Renal Primary Cilia Lengthen after Acute Tubular Necrosis

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ABSTRACT
Renal primary cilia are sensory antennas required for the maintenance of normal epithelial differentiation and proliferation in the kidney, but they also have a potential role in epithelial differentiation during renal injury and repair. In mice, tubular damage causes an increase in the length of renal cilia, which may modify their sensory sensitivity during repair. Here, we investigated whether the alteration of renal cilium length during renal injury is clinically relevant. Using biopsies of human renal transplants that suffered acute tubular necrosis during transplantation, we compared the length of renal primary cilia with renal function. Serial biopsies showed that acute tubular necrosis resulted in more than a doubling of cilium length throughout the nephron and collecting duct approximately 1 wk after injury. Allografts displayed a trend toward normalization of cilium length in later biopsies, and this correlated with functional recovery. A mouse model of renal ischemia-reperfusion confirmed the increase and subsequent regression of cilium length during renal repair, displaying complete normalization of cilium length within 6 wk of injury. These findings demonstrate that the length of renal cilia is a clinically relevant indicator of renal injury and repair.


The renal primary cilium is a sensory organelle that extends into the lumen of the renal tubule and collecting duct. The ability of this organelle to sense fluid flow and initiate calcium-based signaling is important for the maintenance of normal epithelial phenotype in the renal tubule and duct. Wnt signaling and signal transducer and activator of transcription 6 activity may also influence epithelial phenotype and are subject to flow-based regulation in the kidney. The importance of the renal cilium is highlighted by the fact that defects of this organelle lead to cystic kidney disease caused largely by epithelial dedifferentiation and overproliferation (reviewed in references 5 and 6).

Although the role of renal cilium defects in the pathogenesis of cystic kidney disease has been explored in detail, potential roles for this organelle in the injured kidney have received less attention. Similarities in the pathways driving cellular dedifferentiation and proliferation in polycystic kidney disease (PKD) and after epithelial injury have raised questions about the importance of primary cilia during renal repair. We previously demonstrated that mouse models of renal injury exhibit dramatic increases in renal cilium length that are likely to modify cilium-mediated signaling in the epithelial layer during renal repair.

Acute tubular necrosis (ATN) is a common complication of serious illness, occurring most of...
ten in the setting of hospital admission. Resulting largely from a defined ischemia-reperfusion injury (IRI), treatment is limited to replacing renal function with dialysis in severe cases while waiting for intrinsic renal repair to take place. Little is known of the mechanisms leading to repair that may be important for the development of new therapies. In the study presented here, we sought to delineate the role of the renal primary cilium in human ATN by studying renal biopsies from the transplanted human kidney. Delayed graft function due to ATN from an IRI occurs in up to 25% of primary grafts (ANZDATA 200711). The series of biopsy samples that are taken to monitor for rejection in delayed graft function provide a unique opportunity to follow renal cilia during repair in ATN. The distribution and dimensions of renal cilia were assessed in a series of biopsy samples from human renal allografts with histologically proven ATN and an absence of rejection. Renal cilium length was correlated with the clinical parameters of urinary volume and serum creatinine. The dynamics of changes in renal cilium length during recovery from injury, as observed in human biopsy samples, were further explored in a mouse model of renal ischemia-reperfusion.

RESULTS

ATN Results in a Dramatic Increase in Renal Primary Cilium Length

As shown in representative images of cilia from allograft A (Figure 1, A through G), renal cilia stained intensely with anti-acetylated α-tubulin and were detected throughout the aquaporin-1-positive proximal tubule and the aquaporin-1-negative distal tubule and collecting duct of the kidney. The onset of ATN was associated with a noticeable increase in cilium length (Figure 1, B, C, E, and G) compared with cilium length at the time of transplantation (Figure 1, A, D, and F). Quantification of renal cilium length confirmed a dramatic increase in average cilium length with the onset of ATN 5 to 7 d after transplantation (Figure 2). Allografts often displayed a trend toward the normalization of cilium length as repair proceeded. Grafts A, B, C, E, and F showed a reduction in cilium length relative to peak in the last biopsy. This reduction of cilium length occurs throughout the nephron and collecting duct (Figure 2E), but is only significant in the proximal tubule of series B and C (Figure 2, B and C) and distal tubule-collecting duct in series F (Figure 2F). Series D does not show any reduction in cilium length in the last biopsy (Figure 2D). Decreasing serum creatinine and increasing urine output (Figure 2) traced the progress of functional recovery in allografts over time.

Renal Cilia in the Mouse Renal Ischemia-Reperfusion Model

Because the human transplant biopsy series investigated do not offer the opportunity to document renal cilium length through to the completion of repair, a mouse renal IRI model of ATN was used to verify the trend toward the normalization of cilium length observed in human allografts. The histopathology of renal IRI in the mouse was used to assess the correlation between epithelial injury and repair and changes in cilium length (Figure 3). The process of renal repair after IRI has been described in detail elsewhere. Briefly, 1 wk after 45 min of ischemia there was extensive damage to the proximal tubule epithelium, resulting in protein cast formation, epithelial cell

Figure 1. Primary cilia in biopsies from a renal allograft with transplantation-induced ATN. Micrographs shown are from allograft A on the day of transplantation (low power: A; high power: D and F) and 6 d after transplantation (low power: B and C; high power: E and G). Renal cilia (arrows) were stained with anti-acetylated α-tubulin (green) and are shown in the aquaporin-1-positive (red) proximal tubule (A, B, D, and E) and the aquaporin-1-negative distal tubule and collecting duct (A, C, F, and G). Nuclei are counterstained with 4',6-diamidino-2-phenylindole (blue). Compared with the day of transplantation, cilia were longer throughout the proximal tubule and distal tubule/collection duct after ATN. Scale bar in C = 50 μm; A and B are the same magnification as C. Scale bar in G = 10 μm; D, E, and F are the same magnification as G.
death, tubular and collecting duct dilation, and an interstitial inflammatory cell infiltrate (Figure 3B). Two weeks after IRI, there was a reduction in the number and size of protein casts and reduced tubular dilation, indicative of resolving tissue injury compared with the 1-wk time point. There was evidence of re-epithelialization, although this process was often partially complete and many tubules still displayed a disorganized structure (Figure 3C). Six weeks after IRI, there was widespread recellularization of the tubular epithelium and a return of normal architecture (Figure 3D).

Renal cilia in mice subjected to renal ischemia-reperfusion were visualized by immunostaining with anti-acetylated α-tubulin in conjunction with markers for the proximal tubule, the distal tubule/collecting duct (Figure 4). Compared with uninjured control (Figure 4, A, E, H, and K), cilia were noticeably longer (Figure 4, B, C, F, I, and L) and 2 wk (not shown) after injury and appeared normal after 6 wk of recovery (Figure 4, D, G, J, and M). Quantification of cilium length revealed a statistically significant increase in average cilium length 1 wk after injury in the proximal tubule (Figure 5A), the distal tubule (Figure 5B), and the collecting duct (Figure 5C). Average cilium length subsequently regressed throughout the kidney at the 2-wk time point and was not statistically different from control 6 wk after ischemic renal injury (Figure 5).

**DISCUSSION**

Data from human renal transplant biopsies presented here indicate that approximately 1 wk after ATN there was more than a doubling of cilium length throughout the nephron and collecting duct. The lengthening of renal cilia in allograft samples is indiscriminate and of a similar magnitude throughout the entire nephron and collecting duct. Comparable widespread increases in renal cilium length have previously been described in mouse models of injury. These results highlight the fact that cilium lengthening is not restricted to the localized region of cellular injury. Biopsy series also suggest a link between repair and renal cilium length, because grafts generally demonstrate evidence of functional recovery combined with a trend toward the normalization of cilium length in the last biopsy.

Because the transplant biopsy series investigated do not continue through to complete recovery, the increase and subsequent regression of cilium length observed in biopsies was verified in the mouse renal ischemia-reperfusion model of ATN. This mouse study demonstrates that the proximal tubule, the distal tubule, and the collecting duct all undergo increases in cilium length after renal injury. The complete normalization of renal cilium length occurs within 6 wk and coincides with widespread re-establishment of a morphologically normal epithelial layer in the renal tubule. A comparable
normalization of renal cilium length has been reported after the reversal of ureteral obstruction in the mouse.\(^9\) Decreases in cilium length observed during renal repair in mouse models are much more dramatic than those we have observed in human renal allografts. This difference may relate to the more complex and ongoing nature of ATN in human renal allografts. This difference may relate to the more complex and ongoing nature of ATN in human renal allografts in comparison to mouse models\(^{13}\) and/or the nephrotoxicity of immunosuppressive drugs.\(^{14}\)

The exact factors regulating renal cilium length during renal injury and repair have not yet to be determined. The presence of longer cilia throughout the nephron and collecting duct points to factors with the ability to exert an influence on epithelial cells throughout the kidney. There is an established link between ciliogenesis and the cell cycle that is best demonstrated by the in vitro use of serum starvation to induce cell cycle arrest and cilium formation.\(^{15,16}\) However, it is unlikely that a similar process accounts for our in vivo observations of increased cilium length in the suboptimal renal environment induced by injury. Epithelial proliferation in the uninjured kidney is very low,\(^{8,17}\) meaning that cell-cycle-related inhibition of ciliogenesis probably does not limit cilium length under resting conditions. Given that in vitro studies have shown that primary cilium assembly may be inhibited by flow,\(^{18,19}\) the possibility that cilium length increases result from an injury-induced reduction in urine flow must also be considered. However, allograft biopsy data demonstrate that the resumption of urine production after ATN does not necessarily result in a reduction in the length of renal cilia. The distal tubule/collecting duct of series B and C, the proximal tubule of series F, and all segments of series D fail to display a reduction of cilium length with the increase of urine output. These results suggest roles for other injury and repair-related factors in influencing cilium length. Candidates include injury-induced changes in the composition of urine,\(^{10}\) cytokines produced during inflammation,\(^{20}\) and dilution. All are common features of renal injury, including ATN, and have the potential to influence epithelial cells throughout the tubule and collecting duct. In an interesting parallel, we note that there are genetic models of cystic kidney disease that exhibit a long cilium phenotype that appears to be a secondary consequence of cystogenesis rather than a direct effect on cilium assembly.\(^{21,22}\) Thus we suggest that a shared pathogenic feature may be responsible for cilium lengthening in both acute tubular injury and some forms of cystic kidney disease.

Increases in renal cilium length appear to be a common feature of epithelial injury in mouse models and human ATN. This finding has important implications for the modulation of cilium-based signaling during epithelial repair. Cilium-based calcium signaling is dependent on the deflection of the renal primary cilium by flow,\(^1\) and studies have demonstrated a positive correlation between the sensitivity of the cilium to deflection and its length.\(^{19,23}\) Importantly, relatively small increases in cilium length, in terms of what we have observed after injury, are predicted to greatly increase the sensitivity of this organelle to deflection.\(^{24}\) This suggests a transient increase in the sensitivity of flow-dependent signaling after renal injury that will promote a differentiated epithelial phenotype in the repairing kidney. The current consensus is that epithelial repair in the kidney is predominantly facilitated by the controlled dedifferentiation, proliferation, and redifferentiation of intrinsic epithelial cells.\(^{25,26}\) We suggest that increases in renal cilium length and cilium-based signaling act to balance the propensity for epithelial dedifferentiation that exists in the injured kidney and mediate the transition back to a fully differentiated phenotype that is required to re-establish a functional epithelial layer. In keeping with this scheme, our previous study of renal IRI in the mouse demonstrated that cilium lengthening in the proximal tubule follows a burst of repair-related epithelial proliferation.\(^8\) Because flow-mediated signaling appears to be essential for the maintenance of epithelial differentiation, increasing cilium sensitivity may also be particularly important in the reduced-flow environment that often results from kidney injury. A recent study in which renal cilia were conditionally ablated in the kidneys of adult mice supports a role for renal cilia in epithelial repair.\(^{27}\) These mice did not immediately develop a polycystic kidney phenotype unless subjected to renal IRI, pointing to critical defects of epithelial repair in the absence of cilia.

Given accumulating evidence that the renal primary cilium is important during repair, understanding the interplay be-
tween the changes in cilium length and sensitivity in the altered environment of the injured kidney is likely to provide new insights into renal repair. A better understanding of the role of cilia during renal repair is also likely to provide insights into the progression of PKD. The cilium-related defects that cause PKD appear to result in the inappropriate activation/persistence of epithelial repair processes in a manner that leads to cytogenesis.7 PKD in some respects appears to be perceived by the kidney as an injury,28,29 which may in turn cause an escalation of aberrant repair responses that compounds cystogenesis.

**CONCISE METHODS**

**Renal Graft Biopsy Samples**

De-identified renal biopsies from the cortex of transplanted kidneys were obtained from St Vincent’s Hospital, Melbourne, Australia. Only biopsy series that did not show any significant pathology except for ATN were chosen. In particular, samples with acute rejection as assessed by histology and C4d immunostaining were excluded. A total of 21 biopsies from 6 patients (5 men and 1 woman, ages from 52 to 67 yr) diagnosed with ATN after cadaveric renal transplantation (patients A through F) were investigated in the study. Patients were on standard triple immunosuppression therapy of cyclosporin, myco-phenolate mofetil, and prednisolone. Biopsies were obtained 0 to 84 d posttransplantation as the medical need arose, immersion-fixed in 10% neutral-buff ered formalin, and embedded in paraffin. Graft function data including serum creatinine, urine output (to a maximum of 2l), and detailed pathology reports were obtained for each graft biopsy series.

**Induction of IRI in Mice**

All animal experiments were approved in advance by a Monash University Animal Ethics Committee and adhered to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. IRI and kidney collection was as described previously,8 with the exception that the left renal pedicle was clamped for 45 min. Kidneys were collected 1, 2, and 6 wk after the induction of injury, and kidneys from mice that did not undergo surgery were used as controls. Five mice were used per time point. Perfusion-fixed kidneys (4% paraformaldehyde in PBS) were embedded in paraffin and sectioned and for hematoxylin and eosin or immunofluorescence staining.

**Visualizing and Measuring Renal Cilia**

Human biopsy and mouse tissue sections were boiled in citrate buffer for antigen retrieval. Cilia were stained with an antibody against repair by augmenting cilium-based signaling during critical phases of renal repair. A better understanding of the role of cilia during renal repair is also likely to provide insights into the progression of PKD. The cilium-related defects that cause PKD appear to result in the inappropriate activation/persistence of epithelial repair processes in a manner that leads to cytogenesis.7 PKD in some respects appears to be perceived by the kidney as an injury,28,29 which may in turn cause an escalation of aberrant repair responses that compounds cystogenesis.
acetylated α-tubulin (Sigma, St Louis, MO) in conjunction with anti-mouse Alexa Fluor 488. For human biopsy samples, an antibody against aquaporin-1 (Chemicon, Temuluca, CA) in conjunction with a secondary anti-rabbit Alexa Fluor 647 antibody was used to detect the proximal tubule brush border. For mouse samples, the M.O.M immunodetection kit (Vector Laboratories, Burlingame, CA) was used. *Lotus tetragonolobus* agglutinin and *Dolichos biflorus* agglutinin (both from Vector Laboratories) were used as markers for the proximal tubule and collecting duct, respectively, and anti-thiazide-sensitive sodium-chloride cotransporter (Chemicon) with anti-rabbit Alexa Fluor 647 was used to detect the distal convoluted tubule. In human biopsy and mouse tissue samples, 4′,6-diamidino-2-phenylindole staining of DNA was used to visualize nuclei, and sections were mounted in Prolong Gold antifade medium (Molecular Probes, Eugene, OR). Images of cilia completely contained in a single plane of focus were captured from randomly chosen high-power fields (100×, 92 × 62 μm) on a Provis fluorescence microscope (Olympus, Tokyo, Japan) and measured using AnalySIS version 5.0 software (Olympus). For each patient biopsy sample, 50 proximal tubule and 50 distal tubule/collecting duct cilia were measured. For each mouse, 20 cilia were measured per segment.

**Statistics**

Cilium length data were analyzed using a one-way ANOVA with an accompanying Tukey’s post hoc test performing intergroup comparisons. Statistically significant differences within segments examined were defined as P < 0.05 and indicated by the use of different letters. When two letters are assigned to a value, it is not significantly different from values bearing either letter. Values are expressed as mean ± SEM.

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**DISCLOSURES**

None.

**REFERENCES**