Cardiovascular, Pulmonary and Renal Pathology

Diabetic Albuminuria Is Due to a Small Fraction of Nephrons Distinguished by Albumin-Stained Tubules and Glomerular Adhesions

Patricia M. Kralik,* Yunshi Long,* Ye Song,*
Lu Yang,[†] Haiyang Wei,[‡] Susan Coventry,*
Shirong Zheng,* and Paul N. Epstein*

From the Departments of Pediatrics,* Pharmacology and Toxicology,[†] and Anatomical Sciences and Neurobiology,[‡] University of Louisville, Louisville, Kentucky

OVE26 diabetic mice develop severe albuminuria. Immunohistochemical analysis revealed a pattern of intense albumin staining in a small subset of OVE26 tubules. Immunostaining was strikingly heterogeneous; some tubules stained intensely for albumin, but most tubules had weak or no staining. Serial sectioning showed that staining patterns were distinctive for each nephron. Electron microscopy revealed that albumin accumulated in villi and at the base of the brush border. Tubule cell injury, as shown by loss of villi, tubule dilation, and cellular protrusions into the tubule lumen, was unambiguously associated with albumin staining. Examination of albumin staining of proteinuric human kidneys also showed a heterogeneous pattern of staining. Analysis of OVE26 serial sections indicated that all glomeruli connected to albumin-positive tubules were identified by albumin-stained lesions in the tuft that adhered to Bowman's capsule, implicating this as a critical feature of heavy albumin leakage. These results indicate that albumin accumulation provides a marker of damaged nephrons, and confirm that albumin leakage produces significant tubular damage. This study shows that that formation of sclerotic glomerular adhesions is a critical step leading to severe albuminuria. (*Am J Pathol* 2009, 175:500–509; DOI: 10.2353/ajpath.2009.080939)

Diabetic nephropathy (DN) is the leading cause of end-stage renal disease. Unraveling DN pathology is difficult due to our inability to identify which glomeruli leak protein and connect to damaged tubules. Two-photon microscopy has been used to image function in individual nephrons, but this method works only in unusual rats with

glomeruli close to the kidney surface.¹ Thus, we cannot distinguish between functionally normal and abnormal glomeruli in diabetic kidneys. Another weakness of our research effort is the lack of an adequate model of DN. Many hyperglycemic animals have been characterized,^{2,3} but they display only early features of human DN. For example, rodent models of diabetes show only a very modest decline in glomerular filtration rate, but renal failure in humans means that glomerular filtration rate has declined by more than 80%.⁴ Furthermore albuminuria in these established DN models is increased by less than tenfold, whereas albuminuria in human DN is elevated several hundredfold.⁵

We and others described the OVE26 model of DN,^{6–8} which unlike prior DN models, exhibits advanced albuminuria. Here we report that severe albumin leakage can be easily identified by massive accumulation of albumin within proximal tubule epithelial cells. Using this accumulated albumin as a marker we are able to conclude that nephrons leaking excessive protein are rare, that nephron albumin leakage appears to be almost an all-or-none phenomenon, that proximal tubule epithelial cells exposed to high albumin are structurally damaged, and that every tubule with albumin accumulation originates in a glomerulus containing an albumin-stained adhesion between the tuft and Bowman's capsule. Furthermore, proteinuric human specimens show patterns of albumin accumulation similar to that which occurs in OVE26 mice.

Materials and Methods

Experimental Animals

OVE26 diabetic mice on the FVB background were produced in our laboratory and FVB mice were obtained

Supported by National Institutes of Health grant DK072032 and JDRF grant 4-2005-88.

P.M.K. and Y.L. contributed equally to the manuscript.

Accepted for publication April 29, 2009.

Address reprint requests to Paul N. Epstein, University of Louisville, 570 S Preston, Louisville, KY 40202. Email: paul.epstein@louisville.edu.

from Charles River (Frederick, MD). OVE26Nmt mice on the FVB background were produced in our laboratory⁶ and db/db mice on the background C57BLKS were obtained from Jackson Laboratories. All had free access to standard chow and water. Procedures were followed as per the Guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the University of Louisville Institutional Animal Care and Use Committee.

Urinary Albumin Excretion

Individual diabetic mice were placed in metabolic cages for 24 hours with access to chow and 10% liquid diet (Glucerna, Abbott Laboratories, Abbott Park, Illinois), as we have previously described.^{6,7} Urinary albumin was determined using a mouse albumin enzyme-linked immunosorbent assay kit (Bethyl Laboratories, Montgomery, TX) within the linear range of the assay and expressed as μg or $\text{mg}/24$ hours.

Histological Assessment of Renal Damage

Kidneys were removed from anesthetized mice, cut in half sagittally, and fixed in 10% neutral buffered formalin for 16 hours. They were then transferred to 70% ethanol until embedding in paraffin then sectioned at 3 μm . After deparaffinizing in xylene, the sections were stained with Masson's trichrome using standard protocol. Fibrosis in glomeruli was considered to be present if there were enlarged areas of fibrous blue staining in trichrome-stained images as judged by a blind observer. If large glomerular trichrome-stained areas were amorphous and cell-free, they were considered to be nodules. Glomeruli were indicated as positive or negative for fibrosis or nodules and statistical comparisons between groups of 4 FVB and 4 OVE26 mice were made by Mann-Whitney Rank Sum Test.

Immunohistochemistry

Mice were anesthetized with intraperitoneal injection of ketamine (100 mg/kg) and xylazine (32 mg/kg), then perfused through the heart with 30 ml of tyrode solution for 8 minutes, followed by 30 ml of a 10% neutral buffered formalin over 8 minutes. Kidneys were removed from anesthetized mice, cut in half sagittally, and then fixed in 10% neutral buffered formalin for 16 hours. Three-micrometer paraffin sections (prepared as described in the preceding paragraph) were used for immunohistochemical detection of albumin. Briefly, following deparaffinization and hydration, the sections were subjected to antigen retrieval using Dako target retrieval solution in a decloaking chamber (Biocare Medical, Concord, CA) at 125°C for 5 minutes; after cooling to room temperature, endogenous peroxidases were blocked by incubation in 3% H_2O_2 in water for 5 minutes. Nonspecific binding was blocked using 5% goat serum in PBS for 1 hour. The sections were then incubated with horseradish peroxidase-conjugated goat anti-mouse albumin (Bethyl Labs)

at 1:200 dilution for 1 hour at room temperature in a humidified chamber. Peroxidase activity was identified by 3 minutes reaction with 3,3'-diaminobenzidine (Vector Labs, Burlingame, CA) after which the sections were counterstained with hematoxylin (Gill's formula, Vector Labs), dehydrated, and mounted. For double staining of sections for albumin and C3 we used rabbit anti-mouse albumin (Bethyl Labs) followed by Cy3 conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) at a dilution of 1:200 and fluorescein isothiocyanate-conjugated goat anti-mouse C3 (MP Biomedicals) at a dilution of 1:100. For double staining of sections for albumin and IgG we used goat anti-mouse albumin (Bethyl Labs) at a dilution of 1:600 and fluorescein isothiocyanate-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories) at a dilution of 1:200.

Digital Quantitation of Albumin Staining

The sections were examined under light microscopy (Nikon Eclipse E600, Melville, NY) and the entire field of the kidney was digitized under low power ($\times 4$ objective) using a Nikon DS-Fi1 camera and NIS-Elements software; briefly, individual images of the section were captured, and then merged to produce an image of the whole kidney section. Using Adobe Photoshop CS2 software (Adobe Systems, San Jose, CA) the medulla was cropped off leaving just the cortical area of the whole section image. The stained tubule clusters were counted and the number of clusters per total cortical area was determined. Albumin staining in the cortical area was assessed using the color selection tool of the program and the volume of tubular albumin staining per total cortical area was determined. Statistical comparisons were made by one-way analysis of variance with Sigma Stat software (San Jose, CA).

Albumin Immunohistochemistry for Electron Microscopy

The protocol is a modification of that previously reported.⁹ Mice were deeply anesthetized with intraperitoneal injection of ketamine (100 mg/kg) and xylazine (32 mg/kg) then perfused through the heart with Tyrode solution, followed by a fixative of 4% paraformaldehyde in 0.1 M/L sodium phosphate buffer, pH 7.4. The kidneys were removed and cut sagittally, then placed overnight in 4% paraformaldehyde; the following day the tissue was placed in 0.1 M/L sodium phosphate buffer at 4°C until sectioning at 50- μm thickness with a vibratome. The sections were then blocked with 10% normal goat serum and 0.3% Triton 100 in PBS for 30 minutes followed by incubation overnight at 4°C with horseradish peroxidase-conjugated goat anti-mouse albumin diluted to 1/800 in 1% normal goat serum in PBS. After washing with sodium phosphate buffer, then 0.1 M/L sodium acetate buffer, pH 6.0, sections were incubated with filtered, nickel-intensified diaminobenzidine reaction solution for 5 to 10 minutes. The sections were washed again with the sodium

Figure 1. Albumin accumulates in tubule clusters of severely albuminuric OVE26 mice. Albumin immunohistochemistry was performed on kidney sections from OVE26 mice with albuminuria over 10 mg/day (Hi OVE), less than 4 mg/day (Low OVE), or non-diabetic FVB mice (FVB). **A–C:** Typical low power (original magnification, $\times 4\times$) images from Hi OVE, Low OVE, and FVB mice. Examples of some tubule clusters have been circled in (**A**) and all tubule clusters are circled in (**B**). **D:** Average daily urine albumin for the mice used in this analysis. **E and F:** Semiquantitative comparisons for the volume of albumin staining (area multiplied by intensity of staining) and the number of stained clusters normalized to renal cortical area. **Asterisks in D–F** indicate that all groups are significantly different from one another ($P < 0.05$) by one-way analysis of variance on ranks ($n = 4$ mice per group). Vertical bars are the SE.

acetate buffer and sodium phosphate buffer before mounting on slides for examination under bright field microscopy. Sections were postfixed in 2% osmium tetroxide, dehydrated with an ethanol series, and then embedded in Durcupan resin (Ted Pella Co., Redding, CA) between two sheets of Aclar plastic (Ladd Research, Williston, VT). After examining the embedded sections under light microscopy, selected areas of interest were taken to mount on blocks and ultrathin sections (70 to 80 nm, silver-gray interference color) were cut serially with a diamond knife. To avoid visualizing the same cortical area on multiple sections, every tenth section was collected on Formvar-coated copper grids. Selected sections were then stained with 10% uranyl acetate in methanol for 30 minutes before examining under electron microscopy.

Lectin Histochemistry

To localize the structures predominantly staining for albumin, two different biotinylated lectins (Vector Labs) were used to identify proximal and distal tubules in kidney sections adjacent to sections previously stained for albumin. Lotus tetragonolobus lectin (LTA) was used as a proximal tubule marker¹⁰ and peanut agglutinin was used as a distal tubule marker.^{11,12} Briefly, after the sections were cleared of paraffin and rehydrated, they were heated to 98°C in Dako Target Retrieval solution for 20 minutes, rinsed, and then treated with 3% H₂O₂ for 5 minutes. After blocking with 0.1% bovine serum albumin in PBS for 1 hour, the sections were incubated with the biotinylated lectins (20 μ g/ml prepared in 10 mmol/L HEPES, 0.15 M/L NaCl, with 0.1 mmol/L each Ca²⁺,

Figure 2. Consistency of albumin staining in serial sections. **A–D:** Albumin staining in a series of four adjacent 3- μ m sections. The same tubules stain in each section and they display similar morphology and the same pattern of staining in each section. The **red arrows** point to the same dilated tubule with mostly brush border staining. The **black arrows** point to the same dilated tubule with brush border and cytoplasmic staining. The **white arrow** points to the same non-dilated tubule with only cytoplasmic staining.

Figure 3. Diabetic models with low levels of albuminuria have weak tubular albumin staining. **A:** Typical staining from an OVE26Nmt kidney at 5 months of age with albuminuria of 0.3 mg per day and blood glucose over 600 mg/dl. **B:** Typical staining in a db/db diabetic mouse on the C57BLKS background. Despite blood glucose values over 550 mg/dl tubule staining was weak. **C:** Strong albumin staining of one OVE26Nmt mouse at 7 months of age with high albuminuria (11 mg per day). Observations in **(A)** and **(B)** were from three OVE26Nmt mice and three db/db mice between 4 and 5 months of age.

Mg²⁺, and Mn²⁺) for 30 minutes at room temperature. The sections were rinsed in PBS containing 0.1 mmol/L Ca²⁺, Mg²⁺, and Mn²⁺ then incubated with Elite ABC reagent (Vector Labs) for 30 minutes at room temperature, after which reactions were developed with diaminobenzidine and counterstained as mentioned previously.

Results

Tubule Albumin Staining

Most adult OVE26 diabetic mice excrete more than 10 mg of albumin per day.⁷ Kidney sections of OVE26 mice were stained with anti-mouse albumin antibody to determine whether albumin accumulates in renal tubules. As shown in Figure 1A, tubular staining was evident and present in a strikingly heterogeneous pattern: Some tubules stained intensely but most tubules did not stain above background. Higher magnification (Figure 2) revealed that tubules stained in clusters and that within each cluster, the staining pattern of the tubules was similar. This suggests that each stained cluster is made up of tubules from the same nephron. All OVE26 mice develop hyperglycemia above 600 mg/dl, but the onset of severe albuminuria over 10 mgs per day is variable. As shown in Figure 1D some adult OVE26 mice have only moderate levels of albuminuria, between 1 and 4 mg per day. These mice exhibited less albumin staining (Figure 1B) than mice with higher albuminuria, but their staining was clearly different from nondiabetic mice (Figure 1C). It should be pointed out that occasional tubules in most mice, including nondiabetic mice sometimes presented detectable staining for albumin and this could be intensified by more extended exposure to diaminobenzidine chromagen. The impression that there are groups of albumin stained tubules in proteinuric mice was confirmed by semiquantitative analysis of staining in groups of high albuminuric diabetic mice (urinary albumin excretion [UAE] >10 mg/day, average age 7 months), low albuminuric diabetic mice (UAE <4 mg/day, average age 4 months), and nondiabetic mice (typically UAE <0.2 mg/day, average age 5 months). The extent of albumin staining expressed either as the area of staining multiplied by

Figure 4. Albumin staining is in a subset of proximal tubules of OVE26 mice. **A–C:** Serial or near serial sections stained for albumin **(A)**, the proximal tubule marker LTA **(B)**, and the distal tubule marker peanut agglutinin **(C)**. All albumin staining appears to be in LTA-stained tubules, but not all LTA-stained tubules stain for albumin.

Figure 5. Trichrome and albumin staining of tubules in OVE26 serial sections. **A** and **B**: $\times 200$ images of albumin stained tubules that are dilated, thin-walled, and contain casts. **C** and **D**: $\times 1000$ images of the tubules indicated by arrows in (**A**) and (**B**). **E** and **F**: Two albumin-stained tubules (marked by arrows) that appear frayed or mottled, as compared with surrounding tubules. In trichrome staining these tubules are brighter red than surrounding tubules. **G** and **H**: $\times 1000$ images of the lower right tubule.

staining intensity (Figure 1E) or simply as the number of stained clusters (Figure 1F) was higher in low albuminuria OVE26 mice than in FVB mice ($P < 0.05$) and greatest in high albuminuria OVE26 mice ($P < 0.05$). To demonstrate that the heterogeneous albumin staining pattern was not an artifact, serial kidney sections from high albuminuric OVE26 mice were stained for albumin. The same subset of tubules stained in four consecutive sections (Figure 2, A–D) and we have followed stained tubules for up to forty sections. The pattern of staining was also maintained between sections: Different tubules stained predominantly in the cytoplasm, brush border, or both, and the pattern for each tubule showed continuity between sections.

To confirm that albumin accumulation was related to high levels of albuminuria rather than diabetes per se, we stained kidneys from diabetic models with lower levels of albumin excretion than OVE26 mice. A recently generated transgenic mouse (designated Nmt) overexpresses the antioxidant protein metallothionein in podocytes.⁶

Figure 6. Albumin-stained OVE26 tubules also stain for IgG and C3. OVE26 (**A**, **C**, **E**, **G**) and FVB (**B**, **D**, **F**, **H**) stained tubules were double stained for albumin and mouse IgG (**A–F**) or for albumin and mouse complement component C3 (**G–H**) as described in *Materials and Methods*. Images are typical of examples from at least three mice of each type. The antibody and the type of mouse are indicated on each panel. Side by side panels show double staining of the section. Original magnification, $\times 400$.

Nmt mice crossed with OVE26 mice produce double transgenic OVE26Nmt mice with the same hyperglycemia as OVE26 but typically reduced albuminuria. Kidneys from OVE26Nmt mice with UAE levels between 0.3 and 0.7 mg showed weak staining for albumin (Figure 3A). We also examined albumin accumulation in db/db mice with blood glucose levels over 550 mg/dl. All of these mice had UAE values less than 1 mg and none of them showed positive kidney staining for albumin (Figure 3B). One OVE26Nmt mouse did have a UAE level of 11 mg and demonstrated albumin staining typical of what was seen in OVE26 mice with comparable albuminuria (Figure 3C). We next assessed whether OVE26 staining was in distal or proximal tubules by staining with lectin markers: LTA for proximal tubules and peanut agglutinin for distal tubules. As shown in Figure 4, A–C, obvious albumin staining was predominantly in tubules that reacted with LTA; however only a fraction of proximal tubules stained strongly for albumin.

Albumin-Associated Tubule Abnormalities

Trichrome staining was used to assess morphology of albumin-stained tubules (Figure 5). Many stained tubules contained casts that were often lost during immunostaining (compare Figures 5, A–D). Most albumin-containing tubules were dilated and thin walled. However, a few stained tubules were thick but appeared ragged and contained intense red droplets on trichrome images (Figure 5, E–H). These droplets appear identical to the protein droplets found in tubule cells of axotolotol kidney after chronic protein overload treatment.¹³ We also tested whether two other serum proteins, IgG and C3, accumulated in diabetic tubules. This has been observed in nondiabetic models of proteinuria.¹⁴ As shown in Figure 6, A–H, most of the OVE26 tubules that stained for albumin also stained for IgG and C3. Some albumin stained tubules did not stain for C3 as seen in the lower left tubule of panels C and G.

More detailed analysis of albumin associated damage to tubules was performed by immuno-electron microscopy with the same antibody used for light microscopy on five OVE26 mice and four FVB mice. As observed at the light level, FVB tubules (Figure 7A) and most OVE26 tubules (Figure 7B) had weak albumin staining. In FVB and unstained OVE26 tubules the brush border displayed a continuous layer of villi and the lumen was small. Some OVE26 tubules stained strongly for albumin (Figure 7, C–F), especially near the surface of the lumen, corresponding to the villi and base of the brush border. Only stained tubules displayed abnormal morphology; their brush border was less dense and completely absent in patches (Figure 7C). In many cells, the cytoplasm protruded into the lumen (Figure 7D) and these protrusions had the same texture as detached bodies within the lumen (Figure 7E). Positively stained tubules were dilated and frequently adjacent to ragged, thin-walled tubules (Figure 7F), which appear to be damaged distal tubules. These thin-walled tubules may correspond to the damaged tubules visible by light microscopy on the bottom half of Figure 5, C and D.

Dense, cytoplasmic particles were common paralleling the brush border in FVB (Figure 7A) and OVE26 (Figures 7, C–E) proximal tubule epithelial cells. These particles are consistent in shape and location¹⁵ to lysosomes. Because lysosomes are innately electron dense, it is not possible to tell if they stain for albumin. However in OVE26 cells with positive staining, the lysosomes were obviously bigger (400 to 1000 nm diameter) than in FVB tubules (100 to 400 nm diameter).

Albumin Staining in Tubules of Human Samples

Kidney sections from three non-proteinuric individuals, one patient with diabetic nephropathy and five patients with non-diabetic proteinuria were stained with anti-human albumin antibody. Proteinuric sample stained much more strongly for albumin (Figures 8, A–C) than nonproteinuric samples (Figure 8D). Overall, staining was more prevalent and dispersed in the cytoplasm of human

Figure 7. Electron microscopy reveals albumin-associated tubule damage. **A:** FVB tubules did not have positive albumin staining and they had a dense brush border surrounding the lumen (original magnification, $\times 3800$, scale bar = $2\ \mu\text{m}$). **B:** In most OVE26 tubules there was no positive albumin staining and the brush border, and lumen appear normal (original magnification, $\times 1900$, scale bar = $5\ \mu\text{m}$). **C:** In many OVE26 tubules the brush border stained strongly for albumin (original magnification, $\times 3800$, scale bar = $2\ \mu\text{m}$). In these positively stained tubules the brush border density is reduced or absent. **D:** In many positively stained tubules, the cytoplasm protrudes into the tubule lumen (original magnification, $\times 2750$, scale bar = $2\ \mu\text{m}$). **E:** Tubular protrusions are released into the tubular lumen (original magnification, $\times 2750$, scale bar = $2\ \mu\text{m}$). **F:** Thin-walled ragged tubules sit adjacent to positively stained proximal tubules (original magnification, $\times 1475$, scale bar = $5\ \mu\text{m}$).

samples than in OVE26 samples. However there was an obvious distinction between stained and unstained clusters of tubules, similar to what was seen in OVE26 samples.

Identification of Glomeruli Leading to Albumin-Stained Tubules

Positive albumin staining provides a marker to map the connection between tubules that carry excessive albumin and glomeruli that leak albumin. For brevity, we refer to glomeruli that originate stained tubules as “impaired.” A basic example of the tracing procedure is shown in Figure 9A. The positively stained tubule can be followed in each section until it reaches the impaired glomerulus of origin. In this manner, 23 impaired glomeruli from three

Figure 8. Tubules of human proteinuric patients stain with human albumin antibody. Sections are from a chronic diabetic nephropathy patient (A), two nephrotic patients (B and C), and a non-proteinuric patient (D). Original magnification, $\times 200$.

OVE26 mice were identified as the origin of 23 albumin-stained tubules. For comparison, we analyzed another 23 OVE26 glomeruli that did not lead to albumin stained tubules. These will be referred to as OVE26 control glomeruli. Sections passing through the entire glomerulus were examined. The most distinctive feature of impaired OVE26 glomeruli were large, albumin-stained adhesions between the glomerular tuft and Bowman's capsule (Figure 9B) that resemble glomerulosclerotic crescents.¹⁶ The albumin stained adhesions were present in 100 percentage of OVE26 glomeruli that led to stained tubules and 0 percentage of control OVE26 glomeruli (Figure 9C). The cross-sectional area of the glomerulus, measured in the middle section of each glomerulus was 17% larger (Figure 9D) in impaired glomeruli, as compared with control ($P < 0.02$). This difference was not due to enlargement of the area of glomerular tuft (Figure 9E), as this was almost identical in impaired and control OVE26 glomeruli. Rather, the difference was due to expansion of Bowman's space by 258% (Figure 9F, $P < 0.01$). We also examined 782 random glomerular profiles from albumin-stained kidney sections of five OVE26 mice (data not shown). Only 28 glomeruli or 3.9% contained adhesions, which most likely was underestimated since only one section from each glomerulus was examined.

Trichrome staining was used to visualize fibrosis (Figure 10). As previously reported^{6,7,17} OVE26 glomeruli are more fibrotic and larger than FVB glomeruli (Figure 10, A and B). Albumin adhesions often coincided with regions of fibrosis (Figure 10, C and D). We examined the frequency of glomerular fibrosis (Figure 10E) and nodules (Figure 10F). Analysis of more than 300 OVE26 and 300 FVB glomerular profiles showed fibrotic areas in 53% of OVE26 glomeruli, but only 6% of FVB glomeruli. Nodules were found in 13% and 0.3%, of OVE26 and FVB glomeruli, respectively. Thus nodules and fibrosis were more common than adhesions in OVE26 glomeruli.

Discussion

Our results show a striking heterogeneous pattern of albumin staining in kidneys of OVE26 diabetic mice. Clusters of proximal tubules stained strongly, but most nephrons had weak albumin staining. The extent of positive staining was dependent on the level of albuminuria. Accumulation of albumin was associated with tubular dilation, loss of brush border villi, and protrusion of the epithelial cells into the lumen. Proteinuria in human nephropathy patients was also associated with accumulation of tubular albumin. Albumin-stained tubules in OVE26 mice originated in glomeruli distinguished by albumin-stained adhesion and expansion of Bowman's space.

Protein Accumulation in Tubule Cells and Damage to Tubules

The intense but heterogeneous staining of tubules described herein has not, to our knowledge been reported before in a model of DN. The accumulation of tubular albumin in OVE26 mice appears to be a function of their severe albuminuria. By 5 months of age, most OVE26 mice excrete 10 mg of albumin per day. This is 30 times higher than other diabetic models,² and extends over a longer time course than other proteinuria models.¹⁸ Selected OVE26 mice, with albuminuria between 1 and 4 mg per day also had tubular staining, but it was significantly less than that seen in the most albuminuric group, as assessed by semiquantitative analysis. There was also less albumin staining in other severely hyperglycemic, but less albuminuric models of diabetes, such as db/db and OVE26Nmt. Thus it appears that albuminuria rather than extreme hyperglycemia is critical to albumin accumulation in OVE26 tubules.

The major site of albumin accumulation is in the villi and the base of the brush border. This is consistent with the site of albumin binding to the cubulin/megalin complex and absorption of the complex into endocytotic vesicles.¹⁹ After dissociation of the receptor-albumin complex, endocytotic vesicles carry albumin to lysosomes for degradation. Lysosomes of OVE26 tubule cells observed by electron microscopy were enlarged, suggesting accumulation of protein in this compartment. The accumulation of albumin in the villi and enlargement of lysosomes suggest that the capacity to process protein was overwhelmed, but that the uptake of protein from the lumen continued.

Overload of albumin has long been proposed as a primary cause of tubular injury,²⁰ though this is not universally accepted.^{21,22} The heterogeneity of albumin accumulation in separate nephrons provides an opportunity to compare tubules in the same kidney that have dramatically different albumin loads. It was apparent by immuno-electron microscopy that albumin-positive tubules were especially damaged. Extensive areas of brush border villi were absent and in areas denuded of villi the contents of the epithelial cell protruded into the lumen. These protrusions matched the size and texture of bodies

Figure 9. Features of glomeruli leading to albumin-stained tubules. **A:** Example of the method used to find glomeruli that lead to positively stained tubules. The asterisk in each panel marks the mapped tubule and its originating glomerulus (original magnification, $\times 400$). **B:** Typical examples of glomeruli that have been connected to albumin-stained tubules by serial sectioning. An albumin-stained adhesion is evident in each glomerulus. The left most panel also shows a typical OVE26 control glomerulus that does not lead to a stained tubule nor contains an adhesion (original magnification, $\times 100$). **C:** Quantitation of fully sectioned glomeruli that contain albumin-stained adhesions from 23 glomeruli leading to albumin-stained tubules (designated as Impaired OVE26) and 23 glomeruli that do not lead to stained tubules (designated as Control OVE26); $*P < 0.01$ by χ^2 test. **D–F:** Glomerular area, glomerular tuft area, and the area of Bowman's space for the center section of each of the Impaired or Control OVE26 glomeruli (area is in μm^2 , $*P < 0.01$ by student's *t*-test). Results are from three severely albuminuric OVE26 mice.

floating free within the lumen suggesting that these protrusions eventually detach from proximal tubule epithelial cells. In OVE26 tubules, IgG and complement component C3 leak into the same nephrons as albumin and they could be the direct mediators of damage. C3 leakage has been reported in several other models of proteinuria,²⁰ but to our knowledge has not been shown for diabetic nephropathy. We suspect that this is due to the fact that OVE26 mice have far higher levels of protein leakage than other models of DN. Overworked lysosomes could

also increase free radical production²³ or leak lysosomal enzymes. While our results do not provide the mechanism of damage, they show that tubule injury was a function of protein leakage within individual nephrons.

Tubules of proteinuric human samples revealed some similarities to OVE26 tubules, which indicate that these results have relevance beyond OVE26 nephropathy. Human proteinuric samples exhibited higher albumin accumulation than nonproteinuric specimens. Also, albumin accumulation was very heterogeneous, as was seen in

Figure 10. Characterization of OVE26 glomeruli by trichrome staining. In a typical comparison (**A** and **B**), it is apparent that OVE26 glomeruli had more fibrous material and were much larger than FVB glomeruli (original magnification, $\times 400$). **C** and **D**: Consecutive sections of an impaired OVE26 glomerulus stained for albumin and trichrome, respectively (original magnification, $\times 100$). Albumin adhesions often co-localized with fibrous areas (indicated by **arrows**). In OVE26 sections the frequency of fibrotic glomeruli (**E**) and the frequency of glomeruli with nodules (**F**) were higher than in FVB glomeruli ($*P < 0.03$ by Mann-Whitney Rank Sum Test) and much more frequent than albumin adhesions.

OVE26 kidneys. There are at least two nephron subpopulations, one that does not accumulate albumin and another that accumulates a great deal of albumin and exhibits damage. The albumin staining observed in human nephrotic samples was different from that observed in OVE26 samples: Fewer tubules stained in diabetic mouse than in human biopsy samples. Also the subcellular pattern of staining was different; in the OVE26 kidney, albumin staining was primarily near the brush border or in particulate subcellular organelles, and to a lesser extent diffused throughout the cytoplasm. Some tubules of human specimens did display brush border staining (data not shown), but the predominant staining pattern appeared to be diffuse throughout the cytoplasm. We do not know the basis of the different pattern seen in human tubules, which may be due in part to differences in sample preparation.

Glomeruli Leading to Albumin-Stained Tubules

Tracing of albumin-stained tubules in consecutive, serial sections allowed us to make an absolutely certain connection between tubules accumulating albumin and the glomeruli that leak albumin. Every glomerulus containing an albumin-stained adhesion led to an albumin-stained tubule. These glomeruli exhibited additional abnormalities such as fibrosis and enlargement of Bowman's space. Glomerular fibrosis is a common characteristic of human DN and is reported in most experimental models of diabetes. Since glomerular fibrosis occurs in diabetic models even if albumin excretion is only marginally increased,^{2,24,25} and because we observed fibrosis in glomeruli that did not lead to albumin stained tubules, it

seems unlikely that fibrosis was the factor directly responsible for albumin accumulation. It was striking that albumin-stained adhesions were observed in all OVE26 glomeruli leading to albumin-stained tubules and adhesions were not present in any of the glomeruli that did not lead to albumin-stained tubules. The adhesions resemble crescentic glomerular lesions reported in some models of DN. Significantly, those models also exhibit extreme albuminuria,^{26,27} suggesting that adhesions between the glomerular tuft and Bowman's capsule are a pathological marker for profound albuminuria. This is consistent with proposals in nondiabetic proteinuric models.¹⁶ Since we could directly map the connection between albumin-stained tubules and adhesion-containing glomeruli, our findings reinforce this proposition.

Abbate et al^{14,28,29} studying nondiabetic models of proteinuria, previously obtained findings similar to what we observed in diabetic OVE26 mice. They reported renal deposition of albumin, C3 and IgG, which accumulated in glomerular adhesions and proximal tubules. Therapies that reduced proteinuria²⁸ decreased protein accumulation, renal inflammation, and interstitial fibrosis. When their observations were made early in the disease process, before glomerular adhesions were established, they still found scattered deposits of protein in the glomerulus, suggesting that adhesions are not a requirement for protein leakage, rather they may be a consequence of leakage. In OVE26 mice proteinuria develops stochastically anytime between 2 and 7 months of age⁷ making it difficult to study pre-albuminuric stages of the disease. However, if the onset of protein leakage promotes further glomerular damage this would lead to an acceleration of glomerular pathology on an individual

nephron basis. This process of feed-forward pathology could explain the sharp differences in tubular accumulation that we observed between neighboring nephrons in OVE26 mice.

It is not possible to determine in this diabetic model whether the albumin-stained adhesions are a cause or consequence of albumin leakage. However based on our findings that all adhesion-containing glomeruli led to stained tubules, the sharp distinction between populations of albumin-stained and weakly stained tubules, and the damage to albumin-stained proximal tubule epithelial cells, we support the following scheme: A combination of glomerular expansion^{7,30} and podocyte death⁶ reduces podocyte density,³⁰ which may initiate protein leakage. As previously proposed in nondiabetic models of proteinuria,^{16,22} reduced podocyte density also exposes patches of the glomerular basement membrane that is then primed for adhesion to Bowman's capsule. The adhesion triggers local breakdown of the filtration barrier with more profound protein leakage, leading to brush border damage and subsequent nephron damage.

Acknowledgments

We thank Sherry Clark, Yun Huang, and Sallye Burns for their assistance. We also thank Jon B. Klein, Jianxiang Xu, and Edward C. Carlson for valuable discussions of data and conclusions.

References

- Molitoris BA, Sandoval RM: Intravital multiphoton microscopy of dynamic renal processes. *Am J Physiol Renal Physiol* 2005, 288:F1084–F1089
- Qi Z, Fujita H, Jin J, Davis LS, Wang Y, Fogo AB, Breyer MD: Characterization of susceptibility of inbred mouse strains to diabetic nephropathy. *Diabetes* 2005, 54:2628–2637
- Breyer MD: Stacking the deck for drug discovery in diabetic nephropathy: in search of an animal model. *J Am Soc Nephrol* 2008, 19:1623–1624
- K/DOQI clinical practice guidelines for chronic kidney disease: evaluation, classification, and stratification. *Am J Kidney Dis* 2002, 39:S1–266
- Nathan DM: Long-term complications of diabetes mellitus. *N Engl J Med* 1993, 328:1676–1685
- Zheng S, Carlson EC, Yang L, Kralik PM, Huang Y, Epstein PN: Podocyte-specific overexpression of the antioxidant metallothionein reduces diabetic nephropathy. *J Am Soc Nephrol* 2008, 19:2077–2085
- Zheng S, Noonan WT, Metreveli NS, Coventry S, Kralik PM, Carlson EC, Epstein PN: Development of late-stage diabetic nephropathy in OVE26 diabetic mice. *Diabetes* 2004, 53:3248–3257
- Yuzawa Y, Niki I, Kosugi T, Maruyama S, Yoshida F, Takeda M, Tagawa Y, Kaneko Y, Kimura T, Kato N, Yamamoto J, Sato W, Nakagawa T, Matsuo S: Overexpression of calmodulin in pancreatic [beta] cells induces diabetic nephropathy. *J Am Soc Nephrol* 2008, 19:1701–1711
- Bickford ME, Wei H, Eisenback MA, Chomsung RD, Slusarczyk AS, Dankowski AB: Synaptic organization of thalamocortical axon collaterals in the perigeniculate nucleus and dorsal lateral geniculate nucleus. *J Comp Neurol* 2008, 508:264–285
- Hossain Z, Ali SM, Ko HL, Xu J, Ng CP, Guo K, Qi Z, Ponniah S, Hong W, Hunziker W: Glomerulocystic kidney disease in mice with a targeted inactivation of Wwtr1. *Proc Natl Acad Sci USA* 2007, 104:1631–1636
- Raschperger E, Thyberg J, Pettersson S, Philipson L, Fuxe J, Pettersson RF: The coxsackie- and adenovirus receptor (CAR) is an *in vivo* marker for epithelial tight junctions, with a potential role in regulating permeability and tissue homeostasis. *Exp Cell Res* 2006, 312:1566–1580
- Surendran K, Simon TC, Liapis H, McGuire JK: Matrilysin (MMP-7) expression in renal tubular damage: association with Wnt4. *Kidney Int* 2004, 65:2212–2222
- Gross ML, Hanke W, Koch A, Ziebart H, Amann K, Ritz E: Intraperitoneal protein injection in the axolotl: the amphibian kidney as a novel model to study tubulointerstitial activation. *Kidney Int* 2002, 62:51–59
- Abbate M, Zoja C, Corna D, Capitanio M, Bertani T, Remuzzi G: In progressive nephropathies, overload of tubular cells with filtered proteins translates glomerular permeability dysfunction into cellular signals of interstitial inflammation. *J Am Soc Nephrol* 1998, 9:1213–1224
- Yabuki A, Suzuki S, Matsumoto M, Nishinakagawa H: Effects of sex hormones on the development of giant lysosomes in the proximal tubules of DBA/2Cr mouse kidney. *J Anat* 2003, 202:445–452
- Kriz W: The pathogenesis of 'classic' focal segmental glomerulosclerosis—lessons from rat models. *Nephrol Dial Transplant* 2003, 18 Suppl 6:vi39–vi44
- Carlson EC, Audette JL, Klevay LM, Nguyen H, Epstein PN: Ultrastructural and functional analyses of nephropathy in calmodulin-induced diabetic transgenic mice. *Anat Rec* 1997, 247:9–19
- Kriz W, LeHir M: Pathways to nephron loss starting from glomerular diseases—insights from animal models. *Kidney Int* 2005, 67:404–419
- Gekle M: Renal tubule albumin transport. *Annu Rev Physiol* 2005, 67:573–594
- Abbate M, Zoja C, Remuzzi G: How does proteinuria cause progressive renal damage? *J Am Soc Nephrol* 2006, 17:2974–2984
- Le Hir M, Besse-Eschmann V: A novel mechanism of nephron loss in a murine model of crescentic glomerulonephritis. *Kidney Int* 2003, 63:591–599
- Hoffmann S, Podlich D, Hahnel B, Kriz W, Gretz N: Angiotensin II type 1 receptor overexpression in podocytes induces glomerulosclerosis in transgenic rats. *J Am Soc Nephrol* 2004, 15:1475–1487
- Persson HL, Kurz T, Eaton JW, Brunk UT: Radiation-induced cell death: importance of lysosomal destabilization. *Biochem J* 2005, 389:877–884
- Kanetsuna Y, Hirano K, Nagata M, Gannon MA, Takahashi K, Harris RC, Breyer MD, Takahashi T: Characterization of diabetic nephropathy in a transgenic model of hypoinsulinemic diabetes. *Am J Physiol Renal Physiol* 2006, 291:F1315–F1322
- Zent R, Yan X, Su Y, Hudson BG, Borza DB, Moeckel GW, Qi Z, Sado Y, Breyer MD, Vozizyan P, Pozzi A: Glomerular injury is exacerbated in diabetic integrin alpha1-null mice. *Kidney Int* 2006, 70:460–470
- Zhao HJ, Wang S, Cheng H, Zhang MZ, Takahashi T, Fogo AB, Breyer MD, Harris RC: Endothelial nitric oxide synthase deficiency produces accelerated nephropathy in diabetic mice. *J Am Soc Nephrol* 2006, 17:2664–2669
- Inagi R, Yamamoto Y, Nangaku M, Usuda N, Okamoto H, Kurokawa K, van Ypersele de Strihou C, Yamamoto H, Miyata T: A severe diabetic nephropathy model with early development of nodule-like lesions induced by megalin overexpression in RAGE/iNOS transgenic mice. *Diabetes* 2006, 55:356–366
- Abbate M, Zoja C, Rottoli D, Corna D, Perico N, Bertani T, Remuzzi G: Antiproteinuric therapy while preventing the abnormal protein traffic in proximal tubule abrogates protein- and complement-dependent interstitial inflammation in experimental renal disease. *J Am Soc Nephrol* 1999, 10:804–813
- Abbate M, Zoja C, Morigi M, Rottoli D, Angioletti S, Tomasoni S, Zanchi C, Longaretti L, Donadelli R, Remuzzi G: Transforming growth factor-beta1 is up-regulated by podocytes in response to excess intraglomerular passage of proteins: a central pathway in progressive glomerulosclerosis. *Am J Pathol* 2002, 161:2179–2193
- Teiken JM, Audette JL, Laturnus DI, Zheng S, Epstein PN, Carlson EC: Podocyte loss in aging OVE26 diabetic mice. *Anat Rec (Hoboken)* 2008, 291:114–121