Detection of Injury-induced Changes in Gene Expression of the Glomerular Epithelial Cell-specific Marker, Wilm's Tumor-1, by Laser Capture Microdissection

Pu Duann, MD, PhD
Hideki G. Kawanishi, MD
Eugene J. Gross, BS
Prasun K. Datta, PhD
Elias A. Lianos, MD, PhD

Department of Medicine, Nephrology Division, Robert Wood Johnson Medical School, New Brunswick, New Jersey

KEY WORDS: Wilms' tumor, PAN nephropathy, laser capture microdissection, glomerular epithelial cell.

ABSTRACT

The Wilms' tumor 1 gene is a transcription factor expressed in glomerular epithelial cells, where it regulates podocyte cytoskeleton proteins that play a key role in glomerular capillary permselectivity to plasma proteins. It is unknown whether Wilms' tumor 1 gene expression changes in forms of glomerular epithelial cell injury associated with proteinuria. In this study we employed the aminonucleoside of puromycin mediated glomerular epithelial cell injury model in the rat, which histologically resembles human forms of glomerular epithelial cell disease, to assess changes in glomerular Wilms' tumor 1 gene expression. A single intraperitoneal injection of aminonucleoside of puromycin (75 mg/kg) in Sprague-Dawley rats induced proteinuria that peaked 10 days later (urine protein to creatinine ratio = 10 in control compared to 125 in aminonucleoside of puromycin-treated animals). At 4 hours and on days 4 and 10 following aminonucleoside of puromycin injection, glomeruli were either microdissected using a laser capture microdissecting method or were isolated by differential sieving. Total RNA was prepared and analyzed for Wilms' tumor 1 gene levels by RT-PCR. Because podocytes constitute a relatively small portion of total glomerular cells, Wilms' tumor 1 gene messenger RNA levels were factored by those of podocin, a podocyte specific protein found in the slit pore diaphragms, whose messenger RNA levels do not change following aminonucleoside of puromycin induced injury. In microdissected glomeruli there was a 43 % decrement in Wilms' tumor 1 gene expression at 4 hours following administration of aminonucleoside of puromycin. This decrement was attenuated on days 4 and 10. These results were confirmed in glomeruli isolated by differential sieving in which the decre-

ment of Wilms' tumor 1 gene was again most pronounced (28.2%) at 4 hours. These observations indicate that decreased Wilms' tumor 1 gene expression is a very early molecular event in response to glomerular epithelial cell injury.

INTRODUCTION

The Wilms' tumor 1 gene (WT-1) is a tumor suppressor gene that plays a key role in the development of Wilms' tumor, an embryonic kidney cancer. In addition, it is crucial in kidney development during embryogenesis.[1] Thus, WT-1 knockout mice have no kidneys due to failure of the nephric duct to grow out and apoptosis of the metanephric blastema. WT-1 may act as a transcription factor, transcriptional cofactor or post-transcriptional regulator depending on its spliced isoform and cell type. As kidney development and differentiation proceeds during embryogenesis, WT-1 expression in the kidney is down-regulated, except in the visceral glomerular epithelial cells (podocytes) of the mature glomerulus, where expression can be detected throughout life. Glomerular epithelial cells (GEC) line the surface of glomerular capillaries facing the urinary space. They play an important role in maintenance of the glomerular basement membrane, and support the glomerular capillary tuft and control glomerular filtration of protein. WT-1 was shown to be important in maintaining normal GEC function. Thus, WT-1 is mutated in 94% of patients with Denys-Drash syndrome, in whom the most consistent finding is development of glomerular scarring. WT-1 mutations have also been found in patients with nephrotic syndrome and in isolated cases of glomerulosclerosis.[2,3] Reduced WT-1 expression levels in transgenic mice result in glomerular lesions of proliferation or scarring. In these mice, there is also a striking reduction in 2 podocyte-specific genes that encode the cytoskeletal proteins, nephrin and podocalyxin.[4]

While evidence supporting the significance of WT-1 in genetic forms of GEC defects resulting in nephrotic syndrome is solid, studies assessing changes in WT-1 expression in response to non-genetic forms of GEC injury are lacking. Non-genetic forms of GEC injury include drug-induced, immune (ie, membranous nephropathy) and viral (ie, HIV nephropathy). A well-established form of drug induced GEC injury is the one induced by the anti-metabolite aminonucleoside of puromycin (PAN). GEC are uniquely vulnerable to PAN and the ensuing lesion histologically resembles human forms of GEC diseases, such as minimal change disease and focal segmental glomerulosclerosis.[5] This study examined changes in glomerular WT-1 expression levels at early and late stages of PAN-induced GEC injury.

METHODS

Experimental Design and Tissue Processing

Male Sprague-Dawley rats (130 to 150 g) were anesthetized with intraperitoneal injection of 0.1 cc of ketamine: xylazine (1:3 ratio) per 100 g body weight. They subsequently received a single intraperitoneal injection of 75 mg/kg of aminonucleoside of puromycin (Sigma-Aldrich, St. Louis, Mo) to induce glomerular epithelial cell (GEC) injury, as previously described.[6] Control animals were injected with equal volumes of normal saline.

A single single timed urine sample was collected at 4 and 10 days following the PAN injection. Urine protein was measured using a Bio-Rad Dc Protein assay kit (Bio-Rad Laboratories, Hercules, Calif). Urine creatinine was measured using a Sigma-Aldrich kit (Sigma-Aldrich, St Louis, Mo). The

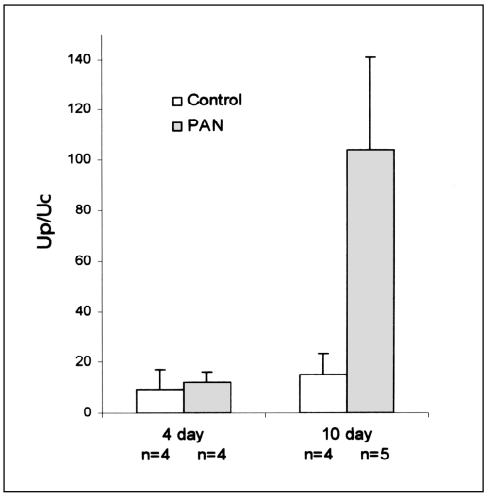

Figure 1. Proteinuria, expressed as urine protein (Up) to urine creatinine (Uc) ratio, in PAN-treated rats at the points of study (days 4 and 10) is shown. There was a marked increase in urine protein excretion on day 10 only. There was no detectable increase in urine protein excretion on day 4. Values shown are mean ± standard deviation (± SD).

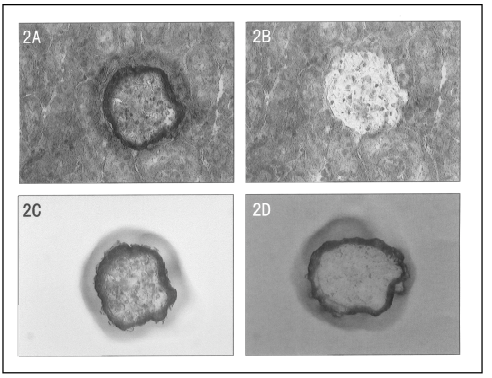

Figure 2. Figure 2A shows a representative glomerulus following laser dissection but prior to its capture on the vinyl film of the LCM cap. Figure 2B shows the empty space on the remaining cortical section following capture and removal of the glomerulus. Note that, following capture and removal of the glomerulus, a number of glomerular cells remained in the space previously occupied by the glomerulus. In 2C, the captured glomerulus on the LCM cap is shown. In 2D, the LCM cap surface following TRIzol lysis of the captured glomerulus is shown.

urine protein to creatinine ratio was then calculated to determine the extent of proteinuria. Following completion of urine collections, rats were euthanised and nephrectomies were immediately performed at 4 hours, 4 days, and 10 days after the PAN injection.

The kidneys were immersed in optimal cutting temperature compound (Tissue-Tek OCT compound, Sakura Finetek, Torrance, Calif) and placed in RNAse free cryomolds. The cryomolds were placed in 2-methlybutane and cooled by immersion in liquid nitrogen until frozen. This process reduces architectural distortion by shortening the freezing time. Kidneys were then stored at -80˚C until processed for laser capture microdissection.

Tissue Sectioning and Staining for Laser Capture Microdissection

Using a Leica CM IS 50 Cryostat, kidneys were sectioned at 6 µm and placed onto Arcturus HistoGene LCM Slides (Arcturus, Mountain View, Calif). The slides were then stained using the Arcturus HistoGene LCM Frozen Section Staining Kit (Arcturus, Mountain View, Calif) according to the manufacturer's protocol, and placed into xylene to achieve dehydration and thereby, optimizing capture. The slides were immediately used for laser capture microdissection (LCM).

Laser Capture Microdissection

The Arcturus PixCell II Laser Capture Microdissection System (Arcturus, Mountain View, Calif) was employed under RNase-free conditions to capture glomeruli. The stained sections were removed from xylene and allowed to air dry at room temperature for 5 minutes to evaporate xylene and was immediately used for laser capture microdissection. Sixty glomeruli from each sample were identified and captured onto Arcturus CapSure HS LCM Caps. The laser settings were as follows: laser spot size 30

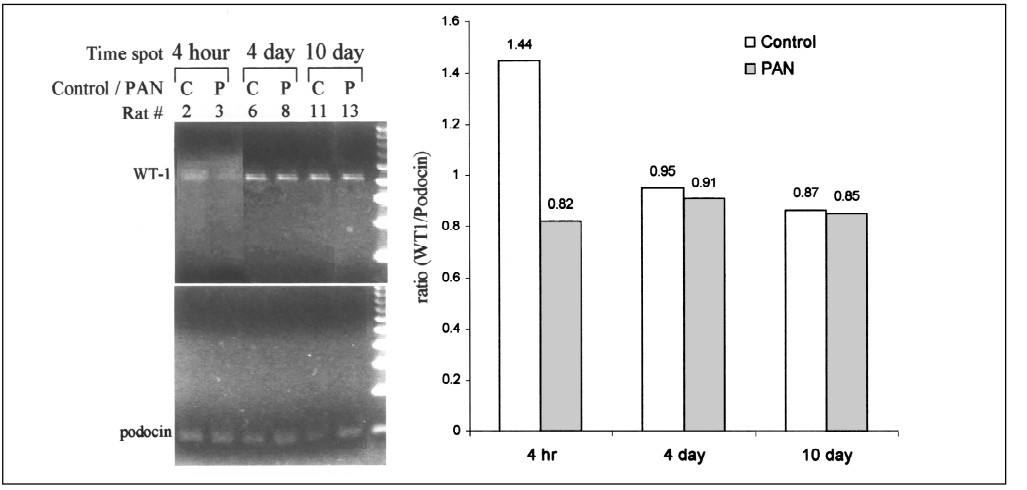

Figure 3. Levels of WT-1 and podocin mRNA detected by RT-PCR performed on total RNA isolated from microdissected glomeruli. The bar graph is densitometric analysis of the gel and shows changes in WT-1/podocin ratio. A decrement in this ratio was most pronounced at 4 hours.

μm, power 50 to 55 mW, and duration of 4.5 to 4.8 micro seconds. The number of pulses to capture each glomerulus varied according to the size of the individual glomerulus. On an average, 4 to 8 pulses were used to capture each glomerulus excluding cells comprising the Bowman's capsule. After completion of the capturing step, sections and caps were examined to ensure adequate transfer of glomeruli to the cap. The time from removal of each slide from xylene to capture onto a HS LCM cap was less than 45 minutes.

Isolation of Glomeruli by Differential Sieving

Glomeruli were isolated according to an established technique first described by Misra.[7] Briefly, the kidneys were removed, and the cortex was separated from the medulla and finely minced. The glomeruli were separated from other cortical tissues by passing the minced cortices through 2 different sizes sieves (106-μm and 75-μm sieves). With repeat flushing, using ice-cold non-serum RPMI 1640 media, the glomeruli were retained on the 75-μm sieve while the non glomerular tissue, mainly cortical tubules, were washed through this sieve. The purity of glomerular preparations was determined microscopically and was routinely 95% to 98%.

Preparation of Total Glomerular RNA and RT-PCR

Total RNA was extracted either from captured or isolated glomeruli, using the TRIzol reagent method (Gibco BRL, Carlsbad, Calif). To isolate total RNA from captured glomeruli, HS LCM caps containing approximately 60 glomeruli were attached to a 0.5 mL eppendorf tube containing 200μL of TRIzol via an adaptor supplied by the manufacturer (Arcturus, Mountain View, Calif). Glomerular RNA was precipitated in –80˚C overnight. To optimize RNA recovery, 20 μg of glycogen (an inert RNA carrier molecule) was added during the overnight precipitation. The RNA was subsequently re-suspended in 5μL of 10 mM Tris-EDTA/DEPC buffer and stored at 70˚C until RT-PCR was performed.

RT-PCR

Messenger RNAs (mRNA) for WT-1, podocin, and glyceraldehyde 3-phos-

phate dehydrogenase (GAPDH) were detected and quantified by reverse transcription-polymerase chain reaction (RT-PCR). Podocin, a podocyte-specific gene, was used as a marker for presence of podocytes in captured glomeruli. Detection of GAPDH RNA served as a marker of comparable extent of capture of glomeruli from each cortical kidney section. Aliquots of 5 µL of RNA were used for complimentary DNA (cDNA) synthesis using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories; Hercules, Calif) according to the manufacturer's instructions. Aliquots of 3 µL of cDNA were used for PCR amplification of WT-1, podocin, and GAPDH using 500 nM of gene specific primers and iQ Supermix (Bio-Rad Laboratories, Hercules, Calif) in a total volume of 25 µL.

WT-1 primers were: forward: 5'-AGCATCTGAAACCAGTGAGAA-3'; reverse: 5'-CAAAATCAGATTTG-GAAGCAGT-3'.

GAPDH primers were: forward: 5'-GTGCTGAGTATGTCGTGGA-3'; reverse: 5'-CACAGTCTTCGAGTG-GCA-3'.

Podocin primers were: forward: 5'-ATTTCCTTGTGCAAACCACTAT-GA-3'; reverse: 5'-CCAAGGCAACCTTTGCATCT-3'.

The PCR cycling profile was 94˚C for 5 minutes to activate iTaq DNA polymerase followed by 1 minute at 94˚C, 1 minute at 56˚C, 3 minutes at 72˚C. Thirty-five-cycle amplifications were performed followed by a final extension at 72˚C for 10 minutes. Fifteen µL of PCR products was separated on a 2% pre-cast agarose gel (Invitrogen, Carlsbad, Calif).

Semi-Quantitative Analysis

Gels were visualized using a gel documentation system (Bio-Rad Laboratories, Hercules, Calif). The optical density of the PCR products were determined using a BioRad Gel Doc 4.4.0 software program and a KODAK ID image software. Because podocytes constitute a relatively small population of total glomerular cells, WT-1 mRNA levels obtained by RT-PCR, as described above, were factored by those of podocin, a podocyte specific gene found in the slit pore diaphragm. The observation that mRNA levels of podocin do not change following PAN induced injury,[8] makes podocin an appropriate marker by which changes in WT-1 levels can be factored.

Relative WT-1 expression was defined as WT-1 mRNA levels, determined by densitometry, factored by those of GAPDH. Relative podocin expression was defined as podocin mRNA levels, determined by densitometry, factored by those of GAPDH. Changes in WT-1 levels were expressed as changes in the relative WT-1/podocin ratio.

RESULTS

Aminonucleoside-Induced Proteinuria

In Figure 1, the degree of proteinuria (Up/Uc) in PAN-treated rats at the points of study (days 4 and 10) is shown. There was a marked increase in urine protein excretion on day 10 only. There was no detectable increase in urine protein excretion at 4 hours or on day 4.

Changes in Glomerular WT-1 Expression Detected by LCM

Figure 2A shows a representative glomerulus following laser dissection but prior to its capture on the vinyl film of the LCM cap. Figure 2B shows the empty space on the remaining cortical section following capture and removal of the glomerulus. Note that, following capture and removal of the glomerulus, a number of glomerular cells remained in the space previously occupied by the glomerulus. In Figure 2C, the captured glomerulus on the LCM cap is shown.

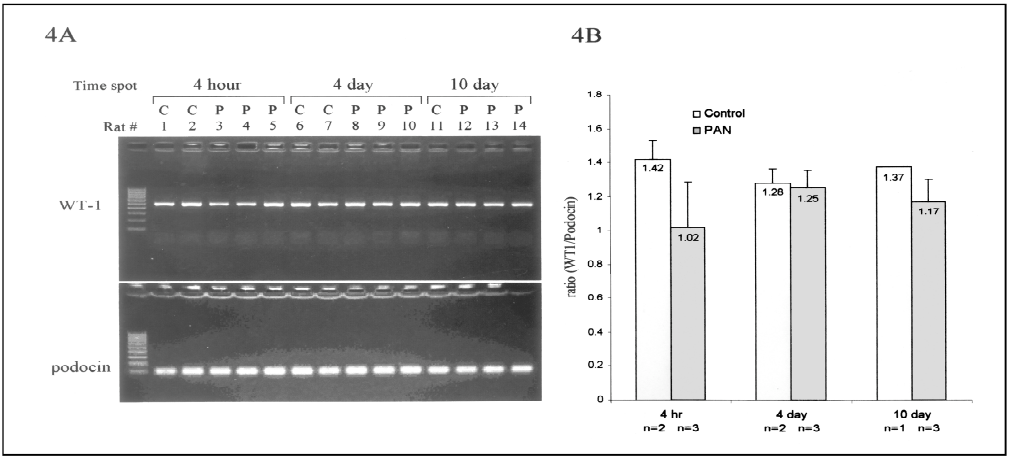

Figure 4. Levels of WT-1 and podocin mRNA as determined by RT-PCR performed on total RNA of glomeruli isolated by differential sieving at 4 hours and on days 4 and 10. The bar graph is a densitometric analysis of the gel and shows the changes in WT-1/podocin ratio determined as described in methods section. The number inside each bar is the actual ratio value. A decrement in WT-1/podocin ratio was most pronounced at 4 hours.

In Figure 2D, the LCM cap surface following TRIzol lysis of the captured glomerulus is shown.

The gel in Figure 3A shows levels of WT-1 and podocin mRNA detected by RT-PCR performed on total RNA isolated from microdissected glomeruli. The bar graph in Figure 3B is the densitometric analysis of the gel in Figure 3A, and shows changes in WT1/podocin ratio. A decrement in this ratio was noted at 4 hours. It returned to near control levels on days 4 and 10.

Confirmation of Changes in WT-1 Expression in Glomeruli Isolated by Differential Sieving

The gel in Figure 4A shows levels of WT-1 and podocin mRNA as determined by RT-PCR performed on total RNA of glomeruli, isolated by differential sieving at 4 hours and on days 4 and 10. The bar graph in Figure 4B shows the densitometric analysis of the gel in Figure 4A, and shows the changes in WT-1/podocin ratio determined as described in the methods section. A decrement in WT-1/podocin ratio was again noted at 4 hours.

Magnitude of Decrement in WT-1 Levels in Isolated Versus Captured Glomeruli

The bar graph in Figure 5 shows the magnitude of the decrement in WT-1 levels in PAN treated animals expressed as the percent change compared to control. The decrement in WT-1 level detected in captured glomeruli was most pronounced (43%) at 4 hours following PAN treatment. The decrement in WT-1 levels detected in glomeruli isolated by differential sieving was also most pronounced (28%) at 4 hours following PAN treatment.

DISCUSSION

Contact of glomerular epithelial cells (GEC) to the glomerular basement membrane (GBM) involves a highly specialized structure and signaling apparatus in their foot processes and slit diaphragms. Key protein constituents of this apparatus include nephrin, podocalyxin, podocin, and the cytoskeleton protein synaptopodin, paxillin, actinin, vinculin, talin, □3/□1 integrin, and CD2AP. Nephrin, podocin, and □-actinin-4 are encoded by genes linked

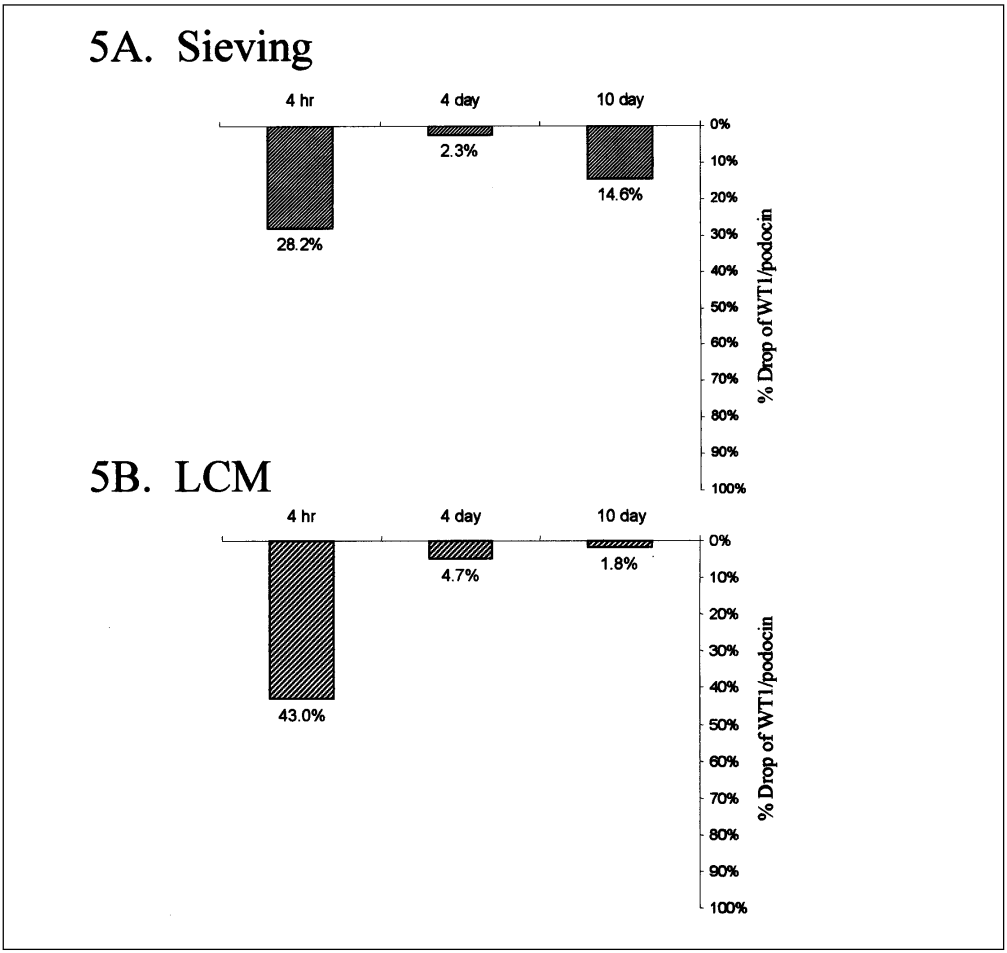

Figure 5. Percent decrement in WT-1 levels in isolated versus captured glomeruli. The decrement in WT-1 levels in PAN treated animals is expressed as percent change compared to control. The decrement in WT-1 level detected in captured glomeruli was most pronounced (43%) at 4 hours following PAN treatment. The decrement in WT-1 levels detected in glomeruli isolated by differential sieving was also most pronounced (28%) at 4 hours following PAN treatment.

to diseases involving GEC and characterized by proteinuria. For example, the congenital nephrotic syndrome gene was mapped to chromosome 19q13, which encodes nephrin, a 185 KD protein that localizes to the GEC slit diaphragm and plays a role in regulating signaling pathways.[9,10] The steroid resistant nephrotic syndrome (autosomal recessive inheritance) gene was mapped to chromosome 1q25-31 which encodes podocin, a 383 amino acid protein that also localizes to the slit diaphragm and interacts directly with nephrin.[11] Identification of factors that regulate podocin and nephrin and characterization of changes in expression of such factors following GEC injury may enhance our understanding of the biology of diseases involving GEC. Such factors may also serve as markers of the extent of injury.

The transcription factor WT-1 was shown to regulate GEC proteins such as the aforementioned nephrin and podocalyxin.[4] WT-1 was cloned based on its role in the development of Wilm's tumor. Two clinical syndromes, the Denys-Drash and the Fraiser syndrome, are caused by WT-1 mutations[1] and both

are characterized by proteinuric glomerular disease. The glomerular lesion in Fraiser syndrome is focal and segmental glomerular sclerosis (FSGS) while that in Denys-Drash is diffuse mesangial sclerosis.[1] This apparent regulatory significance of the WT-1 transcription factor, prompted us to explore changes in its expression at very early and advanced stages of a GEC injury model, induced by the anti-metabolite aminonucleoside of puromycin and resembling human forms of GEC injury, such as minimal changes disease and FSGS.[5] We employed the laser capture microdissection (LCM) method as it can be used in human kidney biopsies, in which a small number of glomeruli are usually available for evaluation.

Although expression of the housekeeping gene GAPDH was detected by RT-PCR in as few as 5 glomeruli, detection of the less abundant WT-1 and podocin genes required capture of at least 60 glomeruli. A technical pitfall of the LCM method is the apparently incomplete capture of all glomerular cells. Thus, as shown in Figure 2A, a considerable number of cells were left behind, following capture of the representative glomerulus shown. This may impact on recovery of GEC genes that are expressed in low abundance. This pitfall and the fact that GEC constitute only a fraction of the glomerular cellularity necessitated factoring of the WT-1 expression values obtained by those of podocin, a GEC specific gene.

Our results indicate that, following PAN-induced GEC injury, there is a decrease in WT-1 expression and that this occurs very early and is transient. Thus, the decrease in WT-1 occurred as early as 4 hours following PAN injection and returned toward control levels by days 4 and 10. Therefore, this event far proceeded proteinuria. Moreover, proteinuria had no further effect on WT-1 expression when urine protein excretion became pronounced (day 10).

A number of studies have demonstrated structural changes in GEC that occur much earlier than the onset of proteinuria. The earliest ultrastructural changes identified at the electron microscopy level in cultured rat GEC are cell rounding, surface blebbing and marked increase in number of microvilli. These changes are observed within 3 hours of exposure to PAN.[12] The earliest ultrastructural change identified at the electron microscopy level in vivo (rat kidney) is a reduction in the number of foot processes and filtration slits with loss of the normal arrangement of interdigitating podocytes and displacement of the slit diaphragm.[5,13] These changes are observed within 2 days following a single dose of PAN, a protocol of PAN treatment similar to the one used in the present studies. Changes in GEC cytoskeleton proteins whose expression could be linked to the WT-1 transcription factor also occur prior to onset of proteinuria. This has been shown for □-actinin, whose levels of expression transiently increase within 1 day post PAN injection and return to control levels by day 3 (prior to onset of proteinuria). □-Actinin expression remains at control levels on day 10 when proteinuria is pronounced,[14] a pattern similar to the one observed with WT-1 expression in the present studies.

To confirm results on changes in WT-1 expression obtained by LCM, we employed the well-established method of glomerular isolation by differential sieving. Purity of typical preparation of glomeruli isolated by this method is 90% to 95%.[7] As shown in Figure 4, a decrement in WT-1 expression was again noted at 4 hours following administration of PAN. This decrement was again transient with expression values returning towards control levels on days 4 and 10.

The significance of the very early decrease in WT-1 expression in GEC

injury remains to be established and to be linked to changes in key cytoskeleton proteins that regulate glomerular capillary permeability to protein. Whether assessment of WT-1 expression is of value in human forms of GEC injury, such as minimal change disease and FSGS, also remains to be elucidated in kidney biopsy material from patients with nephrotic syndrome resulting from these diseases. In this regard, LCM applied to kidney biopsy material may prove to be a valuable tool.

ACKNOWLEDGMENTS

Hideki G. Kawanishi was a recipient of the Kidney-Urology Foundation of America Fellowship. This work was supported in part by a Paul Teschan Research Fund grant from Dialysis Clinic Inc. awarded to Prasun K. Datta, PhD.

REFERENCES

1. Pitchard-Jones K. The Wilm's tumor gene, WT-1, in normal and abnormal nephrogenesis. *Pediatr Nephrol.* 1999;13:620-625.
2. Ito S, Ikeda M, Takata A, Kikuchi H, Hata J, Honda M. Nephrotic syndrome and end-stage renal disease with WT1 mutation detected at 3 years. *Pediatr Nephrol.* 1999;13:790-791.
3. Yang Y, Jeanpierre C, Dressler GR, Lacoste M, Niaudet P, Gubler MC. WT1 and PAX-2 podocyte expression in Denys-Drash syndrome and isolated diffuse mesangial sclerosis. *Am J Pathol.* 1999;154:181-92.
4. Guo JK, Menke AL, Gubler MC, et al. WT-1 is a key regulator of podocyte function: reduced expression levels cause crescentic glomerulonephritis and mesangial sclerosis. *Hum Mol Genet.* 2002;11:651-659.
5. Caulfield JP, Reid JJ, Farquhar MG. Alterations of the glomerular epithelium in acute aminonucleoside nephrosis. Evidence for formation of occluding junctions and epithelial cell detachment. *Lab Invest.* 1976;34:43-59.
6. Koukouritaki SB, Tamizuddin A, Lianos EA. Enhanced expression of the cytoskeletal-associated protein, paxillin, in experimental nephrotic syndrome. *J Investig Med.* 1998;46:284-289.
7. Misra, R. P. Isolation of glomeruli from mammalian kidneys by graded sieving. *Am J Cell Pathol* 1972;58:135-139.
8. Kawachi H, Koike H, Kurihara H, Sakai T, Shimizu F. Cloning of rat homologue of podocin: expression in proteinuric states and in developing glomeruli. *J Am Soc Nephrol.* 2003;14:46-56.
9. Holzman LB, St John PL, Kovari IA, Verma R, Holthofer H, Abrahamson DR. Nephrin localizes to the slit pore of the glomerular epithelial cell. *Kidney Int.* 1999;56:1481-1491.
10. Huber TB, Kottgen M, Schilling B, Walz G, Benzing T. Interaction with podocin facilitates nephrin signaling. *J Biol Chem.* 2001;276:41543-41546.
11. Boute N, Gribouval O, Roselli S, et al. NPHS2, encoding the glomerular protein podocin, is mutated in autosomal recessive steroid-resistant nephrotic syndrome. *Nat Genet.* 2000;24:349-354.
12. Fishman JA, Karnovsky MJ. Effects of the aminonucleoside of puromycin on glomerular epithelial cells in vitro. *Am J Pathol.* 1985;118:398-407.
13. Ryan GB, Rodewald R, Karnovsky MJ. An ultrastructural study of the glomerular slit diaphragm in aminonucleoside nephrosis. *Lab Invest.* 1975;33:461-468.
14. Smoyer WE, Mundel P, Gupta A, Welsh MJ. Podocyte alpha-actinin induction precedes foot process effacement in experimental nephrotic syndrome. *Am J Physiol.* 1997;273(1 Pt 2):F150-F157.