Extensive Podocyte Loss Triggers a Rapid Parietal Epithelial Cell Response

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ABSTRACT
Damage to podocytes is a central pathomechanism of proteinuric kidney disease. However, it is not fully understood how podocyte injury evolves to progressive glomerulopathies such as FSGS or collapsing glomerulopathy. In particular, the role of parietal epithelial cells remains controversial. Here, we show that adriamycin induces DNA damage and podocyte lysis in mice without evidence of autophagy, endoplasmic reticulum stress, or necroptosis. After extensive podocyte loss, activated parietal cells mediated tuft re-epithelialization by two distinct mechanisms. In the majority of glomeruli, vacuolized parietal epithelial cells attached to denuded glomerular basement membrane and, occasionally, disengaged from the parietal basement membrane. Less frequently, parietal epithelial cells covered the denuded visceral basement membrane via formation of proliferative pseudocrescents. Notably, "visceralized" parietal epithelial cells did not express vascular endothelial growth factor but upregulated hypoxia-inducible factor 1 expression. The presence of visceralized parietal epithelial cells in sclerosing and collapsing lesions in a kidney biopsy from a patient with diabetes underscores the human relevance of our findings. In conclusion, repopulation of the glomerular tuft by parietal cells may represent a compensatory response to extensive podocyte loss. Our results suggest, however, that visceralized parietal epithelial cells cannot induce revascularization of the hyalinized tuft, resulting in hypoxic cell death and irreversible destruction of the glomerulus.


FSGS and collapsing glomerulopathy represent related but morphologically distinct patterns of progressive glomerular injury.1 They are generally classified as podocytopathies, but are also seen secondary to other glomerular diseases. Damage to podocytes, specialized terminally differentiated cells, which form an integral part of the kidney filter, is the morphologic hallmark of proteinuria.2 Several attempts were made to explain the evolution from podocyte injury to progressive glomerulopathies,3 including the causative involvement of parietal epithelial cells (PECs), although it is unclear whether a PEC response perpetuates or resolves glomerular injury.4 PECs may serve as a stem cell reservoir for podocytes under physiologic conditions,5 whereas inappropriate activation of PECs was proposed to injure the glomerular filter.6 Another hypothesis proposes a migratory and proliferative podocyte phenotype, which directly results in adhesions and extracapillary proliferations.7 Regardless of such conflicting concepts, the questions of when and how these processes contribute to progressive glomerular injury have not been sufficiently addressed.

Here, we used adriamycin (ADR) nephropathy as a mouse model of FSGS to examine the effects of podocyte injury/loss on PECs, to define the role of PECs in resulting glomerular lesions, and to determine whether PEC activation is detrimental or...
beneficial. We show that ADR induces both sclerosing and collapsing glomerular lesions in the same kidney, and we identify two distinct pathways of PEC activation after extensive podocyte loss.

RESULTS

The Severity of ADR-Induced Proteinuria and Mortality Is Dose Dependent

To examine dose dependency of ADR nephropathy, mice on a mixed Balb/c C57Bl6 background were intravenously injected with ADR at a low dose (11 μg/g body weight), intermediate dose (17 μg/g body weight), or high dose (22 μg/g body weight). Saline-injected mice served as controls. Albuminuria in saline-injected mice remained at baseline (17.9±2.6 μg/24 h) until 4 weeks after injection (19.9±8.0 μg/24 h). ADR caused dose-dependent albuminuria, with a peak at 2 weeks in mice injected with the low ADR dose (11 μg ADR–injected group) (8268±2824 μg/24 h) and a peak at 1 week in mice injected with the intermediate ADR dose (17 μg ADR–injected group) (13,632±2200 μg/24 h). Albuminuria in the 17 μg ADR–injected group dropped to 9294±3456 μg/24 h at 2 weeks (Figure 1A). The high ADR dose (22 μg) induced severe albuminuria (16,335±5838 μg/24 h) within 1 week. Of note, mice in the 11 μg ADR–injected group showed substantial recovery of proteinuria to low levels at 4 weeks (585±231 μg/24 h).

ADR exposure was associated with body weight loss, which became statistically significant in all ADR-injected groups as early as 2 weeks after injection (Figure 1B). The low ADR dose (11 μg) caused 25% lethality by 4 weeks, and >60% of mice in the 17-μg ADR group died within the first 2 weeks (Figure 1C). The high dose of ADR (22 μg) caused severe morbidity and lethality within the first week (Figure 1C). Due to the high mortality rate, severity of weight loss, and overall poor body condition, the surviving mice in the 22 μg ADR–injected and 17 μg ADR–injected groups were euthanized after 1 and 2 weeks, respectively.

ADR Causes Sclerotic and Collapsing Glomerular Lesions in the Same Kidney

Albuminuria results from glomerular filter damage; therefore, we investigated how histologic changes correlate with proteinuria and disease progression in ADR nephropathy. Whereas control kidneys showed no light microscopic changes at 4 weeks (Figure 2A), kidneys of mice in the 11 μg ADR–injected group showed focal loss of renal parenchyma with tubular atrophy, interstitial fibrosis, and scattered proteinaceous tubular casts (Figure 2B). Kidneys of animals in the 17 μg ADR–injected group showed markedly more tubular atrophy and proteinaceous casts at 2 weeks (Figure 2C). In contrast, kidneys of surviving mice in the 22 μg ADR–injected group showed acute tubular necrosis (ATN) at 1 week (Figure 2D).

Figure 1. ADR causes proteinuric kidney disease and mortality in mice. (A) Twenty-four-hour albuminuria at baseline (0) and after ADR injection (1, 2, 3, 4 weeks). Albuminuria in control mice remains at baseline. Mice treated with 11 μg ADR develop mild proteinuria within 1 week, which peaks at 2 weeks and subsequently recovers to low levels. Mice injected with 17 or 22 μg ADR both develop high albuminuria with a peak at 1 week. (B) Differences in total body weight from baseline to 1, 2, 3, and 4 weeks. Mice treated with 11 μg ADR show significant but mild weight loss compared with controls by week 2. Mice treated with 17 or 22 μg ADR exhibit a significant increase in weight loss compared with controls as early as 2 or 1 weeks, respectively. (C) Survival rates of control and ADR-injected mice. Results show that 100% of the control animals survive to 4 weeks, whereas ADR-treated animals show diminished survival rates. Survival appears to be inversely correlated with ADR dosage, with 11 μg ADR–injected mice showing the best and 22 μg ADR–injected mice showing the worst survival. *P<0.05; **P<0.01; ****P<0.0001. (Note: due to death or euthanasia of mice due to severe ADR toxicity, no data on albuminuria, body weight, or survival were obtained on 22 μg ADR–injected mice past 1 week and on 17 μg ADR–injected mice past 2 weeks.)
A detailed quantitative analysis of glomerular lesions showed the following. In control mice, 100% of glomeruli were preserved (Figure 3A, Supplemental Figure 1A). In the 11 mg ADR–injected group, 86% of glomeruli were preserved (Supplemental Figure 1B). Some glomeruli (4.2% ± 3.0%) showed striking PEC vacuolization (Figure 3B). Few chronic glomerular lesions were evident, including 1.8% ± 0.7% segmental glomerulosclerosis (SGS) and 7% ± 3.9% global glomerulosclerosis (GS) (Figures 3, C and D, respectively). A small fraction of glomeruli (1.0% ± 0.5%) revealed epithelial cell proliferation on the tuft as segmental pseudocrescent formation (PCF) (Figure 3, E and F). In kidneys of mice in the 17 μg ADR–injected group, only 36.7% ± 16.4% of glomeruli were preserved at 2 weeks (Supplemental Figure 1C), whereas most glomeruli showed substantial damage, including GS (32.8% ± 12.32%) and SGS (1.8% ± 0.9%). Compared with the 11 μg ADR–injected group, a markedly larger fraction of mice injected with 17 μg ADR demonstrated PEC vacuolization (22.7% ± 5.5%). Some glomeruli showed epithelial PCF (6% ± 2.7%), notably also in a higher percentage than in the 11 μg ADR–injected group. Although the tubulointerstitium in kidneys of mice injected with 22 μg ADR showed severe ATN, the majority of glomeruli were light-microscopically normal (92% ± 4.9%); only 3.5 ± 2.4% of glomeruli exhibited vacuolated PECs, and 2.5% ± 1.3% and 1.5% ± 1.0% were segmentally and globally sclerosed, respectively.

To explore the development of the glomerular lesions observed in the 17 μg ADR–injected mice observed at 2 weeks, we quantified early histologic changes at 1 week (Supplemental Figure 1E). At this time point, only few glomerular lesions were present: 90.7% ± 5.0% of glomeruli appeared normal by light microscopy, 2.4% ± 0.8% showed SGS, 1.6% ± 0.5% exhibited GS, and 5.3% ± 4.1% showed PEC vacuolization. In contrast, pseudocrescents were absent. Thus, high proteinuria at 1 week precedes the development of glomerular lesions.

ADR Induces DNA Damage in Podocytes, but Not Endoplasmic Reticulum Stress or Autophagy
ADR intercalates directly into DNA.9 To examine whether DNA damage contributes to ADR-induced podocyte injury, we analyzed the expression of histone H2AX (H2AX), a marker of double-stranded DNA breaks,10 by immunohistochemistry. Whereas glomeruli of control mice showed only minimal expression of H2AX, ADR-injected mice (17 and 22 mg) showed strong H2AX labeling at 1 week (Figure 4, A and B). The number of H2AX-positive cells markedly decreased at 2 weeks, reflecting loss of damaged podocytes or subsequent DNA repair.

To explore putative roles of endoplasmic reticulum (ER) stress and autophagy in the pathogenesis of ADR nephropathy, we studied the expression of the ER stress chaperone binding immunoglobulin protein (BiP) and the autophagosome marker light chain 3 (LC-3).11,12 The number of BiP-positive cells was not different between controls and ADR-injected mice at any time point analyzed (Figure 4C). Of note, whereas specific LC3-staining was present in distal tubular cells, glomeruli from control or ADR-injected mice were negative for LC3 (Figure 4D).

ADR Causes Podocyte Lysis
We next examined ADR-induced glomerular injury by transmission electron microscopy (TEM). Control kidneys showed healthy podocytes with normal foot processes (FPs), intact slit
glomerular tuft. (C) Glomeruli with sclerosed segments are a frequent mark large vacuoles in PECs, spanning from the parietal basement membrane to the 2 weeks. (B) Glomerulus with parietal epithelial cell vacuolization (PEC Vac). Asterisks injected (17 and 22 mice, and represent the majority of glomeruli in all ADR-injected animals at 1 week. In contrast, few normal glomeruli are observed in 17 µg ADR-injected animals at 2 weeks, whereas they are rare in other ADR-injected groups and absent in controls. (D) Glomeruli with GS are frequent in 17 µg ADR–injected animals at 2 weeks, whereas they are rare in other ADR-injected animals and are absent in controls. (E and F) Glomeruli with single-layered PCF (E) and multi-layered PCF (F). Epithelial cell proliferations covering a collapsed tuft, reminiscent of collapsing glomerulopathy, are encountered in 17 and 22 µg ADR–injected animals. The arrow points to an epithelial bridge (E), and the asterisk marks a multi-layered pseudocrescent (F). Scale bar, 20 µm.

Figure 3. The spectrum of glomerular lesions in ADR nephropathy. (A) Light-microscopically normal glomerulus. Exclusively normal glomeruli are found in control mice, and represent the majority of glomeruli in all ADR-injected animals at 1 week. In contrast, few normal glomeruli are observed in 17 µg ADR–injected animals at 2 weeks. (B) Glomerulus with parietal epithelial cell vacuolization (PEC Vac). Asterisks mark large vacuoles in PECs, spanning from the parietal basement membrane to the glomerular tuft. (C) Glomeruli with sclerosed segments are a frequent finding in ADR–injected animals and are absent in controls. (D) Glomeruli with GS are frequent in 17 µg ADR–injected animals at 2 weeks, whereas they are rare in other ADR-injected animals and are absent in controls. (E) Glomeruli with single-layered PCF (E) and multi-layered PCF (F). Epithelial cell proliferations covering a collapsed tuft, reminiscent of collapsing glomerulopathy, are encountered in 17 and 22 µg ADR–injected animals. The arrow points to an epithelial bridge (E), and the asterisk marks a multi-layered pseudocrescent (F). Scale bar, 20 µm.

diaphragms, regular glomerular basement membrane (GBM), and fenestrated endothelium. Podocytes showed normal nuclei, organelle composition, and cytoskeleton, and were devoid of vacuoles (Figure 5A). In contrast, podocytes of ADR-injected (17 and 22 µg) mice showed widespread FP effacement (FPE) after 1 week (Figure 5B). Effaced podocytes were firmly attached to the GBM, but often contained cytoplasmic vacuoles (Figure 5C). Several podocytes demonstrated beginning cell lysis with rupture of the plasma membrane and leakage of cytoplasmic content, or complete cell lysis (Figure 5D, left and right, respectively). In contrast, PECs were intact. We observed segmental podocyte dropout with denuded GBM at 2 weeks in the 17 µg ADR–injected group. In contrast, low-dose ADR (11 µg) induced only focal FPE, irregular GBM thickening, and segmental loss of endothelial fenestrations. Although podocyte FPE was a global response to ADR, only a subpopulation of podocytes underwent cell lysis. Podocyte lysis occurred in the absence of any PEC changes, suggesting that podocyte loss precedes a PEC response.

rijpi Deficiency Does Not Prevent Glomerular Disease or ATN in ADR Nephropathy

Cell lysis is a hallmark of necrotic cell death. We induced ADR nephropathy in rijpi-deficient (rijpi−/−) mice to examine whether ADR-induced podocyte lysis is mediated by necroptosis, a RIP3-dependent cell death mechanism (Supplemental Figure 2, A and B).13,14 Although rijpi deficiency improved survival, albuminuria and body weight were comparable to wild-type (WT) mice (Supplemental Figure 2, C–E). The distribution and severity of glomerular lesions were also similar to WT mice (Supplemental Figure 3A), indicating that RIP3-mediated necroptosis does not play a role in ADR glomerulopathy. rijpi−/− mice injected with a high ADR dose (22 µg) still developed ATN (Supplemental Figure 3B), suggesting that ATN resulting from ADR is also RIP3 independent. The observed difference in survival between rijpi−/− and WT mice is likely due to a RIP3-dependent effect of ADR in other organs.

PEC Vacuolization Promotes Repopulation of Denuded GBM

A large fraction of glomeruli (22.7% ± 2.5%) in the 17-µg ADR group showed extensive vacuoles in epithelial cells, which spanned Bowman's capsule and connected to the glomerular tuft. To confirm the parietal cell origin of vacuolated cells, we studied these glomeruli by TEM (Figure 6). Virtually all vacuolated parietal cells were located opposite of denuded GBM, and demonstrated an activated phenotype with large nucleoli and chromatin margination. Most of the cells bridged Bowman's space and attached to denuded GBM (Figure 6A). Occasionally, they were disconnected from the parietal basement membrane, leaving a gap in the PEC layer (Figure 6B). We also found hyalinized capillaries covered by cells with morphologic features of PECs (Figure 6C).

To confirm that these cells were activated “viscerIALIZED” PECs (vPECs), and not podocytes, we performed immunostaining for the podocyte marker synaptopodin (synpo) and the PEC marker paired box 2 (PAX2).15,16 In control mice, glomerular PAX2 staining was limited to PECs (Figure 7A), whereas synpo showed intense podocyte staining (Figure 7B, left). Double labeling revealed mutually exclusive synpo and PAX2 staining (Figure 7C, left). In kidneys of ADR-injected
mice, virtually all vacuolated cells were PAX2 positive (Figure 7, A and C, middle) but synpo negative (Figure 7, B and C, middle), thereby identifying them as PECs. In globally sclerosed glomeruli, the few residual PAX2-positive cells (Figure 7, A and C, right) residing within the sclerosed tuft were synpo negative (Figure 7, B and C, right), confirming that they were vPECs.

**PEC Proliferation Promotes Coverage of Denuded GBM in Collapsing Lesions**

To examine whether PECs can form pseudocrescents in collapsing lesions after podocyte loss, we analyzed pseudocrescent-containing glomeruli by TEM (Figure 8A). In collapsed glomeruli, PECs were not vacuolated. Instead they formed a single- or multi-layered bridge of proliferating epithelial cells that stretched onto the hyalinized tuft segment and covered collapsed capillaries (Figure 8A). Remnants of severely injured or lysed podocytes were still trapped between the GBM and vPECs. vPECs in the pseudocrescents were PAX2 positive (Figure 8B) and synpo negative (Figure 8C), confirming their PEC origin. vPECs expressed Ki-67 (Figure 8D), indicating cell cycle entry.17

**vPECs Express Hypoxia-Inducible Factor 1, but Not Vascular Endothelial Growth Factor, Making Them Inadequate Surrogates for Podocytes**

Finally, we started exploring why vPEC-containing glomeruli proceed to irreversible scarring. We hypothesized that, in contrast to podocytes, vPECs do not produce vascular endothelial growth factor (VEGF) required for the development and maintenance of glomerular capillaries.18 In keeping with this, normal glomeruli showed strong VEGF expression in podocytes (Figure 9A). In contrast, glomerular tufts with GS or PCF were largely devoid of VEGF staining (Figure 9, B and C, respectively). Conversely, vPECs in GS (Figure 9B) and PCF (Figure 9C) strongly expressed hypoxia-inducible factor 1 (HIF-1), a marker of hypoxia,19 which was not seen in healthy glomeruli (Figure 9A).

**Detection of vPECs in Human Glomerular Sclerosing and Collapsing Lesions**

To explore the relevance of these findings for human disease, we performed immunostaining for synpo and PAX2 in secondary SGS and collapsing glomerular lesions in a biopsy from a patient with diabetic nephropathy.

We found segmental loss of synpo in the area of sclerosis. In contrast, PAX2 staining was restricted to the sclerotic lesion, suggesting that vPECs replaced lost podocytes (Figure 10A). A collapsed glomerulus showed global reduction of synpo and presence of palisading synpo-negative, PAX2-positive vPECs populating the collapsed tuft (Figure 10B). In contrast, glomeruli with diabetic mesangial changes showed intact synpo was observed in distal tubules, glomeruli are negative for LC-3 in controls as well as in ADR-injected mice. n=50 glomeruli per animal. Scale bars, 20 μm.
staining and absence of PAX2-positive vPECs (Figure 10C). Moreover, similar to mice, both normal human glomeruli and segmentally sclerosed glomeruli were negative for LC-3 (Supplemental Figure 4) and showed no difference in tuft staining for BiP (Supplemental Figure 5).

**DISCUSSION**

This study shows that ADR causes dose-dependent proteinuria and mortality in mice. High doses of ADR are acutely toxic,
induce early, massive proteinuria due to podocyte lysis, and lead to both sclerotic and collapsing glomerular lesions within the same kidney in surviving mice. Acute podocyte injury and lysis in ADR nephropathy are not mediated by ER stress, autophagy, or necroptosis. Moreover, we found no evidence of autophagy in podocytes contrary to previous reports.20 After podocyte dropout, PECs are recruited to the denuded glomerular tuft in an attempt to replace lost podocytes and possibly reduce further protein loss.

Reactive oxygen species and the receptor for advanced glycation end products were implicated in podocyte injury in ADR nephropathy.21,22 Future studies will be required to test whether DNA damage occurs upstream, downstream, or in parallel with reactive oxygen species production. In addition, the glomerular endothelium is known to be an early target of ADR.23,24 Although this is an important finding, this study aims at specifically describing ADR-induced podocyte damage and ensuing progressive glomerular injury.

The relationships between sclerotic and collapsing patterns of glomerular injury and the contribution of PECs are controversial.1 Previous studies suggested that podocyte dedifferentiation and proliferation contribute to collapsing lesions, whereas inappropriate activation of PECs was implicated in the pathogenesis of FSGS.4,6,7 Most recently, tracer studies by Moeller et al emphasized the significance of PECs for glomerular pathobiology and identified PECs as a stem cell reservoir for physiologic podocyte turnover.4,5,25 These findings have sparked a new discussion on whether PEC activation under pathologic conditions is beneficial or detrimental. Our results now point to a targeted activation and recruitment of PECs to areas of denuded GBM, aimed at replacing podocytes, possibly in an attempt to reduce proteinuria and prevent further tuft injury.

Detailed morphologic studies by Kriz et al examined the response of podocytes to secondary processes such as hypertension, which lead to global, slowly progressive podocyte injury.3,26 In this “classic” model of FSGS, the resulting loss of single podocytes leads to an adaptive “pseudohypermorphic” response of the remaining podocytes. This initiates a vicious cycle of increased mechanical stress and single podocyte detachment, which leads to the focal attachment of PECs causing a focal adhesion and finally results in SGS.27 Although these changes can also be seen at later stages of ADR nephropathy, the predominant early manifestations described here follow another pathogenic and morphologic pattern. In contrast to single podocyte dropout in the classic model, ADR induces more widespread podocyte lysis after DNA damage. The rapid and extensive loss of podocytes in ADR nephropathy triggers a massive PEC response, leading to the recruitment of activated PECs to areas of denuded GBM. Of note, this PEC recruitment can occur via two distinct pathways, involving vacuolization leading to glomerulosclerosis, or PEC proliferation resulting in proliferative PCF (Figure 11).
Although both pathways are induced by extensive podocyte loss, the molecular cues serving as a stimulus for PEC activation remain to be established.

Another outcome of this study was the observation that vPECs in ADR nephropathy do not express VEGF but show high levels of HIF-1 expression, indicating hypoxia. As Eremina et al. elegantly showed, podocyte-specific expression of VEGF is required for the development and proper maintenance of glomerular capillaries. Whether the observed hypoxia is due to lack of VEGF expression and leads to a failure of these cells to induce glomerular re-vascularization remains to be addressed.

Our results identify DNA damage as the underlying cause of massive simultaneous podocyte injury. ER stress and autophagy are not involved in the pathogenesis of ADR nephropathy. Necroptosis, a pathway leading to programmed cell death, does not play a role in ADR-mediated toxic podocyte lysis. Moreover, in contrast to studies by Linkermann et al., who recently identified necroptosis as a mechanism of cell death in ischemic and cisplatin-induced renal injury, rip3−/− mice still show severe acute tubular injury after high-dose ADR injection, indicating that ADR-induced ATN is not caused by RIP3-mediated necroptosis. In addition, chronic tubular changes such as tubular atrophy and cystic dilation of tubules are not preventable by rip3 deficiency.

The reparative response after parenchymal loss, such as that seen in tubular atrophy, is usually driven by activation and proliferation of adjacent dormant fibroblasts. Due to its specialized structural and functional properties, a comparable cell reservoir is not available in the glomerulus. Our results raise the intriguing possibility that PECs may serve as “functional fibroblasts” to replace lost podocytes.

Generalized insults to all podocytes occur in human disease as well. Collapsing glomerulopathy is an idiopathic process or an acute complication of HIV, cytomegalovirus, or parvovirus infection, and is also seen in patients undergoing bisphosphonate treatment. Some variants of primary FSGS also show diffuse podocyte injury with global vacuolization of epithelial cells. In their primary form, both patterns of glomerular injury are characterized by massive proteinuria, but can also occur secondary to a variety of glomerular diseases with chronic podocyte damage and lower-grade proteinuria such as diabetic nephropathy. Our results in human biopsies suggest that the two pathways described here may be triggered by extensive podocyte injury in human disease and may contribute to the development of glomerular lesions.

In conclusion, our study unveils the activation and recruitment of PECs in response to podocyte loss from DNA damage.
damage in murine ADR nephropathy. We suggest that although this may form a temporary patch for the leaky filtration barrier, the failure of vPECs to express VEGF may ultimately contribute to hypoxic cell death and promote hyalinosis.

**CONCISE METHODS**

**Experimental Design**

The Massachusetts General Hospital Subcommittee on Animal Research approved the experimental design of this study in adherence to the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. C57/Bl6 mice are far less susceptible to ADR nephropathy than Balb/C mice. Therefore, male rip3+/- mice on a C57/Bl6 background (provided by Dr. M. Whalen, Massachusetts General Hospital, Boston, MA) were backcrossed for three generations to WT Balb/C female mice to generate offspring on a mixed C57/Bl6 Balb/C background, which was sufficient to reach susceptibility to ADR-mediated injury. F3 mice were mated to generate WT and rip3-/- littermates used for experiments at 6–8 weeks of age. Mice were genotyped for rip3 (5'-CGCTTTAGAAGCTTCAGGTTGAC-3', 5'-GCCTGCCCATCACGAAACTC-3', 5'-CCA-GAGGCCACTTTGTTAGCG-3'). Male and female mice were separated into the following experimental groups: WT (controls, n=5; 11 µg ADR, n=11; 17 µg ADR, n=16; 22 µg, n=9) and rip3-/- (controls, n=6; 11 µg ADR, n=6; 17 µg ADR, n=5; 22 µg ADR, n=10). A single dose of 11, 17, or 22 µg/g body weight of ADR (1 µg/µl; Sigma-Aldrich) in 0.9% saline, or saline only, was injected into the retrobulbar plexus of isoflurane-anesthetized mice. Mice were weighed, and 24-hour albuminuria was measured at baseline and at 1, 2, 3, and 4 weeks post-injection using metabolic cages. Of note, mice were fed a 5% sucrose drinking solution during the 24-hour analysis in the metabolic cages. Urine volumes were equal between groups. Ten microliters of urine was boiled in sample buffer and analyzed by SDS-PAGE and Coomassie blue staining. BSA (0.25, 0.5, 1.0, 2.5, and 5.0 µg) served as the standard. Signals were quantified using NIH ImageJ software. Resulting values of the 5 multiplication of the “size of the area” and the “mean gray value” of each albumin standard were used for construction of a standard curve and its associated mathematical function. Values were translated into albumin concentrations and extrapolated to 24-hour urine volume. Results were expressed as the mean ± SEM and assessed by Dunnett’s multiple comparisons test (ANOVA) using GraphPad Prism software. At the end of the experiments, the kidneys were fixed by retrograde vascular perfusion with 4% paraformaldehyde in PBS, removed, and immersed in the same fixative for a maximum of 2 days until further processing for histology, immunohistochemistry, or TEM, as previously described.

**Histology and Immunohistochemistry**

The Brigham and Women’s Hospital Institutional Review Board approved collection and immunostaining of human renal biopsies. Four-micrometer paraffin-processed, formalin-fixed kidney sections were stained with periodic acid–Schiff or, after antigen retrieval (pressure cooker or microwave in citrate buffer, pH 6), with antibodies against H2AX (rabbit pAb, 1:200; Abcam), synaptopodin (mouse mAb, 1:10), PAX2 (rabbit pAb, 1:75; Invitrogen), BiP
(rabbit pAb, 1:500; Cell Signaling Technology), LC-3 (rabbit pAb, 1:100; Abcam), VEGF (goat pAb, 1:200; R&D Systems), or HIF-1α (rabbit pAb, 1:100; Cayman Chemical), followed by horseradish peroxidase–conjugated secondary antibodies (Dako), and counterstained with hematoxylin. One-micrometer sections of resin-embedded tissue were stained with toluidine blue. Images were taken with an Olympus BX53 microscope with DP72 camera and processed using Adobe PhotoShop software.

Electron Microscopy

Ultrathin 80-nm sections of resin-embedded kidney tissue were mounted on copper grids, treated with uranyl acetate and lead citrate, and examined by a pathologist (A.W.) in a blinded fashion using a JEOL 1010 transmission electron microscope.

Histologic Analyses of Mouse Kidneys

Histology of five animals from each group was assessed. Periodic acid–Schiff, toluidine blue–stained sections and electron micrographs were analyzed in a blinded fashion by A.W. or S.H. and classified using standard criteria. For objective quantification of positively stained tuft cells, a custom application for ImageJ software was used. Briefly, positive cells were counted with an ImageJ macro (color threshold: red, 1–243; green, 119–148; blue, 129–185) that provided differentiation between positive and negative cells. Particles with an area >200 pixels² were selected. Localization of positive cells to glomeruli and final counting was done in an overlay picture (Supplemental Material). Results are expressed as the mean±SEM and assessed by Dunnett’s multiple comparisons test (ANOVA) using GraphPad Prism software.

RT-PCR of Renal RIP3 Expression

RT-PCR on whole-kidney mRNA was performed according to standard protocol using primers specific for RIP3 (FP-5′-ACCTGAAGGCTTCTAAAGCG-3′, RP-5′-GCTTTGCTAACAACCTCAGC-3′). RT-PCR for glyceraldehyde 3-phosphate dehydrogenase was performed as a control and showed equal loading.

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Figure 10. Renal biopsy from a patient with diabetic nephropathy (DN), and superimposed segmental sclerosis or glomerular collapse in the same sample, stained with periodic acid–Schiff, and for synpo and PAX2. (A) Glomerulus with mesangial expansion and SGS. The sclerosed segment (asterisk) shows loss of synpo staining and PAX2-positive, synpo-negative vPECs lining the tuft (arrows). (B) Glomerulus with a globally collapsed tuft. The collapsed tuft is repopulated by synpo-negative, PAX2-positive vPECs (arrows). (C) Glomerulus with mesangial expansion only. Note the intact podocyte staining for synaptopodin and absence of PAX2-positive vPECs on the tuft. Scale bar, 50 μm.
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DISCLOSURES
None.

REFERENCES


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Supplemental Figure 1
Quantification of histologic glomerular lesions by light microscopy.
(A) All glomeruli in control animals are light microscopically normal.
(B) In mice treated with 11 µg ADR at 4 weeks, the majority of glomeruli are light microscopically normal, while few show PEC vacuolization (PECVac), segmental glomerulosclerosis (SGS), global sclerosis (GS) and isolated glomeruli bear pseudocrescent formation (PCF).
(C) Mice injected with 17 µg ADR at 2 weeks show a higher incidence of glomerular changes. While only a third of glomeruli are normal, many show PEC vacuolization and global sclerosis, while a smaller fraction exhibit segmental sclerosis. Of note, a higher number of glomeruli demonstrate pseudocrescent formation.
(D) Glomeruli of mice treated with 22 µg ADR at 1 week demonstrate very few glomerular lesions, with the majority of glomeruli being light microscopically normal.
(E) In contrast to the 2 week time point, mice injected with 17 µg ADR at 1 week show a distribution of glomerular changes similar to that seen in 22 µg ADR-injected animals, with very few light microscopic glomerular lesions.
(n = 100 glomeruli/animal)
Supplemental Figure 2
rip3 deficiency does not protect against ADR-mediated proteinuria.
(A) Representative gel electrophoresis of a genotyping PCR to determine RIP3 deficiency. As expected, a single band at 320 bp confirms wild type genotype, a single band at 500 bp confirms homozygosity for rip3 deficiency, and a double band indicates heterozygosity.
(B) Representative RT-PCR for RIP3 in whole kidney cDNA. rip3 mRNA is lost in the kidney of rip3-/- mice, as indicated by absence of the specific band at 300 bp.
(C) 24h albuminuria in rip3-/- mice at baseline (0) and at 1, 2, 3 and 4 weeks after ADR injection. Albuminuria in control animals remains at baseline. Mice injected with 11 µg ADR develop mild proteinuria by 1 week, which decreases to low levels by 3 weeks. Mice injected with 17 µg ADR show high albuminuria with a peak at 1 week (**p ≤ 0.01), which was diminished at 2 weeks (**p≤ 0.05). The results are comparable to wild type mice.
(D) Differences in total body weight in rip3-/- mice from baseline to 1, 2, 3 and 4 weeks after ADR injection. Mice injected with 11 µg ADR show only mildly increased weight loss compared to controls as early as 2 weeks after injection (**p ≤ 0.01). Mice treated with 17 or 22 µg ADR show significantly increased weight loss compared to controls as early as 2 or 1 weeks, respectively (**p ≤ 0.01, ****p ≤ 0.0001, respectively).
(E) Survival rates of saline- and ADR-injected rip3-/- mice. 100% of the controls and the 11 µg and 17 µg ADR-injected mice survived to 4 weeks, while 22 µg ADR treated animals showed diminished survival comparable to that of wild type mice.
Note: No data on albuminuria, body weight or survival were obtained on 22 µg ADR-treated animals past 1 week after injection, and on 17 µg ADR-treated animals past 2 weeks after injection.
Supplemental Figure 3
rip3-deficiency does not prevent histologic changes in the kidney.
(A) rip3-/- mouse kidney 4 weeks after injection of 11 µg ADR. The renal cortex shows mild chronic changes with proteinaceous casts and focal atrophy of the renal parenchyma. Only isolated glomerular changes are present. Morphologic changes are comparable to wild type mice. The lower panel includes a representative glomerulus with global sclerosis (GS).
(D) rip3-/- mouse kidney 1 week after injection of 22 µg ADR. The renal cortex exhibits only isolated glomerular lesions; the majority of tubules demonstrate severe acute tubular injury, characterized by low epithelium and epithelial necrosis, similar to wild type mice.
Scale bars = 100 µm
Supplemental Figure 4
LC3 is not expressed in normal human glomeruli or glomeruli with segmental sclerosis.

(A) Normal human glomeruli show no glomerular staining for LC3, while specific staining is seen in a vesicular pattern in distal tubules only, consistent with autophagosomes.

(B) Renal biopsy from a patient with FSGS (asterisk). A segmentally sclerosed glomerulus shows no specific staining for LC3.

(C) Tubulointerstitium from the renal biopsy shown in (B). Specific staining for LC3 is observed as vesicular staining pattern in distal tubules only, consistent with autophagosomes.

(D) Human brain stained for LC3, serving as positive control. Specific staining is seen in neurons.

Scale bar = 50µm
Supplemental Figure 5
BiP expression in normal and segmentally sclerosed human glomeruli is similar.

(A) BiP staining in a normal glomerulus. There are scattered BiP positive cells present in the tuft and along the PBM.

(B) BiP staining in a glomerulus with FSGS (asterisk) and tuft adhesion to the PBM (double asterisk). Scattered glomerular cells are positive for BiP, similar to the normal glomerulus shown in (A). The staining intensity in PECs varies greatly between glomeruli in both normal renal tissue and FSGS.

Scale bar = 50µm
Supplement to Methods Section:

Programming commands for ImageJ Macro
//Define Folders and get File names
dir=getDirectory("Chose Directory");
list=getFileList(dir);
countDir=dir+" Counted";
File.makeDirectory(countDir);

//Loop
for(j=0; j<list.length; j++) {
    open(dir+list[j]);
    fullName=getTitle();

    // Color Thresholder 1.46f
    // Autogenerated macro, single images only!
    min=newArray(3);
    max=newArray(3);
    filter=newArray(3);
    a=getTitle();
call("ij.plugin.frame.ColorThresholder.RGBtoLab");
run("RGB Stack");
run("Convert Stack to Images");
selectWindow("Red");
rename("0");
selectWindow("Green");
rename("1");
selectWindow("Blue");
rename("2");
min[0]=2;
max[0]=243;
filter[0]="pass";
min[1]=119;
max[1]=148;
filter[1]="pass";
min[2]=129;
max[2]=185;
filter[2]="pass";
for (i=0;i<3;i++){
    selectWindow(""+i);
    setThreshold(min[i], max[i]);
    run("Convert to Mask");
    if (filter[i]=="stop") run("Invert");
}
imageCalculator("AND create", "0","1");
imageCalculator("AND create", "Result of 0","2");
for (i=0;i<3;i++){
    selectWindow(""+i);
    close();
}
selectWindow("Result of 0");
close();
selectWindow("Result of Result of 0");
rename(a);
run("Analyze Particles...", "size=100-2000 circularity=0.0-1.00 show=Outlines clear include summarize");

drawingName=getTitle();
run("8-bit");
saveAs("TIFF", countDir+"/"+drawingName);
drawingName=getTitle();
selectWindow(fullName);
run("Close");
open(dir+fullName);
run("8-bit");
saveAs("TIFF", countDir+"/"+fullName+" bw");
fullName=getTitle();
selectWindow(fullName);
selectWindow(drawingName);
run("Images to Stack", "name=Stack title=[]");
run("Channels Tool... ");
run("Make Composite", "display=Composite");
saveAs("JPEG", countDir+"/"+fullName+" Merged");
run("Close All");

//COMBINED Picture

open(dir+list[j]);
orgName=getTitle();
open(countDir+"/"+fullName+" Merged.jpg");
newName=getTitle();
run("Combine...", "stack1=orgName stack2=newName");
saveAs("JPEG", countDir+"/"+fullName+" Combined");
run("Close All");
}