Podocyte depletion and glomerulosclerosis have a direct relationship in the PAN-treated rat

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Background. Podocytes are highly differentiated glomerular epithelial cells with limited potential to divide. They are responsible for maintaining and supporting the glomerular basement membrane so as to facilitate efficient filtration. The hypothesis tested was whether the development of glomerulosclerosis in the puromycin aminonucleoside (PAN)-treated rat could be attributed to podocyte depletion.

Methods. PAN was injected in Sprague-Dawley rats once, twice, or three times at 30-day intervals. Podocytes were counted in glomeruli using immunoperoxidase histochemistry and antibodies to both GLEPP1 (PTPRO) and WT-1. Podocytes were assayed in urine using reverse transcription-quantitative polymerase chain reaction (RT-QPCR). Glomerular areas were measured by computerized morphometry.

Results. In a preliminary experiment, a single injection of PAN caused a reduction in the glomerular podocyte count by 25%. Additional independent confirmation that podocytes were lost from glomeruli after PAN injection was obtained identifying detached podocytes in Bowman’s space, measurement of nephrin and GLEPP1 mRNA in urine, ultrastructural analysis of glomeruli, and identification of TUNEL-positive apoptotic podocytes in glomeruli. In a second experiment, sequential podocyte depletion by 15, 31, and 53% was achieved by the administration of one, two, or three injections of PAN at 30-day intervals. The region of the glomerulus devoid of podocytes developed glomerulosclerosis, and this area progressively increased as podocytes were progressively depleted. The correlation coefficient ($r^2$) value for the relationship between percent podocyte depletion and glomerulosclerotic area was 0.99. The Y intercept of this plot showed that glomerulosclerosis was initiated when only 10 to 20% of podocytes were lost.

Conclusion. This report supports the growing body of data linking glomerulosclerosis directly to a reduction in relative podocyte number [increased glomerular area per podocyte (GAPP)]. It raises important questions related to the mechanisms of podocyte loss, strategies for prevention of podocyte depletion, and the prevention of progression of glomerular diseases.

The podocyte is a highly differentiated epithelial cell with limited capacity to divide and therefore to be replaced if lost [1–4]. The octopus-shaped podocyte functions to support and maintain the filtration surface (glomerular basement membrane) via major, intermediate, and minor (“foot”) processes that interdigitate between neighboring cells [5]. This support structure serves to counterbalance the hydrostatic filtration force of blood pressure driving the filtration process and at the same time tending to expand glomerular capillaries [6]. The destiny of a podocyte is therefore to cling by its foot processes to the outer surface of the filter, while the thrust of the forces of filtration tends to blow it off its perch. Any factor tending to reduce the ability of a podocyte to remain attached to the glomerular basement membrane (GBM) or to increase filtration forces will therefore tend to promote detachment of podocytes from the glomerulus. Cho, Lumsden, and Whiteside have emphasized via mathematical modeling how partial detachment may tend to lead to further podocyte detachment [7].

Podocytes are particularly susceptible to toxic injury from oxidants. This is demonstrated by the finding that podocyte injury and glomerulosclerosis are the major phenotypes of an antioxidant-defective mouse [8]. Similarly, puromycin aminonucleoside (PAN), which induces oxidant injury in cells via the xanthine oxidase pathway, has been used extensively as a model of podocyte injury [9]. Podocyte injury in this model can be ameliorated by inhibitors of oxidants [10, 11]. Following PAN injection, injury in the rat podocytes is manifest by loss of interdigitating foot processes, detachment from the GBM, pseudocyst formation, reduction in anionic charge, attenuation of the underlying GBM, and associated leakiness of the glomerular filter resulting in proteinuria [12–17]. The PAN model also progresses to a patchy glomerular scarring
process [focal glomerulosclerosis (FGS)] [17]. FGS in humans is commonly associated with progression to end-stage renal disease (ESRD) requiring renal replacement therapy [18].

The maintenance of normal glomerular structure and function requires the interaction between all three glomerular cell types, including podocytes, mesangial, and endothelial cells. As emphasized by Kriz, Gretz, and Lemley, failure of any one of these systems may lead to the same result, namely glomerulosclerosis [3]. Elegant studies in a wide range of experimental models and in humans have suggested that podocyte injury and loss might be a central factor leading to glomerulosclerosis [1–4, 6, 12, 17, 19–30].

Recent information supports the concept that podocyte number may be a key factor dictating the progression of glomerular sclerosis in the type II diabetic Pima Indian population. In these studies, Pagtalunan et al showed that individuals who had more advanced proteinuria (macroalbuminuria) and glomerular matrix accumulation (diabetic glomerulosclerosis) had fewer glomerular podocytes than did individuals who had experienced diabetes for the same length of time but who were not proteinuric and glomerulosclerotic [31]. In contrast, other glomerular cells did not decrease in number in the same glomeruli. In a follow-up report, diabetic Pima Indians with a depleted podocyte population tended to progress toward macroalbuminuria faster than did those who had a greater complement of podocytes four years earlier [32]. Hara et al have shown that in human glomerular diseases podocytes are lost into the urine, where they can be detected by immunofluorescent approaches [33]. This latter report therefore provides direct confirmation that podocytes may be lost from glomeruli into urine in humans.

To understand this process better, we therefore attempted to develop a model in which could sequentially deplete podocytes. We observed that a single dose of PAN caused a limited reduction in podocyte number associated with minor glomerulosclerosis. Therefore, one, two, or three doses of PAN were used in rats administered at 30-day intervals to induce progressive podocyte depletion. A very close relationship between the number of podocytes lost and the degree of glomerulosclerosis was found. The capacity of a podocyte to cover additional territory was limited so that a reduction in podocyte number per unit area [designated as glomerular area per podocyte (GAPP)] above a threshold level was associated with development of glomerulosclerosis. Furthermore, glomerulosclerosis occurred in those areas of the glomerulus in which podocytes, as defined by the specific markers GLEPP1 or WT1, were absent. These data provide support for the concept that podocyte depletion beyond a certain threshold level leads directly to glomerulosclerosis.

**METHODS**

**Animal model of PAN nephrosis**

In an initial experiment, male Sprague-Dawley rats weighing approximately 100 g were administered intraperitoneal PAN (Sigma Company, St. Louis, MO, USA) at a dose of 20 mg/100 g body weight or an equivalent volume of 0.9% saline. Urine was collected over a 24-hour period at day –2 through day 11 and at days 21, 45, 80, and 126. Groups of rats (N = 4 to 5 per group) were sacrificed at day –1 (control) and at days 5, 7, 11, 21, 45, 80, and 126. A second control group was maintained with the experimental group until day 126 for comparison with the experimental day 126 group. In a second experiment, groups of 100 g Sprague-Dawley rats were kept for 90 days before sacrifice. They received either no PAN or PAN once, twice, or three times at 30-day intervals. Thus, the three-injection group received PAN at day 0, day 30, and day 60 and were sacrificed at day 90, which was 30 days after the last injection. For each rat, 24-hour urine collections were performed to measure the 24-hour urine protein excretion and an aliquot of urine was processed for reverse transcription-polymerase chain reaction (RT-PCR) analysis for GLEPP1 and nephrin mRNA measurements (discussed later in this article). At day 90, the rats were killed, and kidneys were perfused with phosphate-buffered saline for two minutes, followed by paraformaldehyde in phosphate buffer for eight minutes at a pressure of 120 mm Hg. After perfusion, kidneys were quickly removed, and 3 to 4 mm sections of kidney were cut for fixation in formalin.

**Immunoperoxidase histochemistry**

Three micrometer-thick formalin-fixed, paraffin-embedded sections were deparaffinized, hydrated, and treated with target unmasking fluid (Signet Labs, Dedham, MA, USA) for two hours at 90°C. Immunoperoxidase staining was performed according to the Vectastatin ABC kit (Vector Laboratories, Burlingame, CA, USA). The primary antibodies used were a monoclonal antibody raised against the rat GLEPP1 extracellular domain (1B4) and a commercially available rabbit polyclonal antibody to Wilms’ tumor-1 protein (WT-1; Santa Cruz Biotechnology, Santa Cruz, CA, USA). These markers are podocyte specific in the mature kidney [34, 35]. The Thy-1 antibody (PharMingen, San Diego, CA, USA) was used as a marker for mesangial and other cells [36]. The detection system used dianaminobenzidine (DAB). Periodic acid-Schiff (PAS) and hematoxylin were used as a counterstain.

**TUNEL assay**

To detect apoptotic cells, the TUNEL assay was performed using the In Situ Cell Death Detection Kit (Boehringer Manheim, Indianapolis, IN, USA). Formalin-fixed, paraffin-embedded sections were stripped of proteins
by incubation with proteinase K (20 μg/mL; GIBCO, Gaithersburg, MD, USA) and stained according to manufacturer’s instructions. Terminal deoxynucleotidyl transferase (TdT) was omitted from the nucleotide mixture as a negative control. Sections treated with DNase to introduce DNA breaks in all nucleoli were used as positive control.

Urine reverse transcription-quantitative polymerase chain reaction analysis

The TaqMan assay has been described previously [37–39]. Briefly, the assay is based on the principle that successful PCR yields a fluorescent signal due to Taq mediated exonuclease digestion of a fluorescently labeled oligonucleotide homologous to a sequence between the two primers. The extent of digestion, which depends directly on the amount of PCR that occurs, can be quantified directly and accurately by measuring the increment in fluorescence that results from decreased energy transfer. This sensitive measurement allows detection in the exponential phase of the PCR reaction that is required for determination of abundance. For urine, this is accomplished by performing the assay on an aliquot of the urine sediment. The urine sediment was prepared from the total urine collected over the course of a day. The urine was spun, and the sediment was collected and resuspended in 0.5 mL RNA later (Ambion, Austin, TX, USA). RNA was isolated by spin chromatography through RNAEasy columns (Qiagen, GmbH, Hilden, Germany) in water and frozen in five aliquots at −80°C.

The steady-state amount of mRNA was analyzed by reverse transcription-quantitative polymerase chain reaction (RT-QPCR). The standard curve method (User Bulletin #2; ABI Prism 7700 Sequence Detection System) was used for the measurement. We derived the RT-QPCR ratio of the podocyte-specific markers, GLEPP1 and nephrin [34, 40], to the constitutively expressed marker, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) at different days after administration of puromycin. Since absolute quantitation of RNA is difficult to achieve, a relative ratio approach was used in our calculation. Thus, all of the measurements in this study were done using a GAPDH marker. This normalization procedure enabled us to compare the expression of a given gene in two urine sediments accurately without needing to know the total amount of analyzable RNA in urine sediment.

Reverse transcription was performed at 60°C for 30 minutes. Following a subsequent ten-minute incubation at 95°C to inactivate reverse transcriptase and activate AmpliTaq Gold, PCR amplification was accomplished 3) 95°C to permit annealing and elongation). To assay the initial concentration of the reactants (the abundance), the number of cycles at which the reaction crosses a threshold value was measured. This number, the C_T value, varies directly with the abundance. To derive the relative abundance measures of two sequences in a given DNA sample, the C_T value derived using the first sequence with the given RNA sample is divided by the C_T value using the second sequence. Derivation of this fraction is independent of RNA sample concentration, eliminating the requirement to measure the RNA concentration accurately.

Morphometric analysis

Images from at least 12 sequential glomerular crosssections at approximately the glomerular equator were collected for each histologic section using the Metamorph Image System (Metamorph, Universal Imaging Corp., Downingtown, PA, USA) by a blinded observer (Y.H.K.). The mean area of each glomerular profile was measured by manually tracing the glomerular outline on a video screen or encircling the area of interest and calculating that area by computerized morphometry using the Image Measurement System (Jandel Sigma Scan 3.0, SPSS, Inc., Chicago, IL, USA). The number of podocytes in each glomerular cross section as defined by GLEPP1 and the WT1 immunoperoxidase staining was counted. The area in each glomerulus that was not stained by the GLEPP1 immunoperoxidase stain (GLEPP1 negative) or alternatively was sclerosed as assessed by PAS staining, was also traced manually, and was measured by computerized morphometry as outlined previously in this article. The number of podocytes per glomerular volume was calculated by assuming that each glomerulus examined was a 3 μm-thick slice of a hemibisected sphere. Thus, the counted number of podocytes present in the volume of this slice was corrected for the complete spherical volume to calculate the number of podocytes per glomerular volume. The GAPP was calculated by measuring the total glomerular area and dividing that number by the counted number of podocytes in that glomerulus. The podocyte covered GAPP (P-GAPP) was calculated by measuring the glomerular area staining for GLEPP1 and dividing this area by the podocyte number counted in that glomerular area.

Glomerular sclerosis was graded on GLEPP1 immunoperoxidase and PAS-stained sections. The scoring system described by Saito et al was used as follows: 0, normal glomerulus; 1, sclerosis involving less than 25%; 2, sclerosis involving 25 to 50%; 3, sclerosis involving 50 to 75%; and 4, sclerosis involving 75 to 100% of glomerular tuft area [41]. The sclerotic index was calculated as follows: S.I. = [(n0 × 0) + (n1 × 1) + (n2 × 2) + (n3 × 3) + (n4 × 4)]/total glomeruli counted. More than 90 glomeruli from each rat were evaluated.

Statistical analysis

The mean number was calculated for each animal. This value was used for statistical analysis using analysis
of variance. All values were expressed as mean ± SEM. A $P$ value of less than 0.05 was considered to be statistically significant.

**RESULTS**

A single intraperitoneal injection of PAN (20 mg/100 g body weight) was used to assess the effect on glomerular podocyte number. Acute podocyte injury and proteinuria was induced as is well described from numerous prior studies (Fig. 1). The structural changes to the podocyte included pseudocyst formation and effacement of foot processes, as assessed by transmission electron microscopy (data not shown).

For initial studies, GLEPP1 was used as a marker of podocytes. A decrease in podocyte number per area was measured as shown in Figure 1. To confirm the observation, a second set of independent measurements was made using WT1 as a marker of podocytes. Very similar results were obtained with both the GLEPP1 and WT1 systems (Fig. 1). Over an 11-day period after PAN injection, the glomerular podocyte count dropped significantly below control levels and remained significantly below control for 126 days.

To address the question of whether this reduction in podocyte count was real or an artifact due to an increase in glomerular size after PAN injection, the number of podocytes per glomerular volume was calculated at the 126 day time point, by which time the glomerular volumes were not different between the experimental and control groups (6.0 ± 0.8 vs. 6.2 ± 0.4 × 10⁵ μm³). At this time, the mean number of podocytes per glomerular volume was 281 ± 18 for PAN-treated and 387 ± 18 for time control, respectively ($P < 0.01$). We concluded that the number of podocytes per glomerulus appeared to have decreased by approximately 27%, and that this reduction occurred between 5 and 11 days following PAN injection.

If podocytes were being lost, then it should be possible to visualize this process by structural analysis. Therefore, histologic sections stained by immunoperoxidase were examined for the podocyte marker GLEPP1 (Fig. 2). GLEPP1-positive cells that appeared to be detached from the GBM were present in Bowman’s space (Fig. 2A). Transmission electron microscopy (TEM) analysis also showed detached rounded up cells in Bowman’s space (Fig. 2C). Toluidine blue-stained one-micron sections of glomeruli were used to assess the degree of glomerulosclerosis. Twenty-four–hour urine protein excretion beginning by day 3 after PAN injection was significantly increased by positive cell count per glomerular area that is significantly increased by day 21 after PAN injection ($P < 0.01$). The Thy-1–positive cell nuclear number was significantly decreased (Fig. 1C), and is present by day 45 after PAN injection.

![Fig. 1. Time-course of variables measured in groups of rats following intraperitoneal injection of puromycin aminonucleoside (PAN).](image-url)
cells were present in Bowman’s space that had features of apoptotic cells (Fig. 2B). Therefore, TUNEL staining for apoptotic cells also was performed at various times after PAN injection. TUNEL-positive cells were present in glomeruli at days five to seven after PAN injection (Fig. 2D). TUNEL-positive cells were not seen either in control glomeruli or glomeruli at later times after the single PAN injection. Taken together, these data support the concept that podocytes are lost from glomeruli in the 5- to 11-day interval following PAN injection in the rat. This loss may be a combination of cell detachment and cell death with apoptosis as one possible mechanism involved.

To examine the hypothesis that podocytes were being lost from glomeruli in the days after PAN injection, the urine was analyzed for evidence of podocyte loss using an RT-PCR approach. Primers for the podocyte-specific proteins GLEPP1 were used, and the results are shown in Figure 3. No signal was detected in urine of normal rats. However, four to five days after injection of PAN, a positive signal GLEPP1 was detected in all of the three rats examined. No further signal was detected in the subsequent days after a single PAN injection. Thus, although detached cells might be in the process of apoptosis, at least some of the detached cells maintained intact mRNA long enough to be detected in the urine in spite of the apoptotic process. The finding that a positive signal was seen on only one day (presumably the peak of cell loss into the urine) suggested that the assay might be insensitive or the signal might be weak possibly due to apoptosis of detached podocytes.

By day 20 after PAN injection, the number of Thy-1–positive cells in glomeruli had increased (Fig. 1C). Since leukocytes as well as mesangial cells are Thy-1 positive, we cannot discriminate between these cell types from this analysis. However, the increase in Thy-1–positive cells...
and mesangial matrix had occurred by day 20, which was after the decrease in podocyte count, which occurred by day 11 in this model. Adhesions to Bowman’s capsule also were present by day 20 after PAN injection. Glomerulosclerosis was not a prominent feature of the single PAN injection model, but was present in increasing amount by day 45 in the model (Fig. 1D). Thus, the temporal sequence of events was first proteinuria (seen by day 3), followed by a reduction in podocyte number (by day 11), and then by an increase in Thy-1–positive cells and early adhesions to Bowman’s capsule (by day 20), and finally the appearance of glomerulosclerosis (by day 45).

If podocytes are being depleted in this model, then it should be possible to reduce the glomerular podocyte count further by sequential injections of PAN. To assess the effect of additional podocyte depletion, a second set of experiments was performed where rats received none, one, two, or three injections of PAN at the same dose per body weight at 30-day intervals, and animals were killed for analysis at 90 days. The results of this study are shown in Figure 4. Each injection of PAN caused an additional amount of podocyte depletion, additional glomerulosclerosis, and an additional degree of proteinuria. Representative histologic sections stained for GLEPP1, WT1, and by PAS for glomerulosclerotic area are shown in Figure 5. The region of the glomerulus that was glomerulosclerotic (PAS positive) corresponded to the area of the glomerulus that did not contain podocytes as defined by specific markers (GLEPP1 and WT1). The correlation between the GLEPP1-negative area and the PAS-positive area (measured independently in different histologic sections) is shown in Figure 6. We conclude that the area of the glomerulus without podocytes (GLEPP1 negative) corresponds closely to the glomerulosclerotic area.

Injury to podocytes results in an increase in glomerular volume, which is manifest by an increase in the glomerular area when seen in a two-dimensional image. The glomeru-
Fig. 5. Sequential histologic sections of control rats (A0, B0, C0), one PAN injection (A1, B1, C1), two PAN injections (A2, B2, C2), and three PAN injections (A3, B3, C3). Sections labeled A show immunoperoxidase staining for GLEPP1 and counterstained with PAS. Sections labeled B show immunoperoxidase staining for WT1. Sections labeled C show PAS staining to identify area of glomerulosclerosis. The arrows indicate areas of glomerulosclerosis in the PAN-treated animals. Arrowheads in A and B sections shows podocytes as identified by GLEPP1 staining (A) and WT1 staining (B).
To determine whether podocytes were capable of serving a larger area of the glomerulus after PAN injury, the glomerular area was expressed in terms of the number of podocytes present in relationship to glomerular area. This can be expressed as total glomerular area (normal plus sclerotic area) per podocyte (GAPP) or as the podocyte-containing glomerular area (GLEPP1-positive area) per podocyte (P-GAPP). As shown in Figure 8A, there was a 60% increase in P-GAPP after the first injection of PAN. However, with subsequent injections of PAN, there was no further increase in P-GAPP. Thus, we concluded that a podocyte can significantly increase the areas it serves by approximately 60%. The reduction in podocyte count after the second and third injection of PAN was not reflected by a further increase in area served by each podocyte (P-GAPP), but rather by an increase in glomerular area without podocytes. Thus, the maximum P-GAPP was approximately 1.6-fold the normal value. In contrast, the GAPP continued to increase with each PAN injection as scarring formed an increasingly large
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Fig. 9. Data expressed as percent of normal glomerular podocytes per glomerular volume and plotted against glomerulosclerosis as measured by PAS-positive area (A) or GLEPP1-negative area (B). In each case, there is a high correlation between these variables for the groups of rats treated by one, two or three injections of PAN ($r^2 > 0.9$). The Y intercept indicates the proportion of the normal podocyte complement that would need to be lost per glomerulus before glomerulosclerosis would occur. This value is approximately 10 to 20%.

proportion of the glomerular area (Fig. 8B). Thus, glomerulosclerosis developed beyond a particular threshold value for GAPP (total GAPP) of approximately 1.6 times the normal value.

To evaluate the relationship between podocyte depletion and glomerulosclerosis in a continuous way, the data for the groups of animals were plotted with percent normal podocyte number per glomerular volume against the proportion of the glomerulus that was glomerulosclerotic as measured either by PAS-positive area or by GLEPP1-negative area (Fig. 9). The $r^2$ values show that there was a very close relationship between the proportion of normal podocyte number remaining after each PAN injection and amount of glomerulosclerosis, as assessed by either method. Furthermore, the intercept on the Y axis at approximately 80 to 90% for the two methods of analysis indicates that there is a theoretical threshold of podocyte number below which glomerulosclerosis would be expected to occur. This number was at approximately 80 to 90% of the normal podocyte number for the rats studied. Thus, after the first PAN injection, the glomerular area increased by 1.3-fold (Fig. 7), and the podocyte count decreased by approximately 20% with small amount of glomerulosclerosis occurring. This combination of increased glomerular area and decreased podocyte number resulted in each podocyte having about a 1.5- to 1.6-fold increase in its normal territory (1.3 divided by 0.8) as is shown in Figure 8. Above this range, glomerulosclerosis became prominent.

**DISCUSSION**

This study used immunohistochemical markers (GLEPP1 and WT1) to define a podocyte. Under some circumstances in human renal biopsies, podocytes may lose these markers, as has been described in collapsing and human immunodeficiency virus (HIV)-associated glomerulosclerosis [30, 42] and possibly also in crescentic nephritis [43]. In both of these cases, podocytes appear to undergo a phenotypic switch that may be analogous to dedifferentiation toward a phenotype that possibly has the capacity to undergo cell division [4, 30]. In our study in rats, there was an apparent reduction in the number of podocytes per glomerulus following PAN injection as measured by WT1 and GLEPP1 staining. It is therefore possible that these markers could merely have been absent from damaged podocytes and therefore not visible under the conditions used, which would render the conclusions and interpretation of these studies invalid. We think this is unlikely for the following reasons. First, the experiments described here were originally performed with the hypothesis based on our previous report in human renal biopsies that identified phenotypically changed cells that had lost their markers (GLEPP1 and WT1) following PAN injection [42, 44]. Thus, we looked carefully for such cells to attempt to support this hypothesis, but were unable to find them. Second, if cells do lose their markers in association with injury, we would expect them to regain their markers after time when the injury had diminished as assessed by decreased proteinuria to the normal range. As shown in Figure 1, even after 126 days following PAN injury, there was no increase in number of podocytes back toward the control range, as identified by the WT1 and GLEPP1 markers. Third, podocytes (positive for GLEPP1) were identified as becoming detached from glomeruli and having an apoptotic phenotype as assessed by TUNEL staining, and podocyte-specific mRNA appearing in the urine was independent evidence that podocytes were being lost from the glomeruli. Thus, we conclude that the data cannot be explained in terms of a phenotypic switch to a podocyte without the GLEPP1 and WT1 markers as we had originally hypothesized. Rather, there was a real loss of podocytes from glomeruli following PAN injection.

As has been suggested by Kriz, Gretz, and Lemley, an important biological question is whether podocytes are able to cover adequately the area of GBM “assigned” to
them and thereby maintain the filtration characteristics of the glomerular filter [1–5]. Failure to do this results in “nude areas” causing in a predisposition to form adhesions to Bowman's capsule and probably also to mesangial expansion, which are the hallmarks of glomerulosclerosis. Two related events occur following injury to the podocyte by PAN. One event is a decrease in podocyte number, as shown previously in this article, and the other is an increase in glomerular size. With this concept in mind, the most important variable to measure is one that takes both these factors into account. This is conveniently considered as the number of podocytes per unit area. While it is very difficult to measure glomerular basement membrane area directly because of the three-dimensional convoluted structure of the GBM, it is not difficult to measure a derivative of it, namely, the number of podocytes per unit area on a glomerular tuft cross-section. In this report, we have designated this variable as the GAPP. A further useful and related variable is the glomerular area actually served by podocytes or P-GAPP. The P-GAPP was measured using the marker GLEPP1 to define the glomerular area served by podocytes. The GAPP and P-GAPP therefore provide different information. These two variables are useful as we try to understand podocyte pathology in the context of the glomerulus.

A third question is the measurement of the absolute number of podocytes per glomerulus. Since glomeruli are in general spherical, it is assumed that a cell count made at the equator of a glomerulus in a section of known thickness and measured diameter can be used to calculate the number of podocytes per whole glomerular volume. This method was used for some of the analyses (Fig. 4) where the data were expressed as podocytes per glomerular volume. Because these counts were made by a blinded investigator, were done for twelve consecutive glomeruli, and were used to compare animals between treatment groups, this is a reasonable approach. However, as with any method that relies on projections based on a sample of the whole, errors will tend to be multiplied.

To partially address this question, we chose to compare the podocyte counts in glomeruli of rats after 126 days when the size of glomeruli was not different between the treated group and age-matched animals from the same batch of rats. The podocyte counts were significantly reduced (by 27%) in the PAN-treated group. Thus, at least in this case, glomerular size did not confound the estimate of the total podocyte calculation. Other experimental approaches that use ultrastructure are limited by the number of podocytes that can be measured practically, and therefore by the sample size and selection bias, as well as by three-dimensional limitations and difficulties in deciding where one podocyte begins and another ends [31]. Both methods have resulted in similar conclusions, namely that podocyte counts per glomerulus are reduced under certain pathologic conditions.

The experimental results described in this report are compatible with the hypothesis that podocyte damage leading to podocyte depletion itself results in glomerulosclerosis in the rat PAN model. The observation that glomerulosclerosis occurred in that area of the glomerulus where podocytes were absent (as defined by GLEPP1 and WT1 markers) would be compatible with the concept that glomerulosclerosis might be the direct result of the absence of podocytes at a particular site in the glomerulus. The finding that the glomerular area that can be served per podocyte (P-GAPP) remained constant over the different levels of injury suggests that each podocyte has limited capacity to serve a larger glomerular area. The data provided suggest that the threshold above which glomerulosclerosis develops is about 1.5- to 1.6-fold the normal value for GAPP. However, this conclusion must be tempered by the consideration that all podocytes were injured by PAN in this model, so the recovered podocytes may have sustained damage that could affect the capacity to cover expanded territory.

Podocyte injury/loss and glomerular expansion was completed by day 11 of the PAN model. By day 20, the number of Thy-1–positive cells and mesangial expansion had increased, and adhesions between Bowman’s capsule and the outer glomerular capillary loops had occurred. Thy-1–positive cells are largely mesangial cells, although it is possible that the count could include some macrophages or T cells that could participate in driving the sclerotic process in this model [36]. The process of formation of adhesions between capillary loops and Bowman’s capsule as a consequence of denudation of the glomerular filtration surface has been described and modeled by Kriz, Gretz, and Lemley [3, 4]. The time course of mesangial expansion could reflect a similar process occurring in the mesangial compartment due to loss of podocytes from the capillary loop in areas adjacent to the mesangium. Thus, the same process (podocyte loss/injury) resulting in an increased GAPP might lead to both adhesions to Bowman’s capsule and mesangial expansion depending on where in the glomerulus the podocyte loss occurred. This concept would help explain the focal nature of focal segmental glomerulosclerosis (FSGS), a poorly understood but obviously important phenomenon seen in many glomerular diseases.

The concept of a variable degree of podocyte reserve and depletion of podocytes to a critical level beyond which glomerulosclerosis occurs is a potentially important one. Such a concept would complement the results of Pagtalunan et al, who measured podocyte number in diabetic Pima Indians [31, 32]. There appeared to be a threshold for GAPP in the Pima Indian population. Below this GAPP threshold, there was no proteinuria or glomerulosclerosis. Above this range, proteinuria and glomerulosclerosis developed.

Further studies are required to determine how differ-
ent genetic factors affect the glomerular complement of podocytes at birth and over a lifetime, and indeed whether decrements in podocyte number do in fact accompany the glomerulosclerotic process in human glomerular diseases. If particular human individuals or groups were to have a lower podocyte reserve on the basis of genetic or environmental influences, then this might help to explain susceptibility to progression to ESRD from hypertension, diabetes, FSGS, and other glomerular diseases.

Factors that might facilitate detachment of podocytes from the GBM would likely play a permissive role in podocyte depletion over time. Drenckhahn and Franke, and Whiteside et al have already shown that podocyte detachment occurs in the PAN model in the rat and provided an interesting mathematical analysis of how partial detachment would predispose to further detachment [5, 12]. We know from other cell systems that epithelial cell detachment from the underlying matrix triggers apoptosis [45]. Therefore, the finding of TUNEL-positive cells and detached cells with nuclear features suggestive of apoptosis is not surprising. Hara et al have also documented podocyte loss into the urine in human glomerular diseases [33]. Factors such as intraglomerular hypertension, immune complexes, and insertion of C5-9 into podocytes might well promote podocyte detachment/apoptosis. Factors that affect the GBM such as diabetes, amyloidosis, and immune complexes also could affect the ability of podocytes to remain attached to the GBM through integrins and other mechanisms. In this setting, underlying genetic factors that reduce adherence of podocytes to the filter surface might predispose an individual to develop glomerulosclerosis. One such factor has recently been described as the cause of late onset familial FSGS in humans [46]. In this case, the mutated protein is α-actinin 4, a protein responsible for linking the integrin molecule to the cytoskeleton of the podocyte. Integrins play a key role in attaching the podocyte to the GBM [47, 48]. A defect in this attachment mechanism could affect the degree to which podocytes adhere and therefore the rate at which they might be lost from the glomerulus. Further studies are required to determine whether families with these and other genetic mutations who develop FSGS also lose their podocytes at an accelerated rate. Such a process would help explain the variable time delay in onset of disease.

This report shows that mRNA in urine can be analyzed by RT-QPCR. This indicates that there is mRNA at least several hundred base pairs long in urine sediment cells. If these cells are apoptotic cells, then their mRNA is not fully degraded by the time it is excreted in urine. Alternatively, there are nonapoptotic cells in addition to apoptotic cells in the urine as has been shown by Hara et al using immunologic approaches [33]. In the studies described GLEPP1 RT-QPCR assay the urine was positive for only one day (day 4 to day 5) after PAN injection. It would be expected that cells were being lost into the urine over several days after PAN injection. This suggests that the method, as it was set up in this set of studies, was rather insensitive in that it only detected the peak day of podocyte excretion. With further refinement, the detection and characterization of mRNAs in urine may prove to be a useful adjunct to traditional methods of urine analysis and in particular might prove useful for monitoring podocyte excretion in the clinical setting using automated techniques.

The concept of progressive podocyte loss, finite podocyte reserve, and development of glomerulosclerosis as a consequence of podocyte loss beyond a critical threshold would also provide support for a rational explanation of why angiotensin-converting enzyme inhibitors are so effective at preventing progression in many forms of glomerular disease including diabetes [49]. The effect of angiotensin-converting enzyme (ACE) inhibitors to decrease intraglomerular pressure would likely be important in reducing the rate of podocyte detachment. Indeed, in the PAN model, ACE inhibitors have been shown to reduce proteinuria and the degree of glomerular injury [50]. The ACE inhibitor effect could well occur via several mechanisms, but reduction in podocyte detachment is an attractive and plausible one. This mechanism would complement the hyperfiltration concept promoted by Brenner, Lawler, and Mackenzie [51]. A loss of podocytes over time could also be an important factor helping to explain the remarkable increase in incidence of ESRD seen as the human population ages, as has been noted for the aging Milan normotensive rat [29].

Taken together, the results from this report provide support for a potentially important mechanism underlying progression of glomerular injury, and draws attention to the need to understand podocyte biology and the role of podocytes in glomerular diseases better.

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REFERENCES