic examination revealed that PPGS originated from microvillous or vesicle-like structures on injured podocytes in the glomerulus. For examining the origin of the PPGS, apical, slit-diaphragmatic, and basal portions of the podocytes were specifically stained, revealing that PPGS are composed primarily of apical podocyte membranes. Several newly developed antibodies that are reactive with various segments of the PCX molecule were used to analyze more detailed membrane structures, and it was found that PPGS contained intact PCX molecules, indicating that cell membrane structures are excreted in urine. The quantification of PCX content and podocyte numbers revealed that urinary sediment PCX (u-sed-PCX) content per urinary podocyte was much higher than PCX content per podocyte from isolated glomeruli of normal controls, suggesting that u-sed-PCX are derived from sources other than just the cell debris of detached podocytes. Analysis of the correlation between u-sed-PCX and renal histology revealed that the presence of PPGS reflects acute glomerular injury. In conclusion, podocyte apical cell membranes are shed into the urine from injured podocytes, indicating a previously unrecognized manifestation of podocyte injury.

J Am Soc Nephrol 16: 408–416, 2005. doi: 10.1681/ASN.2004070564

The podocyte is a highly differentiated cell that has characteristic interdigitating foot processes that cover the outer surface of the glomerular basement membrane (GBM) in the kidney (1). The turnover rate of podocytes is very low under normal and various pathologic conditions compared with that of other glomerular cells (2,3). Meanwhile, podocytes contribute to the hydraulic permeability of the glomerulus and play a crucial role as a filter for macromolecules (1). Because of these biologic and morphologic characteristics of podocytes, injuries to podocytes are accompanied by characteristic changes in morphology, as observed by electron microscopy (EM), including effacement of foot processes, microvillous transformation, and occasional detachment from the GBM (4–7). In several immunologic and nonimmunologic forms of glomerulonephritis, the podocyte is the primary target of injury (8,9). Podocyte injury is also a key event leading to glomerular sclerosis. Recent studies have revealed that the denuded GBM left behind after a podocyte becomes detached and subsequently adheres to parietal epithelial cells, resulting in the formation of a synechia of the glomerular tuft to Bowman's capsule, which represents the earliest stage of segmental sclerosis (10,11).

We recently demonstrated the presence of podocytes and their cell fragments in the urinary sediment of patients with glomerular diseases, in an immunofluorescence (IF) study using a specific monoclonal antibody against podocalyxin (PCX), a glycoprotein that is prominently expressed on podocytes (12). Quantification of urinary podocytes has clinical significance in its ability to predict acute glomerular lesions (13). In addition to urinary podocytes, urine sediments from nephritic patients contain PCX-positive granular structures (PPGS) in or around the urine casts. We hypothesized that these structures represent urinary podocytes and their cell debris. We subsequently found that PPGS are excreted in the urine in far greater numbers compared with urinary podocytes. However, because we also found PPGS in the urine of patients without any urinary podocytes, we questioned whether these structures truly represent cell debris from detached podocytes. Thus, the purpose of the present study was to trace PPGS to their origin immunohistochemically.

Materials and Methods

Patients, Urine Samples, and Kidney Specimens

Urine samples voided in the morning were obtained from 50 healthy children and adolescents (25 male and 25 female; mean age, 12.3 yr; range, 3 to 20 yr) and 53 patients with active glomerulonephritis or nephrotic syndrome (29 male and 24 female; mean age, 11.3 yr; range,

Received July 16, 2004. Accepted November 16, 2004.

Published online ahead of print. Publication date available at www.jasn.org.

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ISSN: 1046-6673/1602-0408

3 to 23 yr) during the period 1997 to 2001. The renal diseases included IgA nephropathy (IgAN; 20 cases; mean proteinuric level, 0.78 g/d; range, 0.31 to 2.56 g/d), nephrotic syndrome (15 cases, steroid sensitive; mean proteinuric level, 1.32 g/d; range, 0.89 to 5.2 g/d), lupus nephritis (LN; 6 cases; mean proteinuric level, 1.13 mg/dl; range, 0.56 to 5.6 g/d), and Henoch-Schoenlein purpura nephritis (HSPN; 12 cases; mean proteinuric level, 1.25 g/dl; range, 0.45 to 3.14 g/d). In addition to routine urinalysis, urine samples were processed for urinary podocyte analysis, quantification of urinary sediment PCX (u-sed-PCX), IF, and immunoelectron microscopic (IEM) studies of urine sediments. Renal biopsies performed during this study period included 26 patients: IgAN (n = 15), HSPN (n = 7), and LN (n = 4). As a normal control, the kidney specimens from autopsy or normal parts of nephrectomized kidney were used. This study was approved by the ethics committee of the Yoshida Hospital. Informed consent was obtained from the patients or the parents.

Immunofluorescence

The urine sediments and frozen sections from renal biopsy specimens were stained using the various primary and secondary antibodies in the same manner as described previously (12). The primary antibodies used were as follows: anti-podocalyxin: monoclonal (mouse), PHM5 (Australian Monoclonal Development, Artarmon, Australia) (14); anti-CR1: monoclonal (mouse), clone; To5 (DAKO, Glostrup, Denmark); anti-GLEPP1: monoclonal, clone; 5C11, a gift from Dr. Roger Wiggins (15); anti-nephrin: polyclonal (rabbit), supplied by Dr. Hiroshi Kawachi (16); anti–ZO-1: polyclonal (rabbit; Zymed Laboratories, South San Francisco, CA); anti-α3 integrin: polyclonal (rabbit; Chemicon, Temecula, CA); anti-ezrin: monoclonal (rat), clone; M11, (Sanko Junyaku, Tokyo, Japan); and anti–β-actin: monoclonal (mouse), clone AC-15 (Abcam, Cambridge, UK). The secondary antibodies used were as follows: FITC-labeled anti-mouse IgG (Cappel, Chester, PA), TRITC-labeled anti-mouse IgG (Cappel), FITC-labeled anti-rat IgG (Cappel), FITC-labeled anti-rabbit IgG (Cappel), and Cy3 conjugated anti-mouse IgG+IgM (Chemicon). Alexa488-conjugated phalloidin (Molecular Probes, Eugene, OR) was used.

To determine whether PPGS come from a particular subcellular portion of podocytes, we performed IF study on urine sediments using various antibodies that react with three distinct subcellular regions of podocytes: (*1*) the apical region of podocytes using anti-PCX, anti-GLEPP1, and anti-CR1 antibodies; (*2*) the slit diaphragm region using anti-nephrin and anti–ZO-1 antibodies; and (*3*) the basal region using the anti-α3 integrin antibody. To examine the detailed relationship between PCX and cytoskeletal molecules, we performed IF study to detect ezrin and actin in urine sediments using antibodies to ezrin, β-actin, and Alexa488-labeled phalloidin.

Histologic Examination (LM, EM, IEM)

Twenty-six renal biopsy specimens were analyzed histologically from patients in whom u-sed-PCX levels were measured at the time of renal biopsy. The following pathologic parameters were analyzed according to the criteria of Shigematsu *et al.* (17): extracapillary change (acute and chronic), intracapillary change (acute and chronic), and tubulointerstitial change (acute and chronic). Pathologists who were blinded to u-sed-PCX levels performed the pathologic examination. Among 26 renal biopsy specimens, 10 specimens including five cases with IgAN, three cases with HSPN, and two cases with LN, were processed for EM examination. The ultrastructural configuration of podocytes in the urine sediments obtained from five patients with HSPN (two cases) and IgAN (three cases) was examined by IEM examination as reported previously (12).

Quantification of Urinary Podocytes (U-Podocyte Test)

The number of urinary podocytes was counted as reported previously (18). The number of podocytes in urine was expressed as cells/ml.

Production of Monoclonal Antibodies to PCX

cDNA coding for the human PCX was obtained from the human kidney cDNA library using reverse transcriptase–PCR based on the method by Kershaw *et al.* (19). Two kinds of GST fusion protein (PC-46, whole portion of PCX; PC-35, extracellular portion) were obtained to produce monoclonal antibodies and characterization of antibodies. The GST fusion protein that contained the intracellular portion of rabbit PCX (RBT-Intra) was provided by Dr. David Kershaw (University of Michigan, Ann Arbor, MI).

Monoclonal antibodies against human PCX were produced from BALB/C mice that were immunized with purified PC-46 by standard methods. The resulting hybridomas were grown in 96-well plates and selected and subcloned on the basis of IF pattern assayed on cryostat sections of human renal cortex or ELISA using the polystyrene multiwell plates coated with a wheat germ agglutinin-binding fraction in a Triton X-100 glomerular lysate as previously reported (20). Among 12 monoclonal antibodies, two clones (70-4, No45) were chosen and further characterized by Western blotting and ELISA. The monoclonal antibody (PHM5) that reacts with the carbohydrate portion of human podocalyxin was purchased.

The IgG fraction of PHM5 was affinity-purified using MEP Hyper Cel (Invitrogen, Carlsbad, CA). After purification, the IgG fraction was FITC-labeled using standard techniques (21).

ELISA

ELISA system was used to quantify PCX in various samples as previously reported (20). This system gave a linear plot over the range of 12.5 to 800 ng/ml. The detection limit of this ELISA assay was 6.25 ng/ml.

For screening, polystyrene multiwell plates were coated with a wheat germ agglutinin-binding fraction in a Triton X-100 glomerular lysate served as a standard in the ELISA assay above at the concentration of 5 μg/ml. After blocking of the plate, the hybridoma culture supernatant was added and processed to the reaction of peroxidase (POD)-labeled anti-mouse IgG (Cappel) and the development of POD.

For the characterization of monoclonal antibodies, the polystyrene multiwell plates were coated with PC-46, PC-35, and RBT-Intra (0.2 μg/ml, respectively). The plate was incubated with 70-4, No45, and PHM5 (5 μg/well, respectively) at 37°C for 1 h. After washing, the plate was further incubated with POD-labeled anti-mouse IgG (Cappel). The development of POD was processed using standard methods.

Western Blot

Detection of protein in the human glomerular lysate was analyzed by Western blotting according to the procedures described previously (20).

Quantification of Podocyte Number per Glomerulus

The kidney specimens were obtained at the time of autopsy from three patients (72-yr-old man, 78-yr-old woman, and 82-yr-old woman) who died of diseases other than kidney diseases. The number of podocytes per glomerulus was calculated on the basis of the method of Hishiki *et al.* (22).

Quantification of PCX of Isolated Glomeruli

Isolated glomeruli were obtained from the kidney specimens above using mesh sieving. The number of glomeruli in the suspension was counted, and after centrifugation, the pellet was dissolved in 0.2%

Triton X-100 in PBS (23) and left to stand for 1 h at room temperature. After centrifugation at 15,000 rpm for 5 min, the PCX content in the supernatant was measured in the same manner as in quantification of PCX in urine sediments.

Statistical Analyses

All data were expressed as the mean ± SEM. A comparison between the groups was made by unpaired *t* test. A comparison between PCX content/podocyte from urinary sediments and PCX/podocyte from isolated glomeruli was made by Mann-Whitney test because of a small number of samples. Differences between groups were considered to be significant at $P < 0.05$.

Results

PPGS Are Derived from Injured Podocytes

Urine sediments that stained with anti-PCX antibody were classified into two structures: (*1*) PCX-positive cells (podocytes) and (*2*) PPGS in any cases with nephrotic syndrome, HSPN, IgAN, and LN, as previously reported (12). PCX-positive cells and PPGS were frequently seen in the urine in cases during the acute phases of the diseases. A representative finding is shown in Figure 1. The location of the PPGS was frequently in the casts and occasionally around the cast in a scattered manner.

When frozen sections from normal and nephritic kidneys were stained with anti-PCX antibody (PHM5) and carefully examined, a slightly more granular appearance of the glomerular staining was found in nephritic kidneys compared with the glomeruli of normal kidneys. A representative figure of normal and nephritic glomeruli is shown in Figure 2, A and B, respectively. In addition, fine granular structures similar to urinary PPGS were occasionally found in the tubular lumen in several cases with HSPN with severe glomerular lesions (Figure 2C).

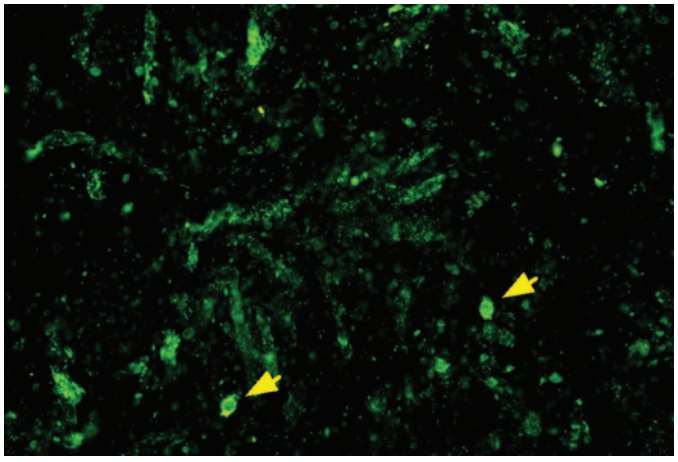

Figure 1. Immunofluorescence (IF) of urine sediments from a patient with Henoch-Schoenlein purpura nephritis (HSPN). Urine sediments that were cytospun on slide glass were stained with anti-podocalyxin (anti-PCX) antibody. Urine sediments contain numerous PCX-positive granular structures (PPGS) mainly in casts and also around casts. Several podocytes are seen (arrow). Magnification, ×200.

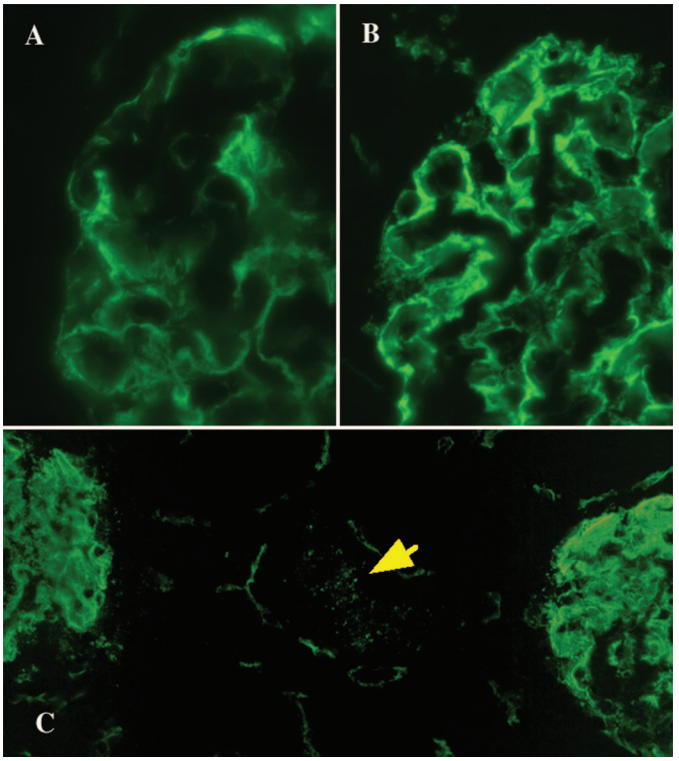

Figure 2. IF of kidney section from a patient with HSPN (A) and normal control (B). Podocytes in nephritic glomeruli were more granular in appearance than those of the normal kidney. The fine granular structures similar to PPGS (arrow) were found in the tubular lumen in a case with HSPN (C). Magnification, ×800 in A and B, ×200 in C.

PPGS Have Cell Polarity

IF study was performed on urine sediments using various antibodies that react with three distinct subcellular regions of podocytes: (*1*) the apical region of podocytes using anti-PCX, anti-GLEPP1, and anti-CR1 antibodies; (*2*) the slit diaphragm region using anti-nephrin and anti–ZO-1 antibodies; and (*3*) the basal region, using the anti-α3 integrin antibody. Urine sediments from various types of nephritis stained with anti-PCX intensely, with only occasional weak staining with anti-CR1 and GLEPP1. On the contrary, the urine sediments did not stain with anti-nephrin, anti–ZO-1, and anti-α3 integrin, suggesting that only apical portions of podocyte cell membranes ware excreted in the urine (Figure 3, A through C). Positive controls for all of these antibodies in normal kidney sections confirmed good staining with each antibody (Figure 3, A′ through C′).

Urine Sediments Have Vesicles of Podocytes Similar to Microvilli

EM of biopsy specimens demonstrated protuberant apical cell membranes on the podocyte surface, consistent with microvillous transformation. These changes of apical cell membranes were seen in several types of nephritis in this study and were most prominently in cases with heavy proteinuria (Figure 4).

IEM of urine sediments showed that urinary podocytes have numerous microvillous structures or vesicle-like structures on

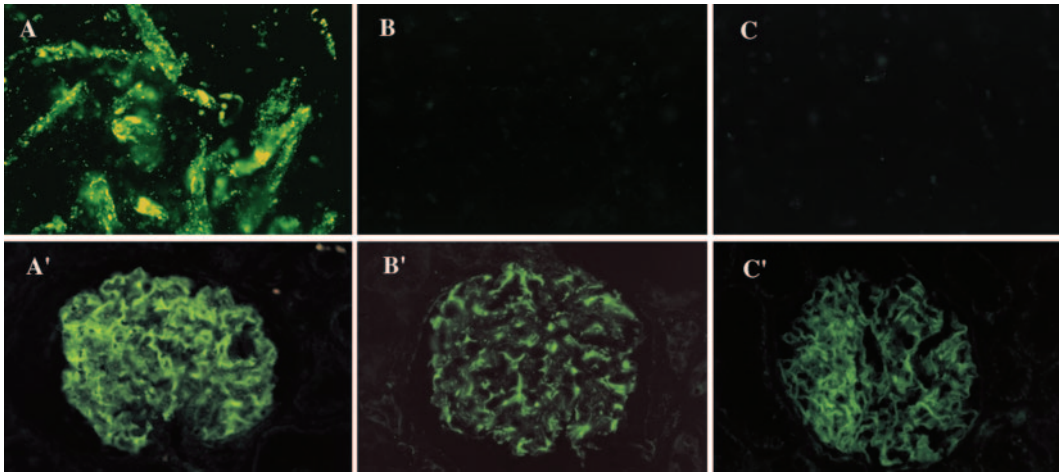

Figure 3. IF of urine sediment from a patient with IgA nephropathy using antibodies that react with different subcellular regions of podocyte: anti-PCX (reacting with apical cell membrane of podocyte), anti-nephrin (reacting with slit diaphragm region), and anti-α3 integrin (reacting with basal region of podocyte). Casts in the sediment are stained with anti-PCX (A) but not with anti-nephrin (B) and anti-α3 integrin (C). IF of kidney sections from normal control are in the lower panel, with anti-PCX (A′), anti-nephrin (B′), and anti-α3 integrin (C′). Magnification, ×400.

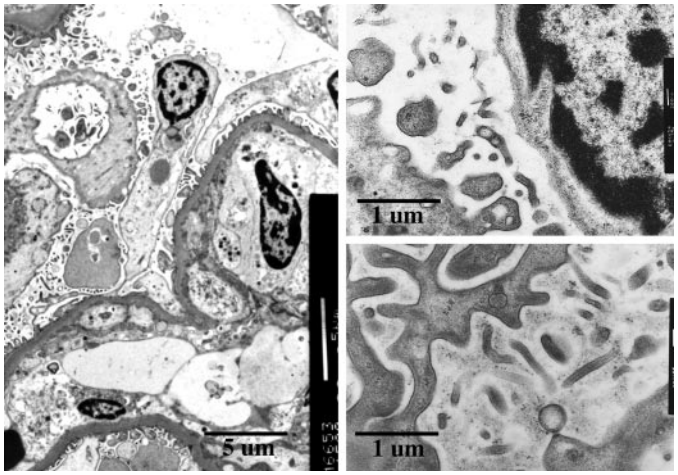

Figure 4. Electron microscopy (EM) of podocyte in the kidney section from a patient with HSPN. Numerous protuberant apical cell membranes on the podocyte surface (microvillous transformation) are seen. Size bar is indicated.

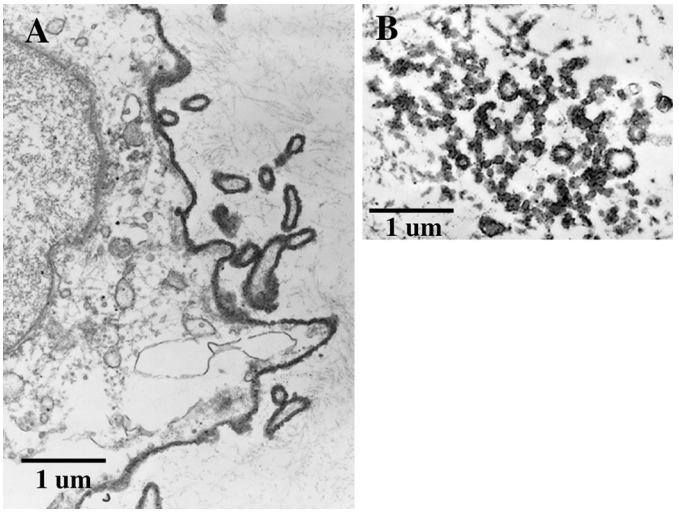

Figure 5. Immunoelectron microscopy (IEM) of urine sediments from a patient with IgA nephropathy. Numerous vesicles are seen on the cell surface of urinary podocytes (A). The vesicles with different size of double-layered cell membrane structures similar to those of urinary podocytes were observed as a cluster in the urine sediments (B). Size bars are indicated.

their cell surfaces. Vesicles that contained different sizes of double-layered cell membrane structures, similar to those seen near urinary podocytes, were often observed in clusters in the urine sediments (Figure 5).

PPGS Have Whole-Cell Membrane Structures

Antibody Characterization. Among several monoclonal antibodies raised in this study, we developed two kinds of antibodies (70-4 and No45). Western blot analysis of 70-4, No45, and PHM5 showed the bands with the same molecular weight, approximately 160 to 170 kD (Figure 6A). The examination using ELISA in which various antigens, including full-length podocalyxin (PC46), extracellular portion (PC35), and intracellular portion (RBT-Intra), were bound showed that (*1*) 70-4 are reactive with both PC46 and RBT-Intra and negative with PC35 and (*2*) No45 are reactive with PC46 and PC35 and negative with RBT-Intra, whereas PHM5 all are negative with PC46, PC35, and RBT-Intra (Figure 6B). These results clearly showed that 70-4 is a monoclonal antibody that recognizes the intracellular region of PCX, whereas No45 is a monoclonal antibody that recognizes the extracellular region of PCX. PHM is a monoclonal antibody that does not react with the protein portion of PCX. PHM5 is demonstrated as a clone that reacts with carbohydrate of podocalyxin (14).

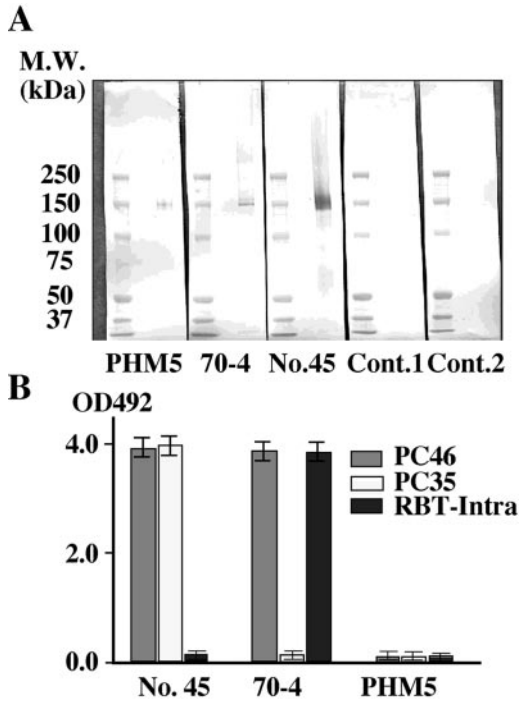

Figure 6. (A) Immunoblot analysis of glomerular lysates with PHM5 (recognizing sugar component of PCX), 70-4 (recognizing intracellular peptide of PCX), and No45 (recognizing extracellular peptide of PCX) showed the bands of PCX with molecular weight of approximately 160 to 170 kD. Antibody of control (RVG1, Cont. 1; and PBS, Cont. 2) are totally negative. (B) ELISA using plates coated with three kinds of antigens: full-length PCX (PC46), extracellular portion of PCX (PC35), and intracellular portion of PCX (RBT-Intra). Monoclonal antibody (70-4) is reactive with both PC46 and RBT-Intra and negative with PC35. Monoclonal antibody (No45) is reactive with PC45 and PC35 and negative with RBT-Intra. PHM5 are negative with PC46, PC35, and RBT-Intra. These results clearly showed that 70-4 is a monoclonal antibody that recognizes the intracellular portion of PCX, and No45 is a monoclonal antibody that recognizes the extracellular portion of PCX. PHM is a monoclonal antibody that does not recognize both protein portions of PCX.

IF Study of Urine Sediments Using Antibodies that React with Different Regions of PCX. IF using different antibodies to PCX, including anti-sugar components (PHM5), anti-extracellular peptide (No45), and anti-intracellular peptide (70-4), demonstrated nearly the same localization pattern with these three antibodies (Figure 7), indicating that PPGS contained whole-cell membrane structures.

PPGS Are not Derived from Cell Debris of Detached Podocytes

Three kidneys were obtained at the time of autopsy. A portion of renal cortex was processed for light microscopic examination to count the number of podocytes per glomerulus, and the remaining portion of the kidney was used for glomerular isolation. The actual values of podocytes per glomerulus were 412, 388, and 420, respectively. The numbers of glomeruli sieved were 120,000, 200,000, and 170,000, respectively. The u-sed-PCX and the number of urinary podocytes from 15 cases with HSPN, IgAN, and LN were also examined.

The quantified values of PCX from isolated glomeruli were divided by the total number of podocytes (cells) from isolated glomeruli in each kidney. The values of u-sed-PCX (ng/ml) were also divided by the number of urinary podocyte (cells/ml). The mean ± SEM of PCX/podocytes from isolated glomeruli was 4.0 ± 0.35 pg/podocyte, and PCX/urinary podocyte was 16.1 ± 4.1 ng/cell ($P < 0.01$). The results are summarized in Table 1.

PPGS Are not Associated with Actin Filaments

Recent studies demonstrated that the PCX molecule binds to actin filaments via NERF 2 and ezrin. Therefore, an IF study was performed to detect ezrin and actin in urine sediments using antibodies to ezrin, β-actin, and Alexa488-labeled phalloidin. Urinary PPGS were negative for ezrin, β-actin, and phalloidin (Figure 8), suggesting against the presence of these PCX-binding proteins in PPGS.

PPGS Reflect Acute Podocyte Injury

Urinary Sediment PCX *versus* Urinary Podocytes and Proteinuria. The levels of u-sed-PCX, proteinuria, and urinary podocytes in three groups of patients are shown in Table 2. Although a small amount of u-sed-PCX was detected in the normal control group, the levels of u-sed-PCX in both the nephrotic syndrome and nephritis groups were significantly higher than in the control group ($P < 0.0001$ and <0.0002, respectively, *versus* control), and the level in the nephritis group was significantly higher than the level in the nephrotic group ($P < 0.03$). The level of proteinuria in the nephrotic group was significantly higher than that in both the normal control and nephritic groups ($P < 0.0001$ and $P < 0.006$, respectively). Urinary podocytes were detected only in the nephritis group, despite the finding of higher levels of proteinuria in the nephrotic group.

Urinary Sediment PCX *versus* Histology. Twenty-six renal biopsies, including IgAN ($n = 15$), HSPN ($n = 7$), and LN ($n = 4$), were analyzed histologically. The cases with acute extracapillary changes had a significantly higher u-sed-PCX level than those without these changes (37.41 ± 6.11 *versus* 12.53 ± 6.52 ng/ml; $P < 0.05$). There was no difference between the cases with or without other changes, such as chronic extracapillary changes and tubulointerstitial changes. There was no correlation with the degree of mesangial proliferation.

Discussion

Podocytes are injured in many forms of human and experimental glomerular diseases. Independent of the underlying disease, if the early structural changes in podocytes are not reversed, then severe and progressive glomerular sclerosis develops. These changes include podocyte vacuolization, pseudocyst formation, microvillous transformation, and detachment of podocytes from the GBM, resulting in podocyte loss from the glomeruli. Previous studies of human diabetic nephropathy and IgAN have provided convincing evidence for a correlation

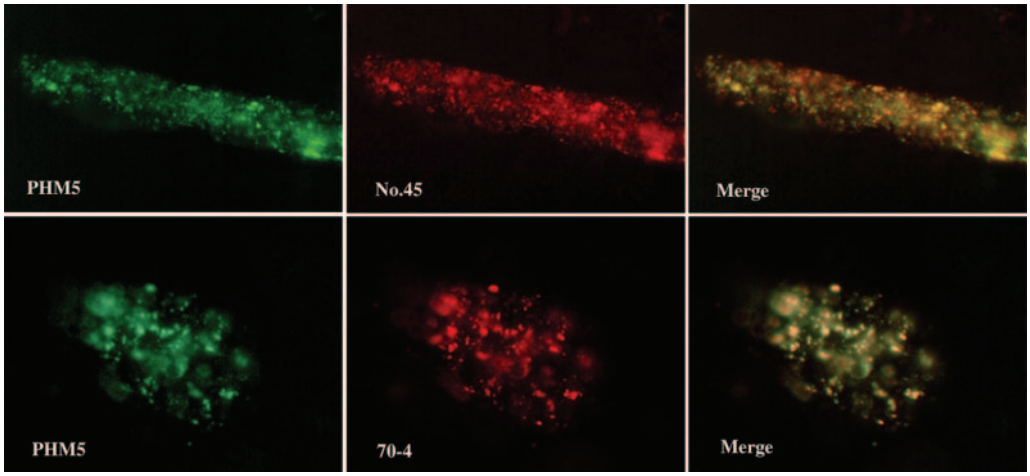

Figure 7. IF of urine sediments from a patient with IgA nephropathy using antibodies that react with different portions of PCX. Upper channel: PHM5 (reacting with sugar components of PCX) and No45 (reacting with extracellular portion of PCX). The picture of merge shows nearly the same localization of both antibodies. Lower channel: PHM5 (reacting with sugar components of PCX) and 70-4 (reacting with intracellular portion of PCX). The picture of merge shows nearly the same localization of both antibodies. These findings clearly indicate that PPGS contain whole-cell membrane structures. Magnification, ×400.

Table 1. Ratio of PCX to podocyte number in urine sediments and isolated glomeruli[a]

	PCX Content	No. of Podocytes	PCX/Podocyte
Isolated glomeruli[b]	170.0–350.0 μg	49,440,000–77,600,000 podocytes	4.0 ± 0.35 pg/podocyte
Urinary sediments[c]	3.6–321.0 ng/ml	0.5–43.0 podocytes/ml	16.1 ± 4.1 ng/podocyte[d]

[a]Data are expressed as means ± SEM. PCX, podocalyxin.
[b]From three kidneys.
[c]From 15 cases with IgA nephropathy (IgAN), Henoch-Schoenlein purpura nephritis (HSPN), and lupus nephritis (LN).
[d]$P < 0.01$ *versus* isolated glomeruli.

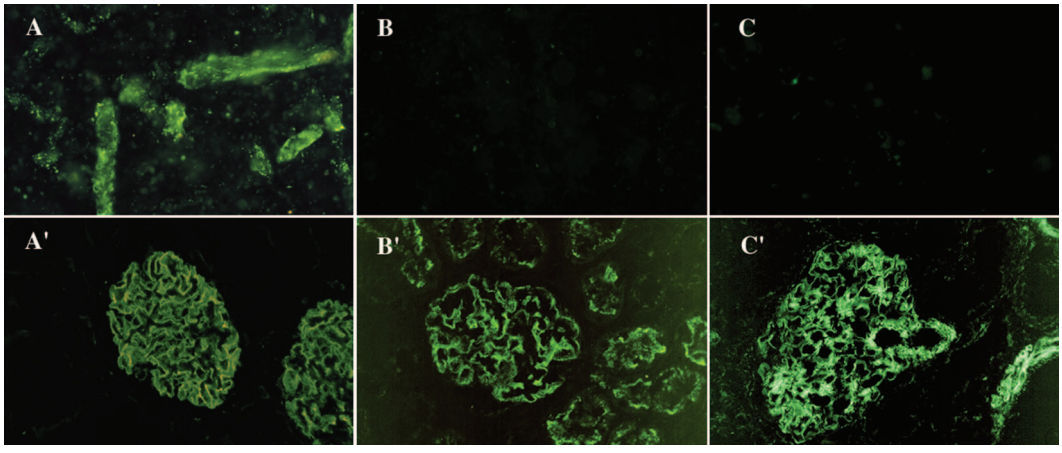

Figure 8. IF of urine sediments using antibodies that react with the molecules between PCX and actin filaments: anti-PCX (A), anti-ezrin (B), and phalloidin (C). Casts in the sediment are stained with anti-PCX but not with anti-ezrin and phalloidin. IF of kidney sections from normal control stained with anti-PCX (A′), anti-ezrin (B′), and phalloidin (C′) in the lower panel. Magnification, ×400 in A through C; ×200 in A′ through C′.

between the loss of podocytes and the progression of glomerular diseases (24,25). The detection of urinary podocytes in various glomerular diseases, including IgAN and diabetic nephropathy, is consistent with the podocyte loss from the glomeruli (13,26). Meanwhile, in addition to urinary podocytes, numerous podocyte-related structures such as PPGS are found in urine. Appearance of these structures in the urine seems to reflect closely the events ongoing in the glomerulus (27). There-

Table 2. U-sed-PCX, proteinuria, and u-podocyte levels in normal controls, nephrotic syndrome, and IgAN/HSPN/LN[a]

	U-sed-PCX (ng/ml)	Proteinuria (mg/dl)	U-Podocytes (cells/ml)
Normal controls ($n = 50$)	0.13 ± 0.04	8.6 ± 0.5	0
Nephrotic syndrome ($n = 15$)	1.59 ± 0.43[b,c]	605.3 ± 135.6[e,f]	0
IgAN/HSPN/LN ($n = 15$)	50.63 ± 20.24[d]	176.9 ± 40.6[g]	6.0 ± 2.8

[a]Data are expressed as means ± SEM. u-sed-PCX, urinary sediment PCX.
[b]$P < 0.0001$ *versus* normal controls.
[c]$P < 0.03$ *versus* IgAN/HSPN/LN.
[d]$P < 0.0002$ *versus* normal controls.
[e]$P < 0.0001$ *versus* normal controls.
[f]$P < 0.006$ *versus* IgAN/HSPN/LN.
[g]$P < 0.0001$ *versus* normal controls.

fore, we performed a detailed analysis of these podocyte-related substances in the urine in an attempt to improve our understanding of the mechanism(s) of podocyte injury during glomerular disease.

To trace PPGS to their origin immunohistochemically, we first examined PPGS morphologically, analyzing both urine sediments and kidney sections. We carefully examined kidneys sections from nephritic patients and normal controls stained with anti-PCX antibody and found the development of a fine granular appearance on the outer surface of capillary walls in sections with nephritis compared with controls, suggesting that PPGS originate from these granular structures on the capillary walls. Similar-sized granular structures to PPGS were also found in the luminal space of renal tubules, suggesting that the PPGS originated from the glomerulus and flowed into the tubular lumen. EM examination of biopsy samples from patients with nephritis revealed fine microvillous or vesicle-like structures on the apical surface of injured podocytes, in addition to other morphologic alterations. IEM of urine sediments from nephritic patients demonstrated the microvillous or vesicle-like structures on the surface of urinary podocytes that were similar to the changes found on podocytes *in situ*. Clustering of vesicle-like structures with different sizes was detected in urine sediments, findings also very similar to the structures on podocytes in the urine or glomerulus. The close similarity of these microvillous or vesicle-like structures at both the light microscopic and EM levels strongly indicated that PPGS in the casts in the urine sediments originated from the microvillous or vesicle-like structures on injured podocytes in the glomerulus.

We further examined the subcellular portions of podocytes from which these vesicle-like structures originated. Although our first observations of urine sediments that immunostained with an anti-PCX antibody suggested that PPGS were derived from the cell debris or the destroyed cell membranes of detached podocytes, we subsequently found that the number of PPGS was disproportional compared with the number of podocytes, giving doubt to our speculation of the origin of PPGS in urine. To clarify this, we immunostained urine sediments with antibodies that recognize different subcellular regions of podocytes, including the apical, slit diaphragm, and the basal regions. The IF study of urine sediments clearly demonstrated that PPGS were positive only for anti-PCX antibody, indicating a definite polarity of PPGS. If the PPGS were derived from the general cell debris of detached podocytes, then PPGS should have stained with any of these region-specific antibodies. This finding indicated that PPGS are excreted into urine not as a result of passive cellular destruction during or after the detachment of podocytes from the GBM but as a result of other mechanisms, such as active cell membrane shedding or vesicle release, that might be reflective of biologic activities of injured podocytes.

Next we examined whether the PPGS truly originated from the cell membranes of podocytes or not. To exclude the possibility that PPGS originate from the cell membranes of other cells such as tubular cells on which PCX molecules are bound, we examined PPGS using monoclonal antibodies that recognize three different regions of the PCX molecule. The IF study revealed that PPGS included intact molecules of PCX, including the intracellular, extracellular, and sugar components, clearly indicating that PPGS originated from the cell membranes of podocytes. Additional evidence of cell membrane structures was based on morphologic findings. IEM of urine sediments from patients with glomerular diseases demonstrated double-layered structures, suggestive of typical cell membranes.

Our next investigation of PPGS was to clarify their relationship to the cytoskeleton of podocytes. Recent studies revealed that the podocyte actin cytoskeleton is connected to apical integral membrane molecules, including PCX (28,29). We examined this association using antibodies that recognize ezrin and β-actin, and phalloidin to label actin. The results were negative for each of these molecules. These results showed that PPGS contained only membrane-bound podocyte proteins and apparently lacked cytoskeletal components. Takeda *et al.* (30) demonstrated that the PCX/NHERF2/ezrin/actin cytoskeleton association was disrupted in damaged podocytes. Thus, it is likely that the PPGS lack ezrin and actin filaments in their structures.

Urinary PPGS are numerous and definitely disproportional compared with the number of urinary podocytes, so we also

examined the ratio of PCX content per podocyte in both urine sediments and isolated glomeruli. Calculation of the PCX/podocyte ratio demonstrated a markedly higher ratio of PCX/podocyte from urine than PCX/podocyte from isolated glomeruli. If the PPGS in urine sediments originated from the cell debris of detached podocytes, then the ratio of PCX/urinary podocyte should be roughly equivalent to the PCX/podocyte from isolated glomeruli. The markedly higher ratio of PCX/urinary podocyte indicated that the PCX in urinary sediments is derived from not only cell debris of detached podocytes but also other source(s). In addition, significant amounts of PCX were detected in urine sediments that did not contain urinary podocytes in patients with nephrotic syndrome, which further supports the concept that the PCX in urine sediments does not originate only from detached podocytes.

EM examination revealed a close similarity between the microvillous structures on the podocyte surface in the kidney and the vesicle-like structures in urine sediments, suggesting that these microvillous structures in the glomeruli are excreted into the urine by shedding from podocytes. By definition, shedding is the release of soluble or vesicle-associated cell surface constituents, without affecting cell viability (31). Shedding membrane vesicles from the cell surface is generally a selective process that is widespread in both normal and diseased cells. The excretion of vesicle-like structures from injured podocytes found in this study is clearly consistent with the process of shedding in general. The idea of shedding has been previously proposed by several authors. Wiggins *et al.* (32) observed a similar phenomenon in experimental nephritis, although a specific marker for podocytes was not used in their studies. Pascual *et al.* (33) identified membrane-bound CR1 (CD35) in human urine and demonstrated the evidence for its release by podocytes. Lehto *et al.* (34) demonstrated urinary excretion of protectin (CD59) in membranous glomerulonephritis. The authors on both of these papers (33,34) used CR1 (CD35) or CD59 for a podocyte marker; however, the expression of CR1 or CD53 could be altered depending on the level of complement activation or phenotypical change of podocytes in diseases. These molecules might be less appropriate for the purpose of examining urinary excretion. On the contrary, PCX is expressed primarily on the apical cell surface of podocytes, and the expression of PCX in glomeruli was not altered in various kinds of nephritis (35,36). In the present study, we successfully observed extensive urinary shedding of podocyte apical cell membranes using PCX as a podocyte marker. We believe that this shedding process represents an active biologic process that includes altered membrane dynamics of podocytes. Extensive microvillous transformation in the injured podocytes is also representative of similar membrane dynamics (37).

In conclusion, we have found that podocyte apical cell membranes are shed into the urine after acute podocyte injury and that this represents a previously unrecognized manifestation of podocyte injury.

Acknowledgments

Portions of this study were presented as a poster at the 2003 American Society of Nephrology national meeting, November 12–17, 2003, San Diego, CA.

We thank Dr. William E. Smoyer for thoughtful review of this manuscript.

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