Acquired resistance to rechallenge injury after acute kidney injury in rats is associated with cell cycle arrest in proximal tubule cells

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Am J Physiol Renal Physiol 310: F872–F884, 2016. First published January 28, 2016; doi:10.1152/ajprenal.00380.2015.—Rats that have recovered from severe proximal tubule (PT) injury induced by uranyl acetate (UA) or cisplatin (11, 12, 22, 23, 34, 36, 37) and found that heat shock protein (12, 23), growth/proliferative factor (11, 37), cell cycle-mediated factors (22, 34), and factors related to DNA repair (22, 23, 34) may play roles in the acquired resistance. Mitigation of AKI in animal models by induction of heme oxygenase-1 (25) or hypoxia-inducible factor (HIF)-1α (20) has also been reported. However, the exact mechanisms of acquired resistance remain unknown.

Proximal tubule (PT) cells are vulnerable to ischemic and nephrotoxic injury. Recently, it has been reported that the activation of p21, a cyclin-dependent kinase inhibitor (CDKI), is associated with resistance to PT injury (26, 32, 41). Upregulation of p21 in PT cells may induce G1 arrest during the development of ischemic or nephrotoxic AKI (21, 38). Furthermore, DNA damage is repaired during this period, allowing the cell to evade apoptotic cell death (31). Amelioration of ischemia-reperfusion AKI or cisplatin-induced AKI by small-molecule cell cycle inhibitors was recently reported (6, 30).

We recently demonstrated that PT cells have a considerable ability to proliferate in response to toxic stimuli in terms of the cell cycle (15). The findings suggested that a high ratio of G1 to G0 phase cells among normal PT cells in rats, a rapid accumulation of G1 phase cells, and G1 arrest before S phase progression in response to toxic stimuli are biological strategies for safe, timely, and explosive cell proliferation. We also found that a CDKI, p27, was associated with G1 arrest. Normal PT cells seemed to induce cell cycle arrest to cope with toxic stimuli; however, this is not enough to protect PT cells against severe injury.

Prevention of acute kidney injury (AKI) is an urgent issue since patients who have experienced AKI have higher short- and long-term mortality rates than those who have not. However, there are currently no specific treatments for the prevention of AKI.

Although it is not universal (42), animals that have recovered from AKI are resistant to subsequent treatments with nephrotoxins and this phenomenon is termed acquired resistance to rechallenge injury (13). This phenomenon of resistance to injury after prior exposure and subsequent recovery has been shown by many investigators in diverse forms of injury, including ischemia-reperfusion (16, 28), oxidants (29), and alkylating agents (17, 18). We have examined the mechanisms of the acquired resistance in rats that have recovered from AKI induced by uranyl acetate (UA) or cisplatin (11, 12, 22, 23, 34, 36, 37) and that found heat shock protein (12, 23), growth/proliferative factor (11, 37), cell cycle-mediated factors (22, 34), and factors related to DNA repair (22, 23, 34) may play roles in the acquired resistance.

Mitigation of AKI in animal models by induction of heme oxygenase-1 (25) or hypoxia-inducible factor (HIF)-1α (20) has also been reported. However, the exact mechanisms of acquired resistance remain unknown.

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Repair of PT injury is now believed to be brought about by proliferation or regeneration of intrinsically surviving PT cells that dedifferentiate in response to ischemic or nephrotoxic injury (3, 9, 39). In rats with UA-induced AKI, we found that the number of PT cells progressively decreased by day 5, then increased along with PT cell proliferation, and reached a peak level at day 7, which exceeded the baseline level. Subsequently, the number of excess hyperplastic PT cells gradually regressed by cell desquamation and apoptosis to adjust the number of PT cells (34). Thus even phenotypically differentiated PT cells 14 days after recovering from AKI may be in a state of cell cycle inhibition.

We hypothesized that regenerated PT cells that have recovered from AKI can markedly stimulate G1 arrest in response to subsequent injury compared with normal PT cells and that the induced G1 arrest is associated with acquired resistance to rechallenge injury. This is an extension of our previous study (15) and aimed to demonstrate the hypothesis that enhanced G1

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arrest in PT cells, which recovered from UA-induced AKI, is crucial for cytoprotection and acquired resistance to rechallenge injury in rats.

Moreover, we analyzed cell cycle status and cell cycle progression in isolated PT cells similarly to our previous study (15). In addition to p21, we also examined the factors related to G1 arrest, the activation of p27, and cyclin D1. We found that PT cells that have recovered from AKI acquired the ability to inhibit cell cycle progression with enhanced G1 arrest, in association with sustained p21 and increased p27 expression, in response to UA or proliferative stimuli.

MATERIALS AND METHODS

Rats

Male Sprague-Dawley rats weighing, 230–300 g (SLC, Shizuoka, Japan), were provided with standard rat chow and drinking water ad libitum. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of the Hamamatsu University School of Medicine.

Reagents

UA dihydrate (purity >98.0%) was purchased from Fluka (Buchs, Switzerland). Lead acetate was purchased from Wako Pure Chemical Industries (Osaka, Japan). Collagenase type II was from Worthington Biochemical (Lakewood, NJ). Percoll was purchased from GE Healthcare UK (Little Chalfont, Buckinghamshire, UK). Trypan blue solution, propidium iodide, Hoechst 33342, and pyronin Y were purchased from Sigma-Aldrich (St. Louis, MO). Hank’s balanced salt solution (HBSS) was from Invitrogen (Carlsbad, CA). Can Get Signal was from Chemicon-Millipore (Temecula, CA). The antibodies listed in Table 1 were used as primary antibodies. Alexa Fluor 633-conjugated donkey anti-goat IgG (Invitrogen) and Alexa Fluor 546-conjugated goat anti-rabbit IgG (Invitrogen) were used as secondary antibodies. Histofine Simple Stain Max PO Kit was from Nichirei Bioscience (Tokyo, Japan). An ApopTag Plus In Situ Apoptosis Detection Kit was from Chemicon-Millipore (Temecula, CA). The antibodies listed in Table 1 were used as primary antibodies. Alexa Fluor 633-conjugated donkey anti-goat IgG (Invitrogen) and Alexa Fluor 546-conjugated goat anti-rabbit IgG (Invitrogen) were used as secondary antibodies. Histofine Simple Stain Max PO Kit was from Nichirei Bioscience. Protease Inhibitor Complete Mini and the phosphatase inhibitor PhosSTOP were from Roche (Mannheim, Germany). ImmunoPure Lane Marker Reducing Sample Buffer was from Thermo Fisher Scientific (Waltham, MA).

Experimental Protocol

In total, 152 rats were used in the present study. All experimental protocols performed are depicted in Fig. 1. We used a reversible AKI model in rats injected with 1 mg/kg of UA dissolved in saline (1 ml/kg) intravenously. In this AKI model, renal function was judged according to serum creatinine levels, which significantly declined and was lowest at days 5–7 and recovered to normal levels by day 14, while PT injury with apoptotic and necrotic PT cells peaked at day 5 and was recovered by regenerated and redifferentiated PT cells by day 14 (10, 36). In this manner, recovery from AKI induced by 1 mg/kg of UA was confirmed.

Experiment 1. To examine whether cell cycle status and cell cycle-associated proteins in the PT cells of rats recovered from AKI are different from those of normal rats, renal damage was induced in 12 rats by administering 1 mg/kg of UA dissolved in saline (1 ml/kg) intravenously (causing severe PT injury with significant renal dysfunction) (36). Fourteen days after recovery, rats were anesthetized intraperitoneally with ketamine (75 mg/kg) and xylazine (10 mg/kg) and euthanized for the isolation of tubular cells (n = 6) and for histological examinations (n = 6). Similarly, 12 nontreated rats were used as normal controls for the isolation of tubular cells (n = 6) and for histological examinations (n = 6).

Experiment 2. Our previous study demonstrated that rats pretreated with 1 mg/kg of UA had acquired resistance to subsequent treatment with 4 mg/kg of UA 14 days after the first treatment (36). To investigate the cell cycle kinetics of PT cells in rats recovered from AKI-accelerated G1 arrest in response to nephrotoxic stimulus, 68 rats were treated with saline (vehicle group) or 1 mg/kg of UA dissolved in saline (AKI group). Fourteen days after the first treatment, rats were injected intravenously with 4 mg/kg of UA and euthanized from 12 to 36 h after treatment for the isolation of tubular cells (n = 6 at each time point).
time point) and from 12 to 48 h after treatment for histological examinations \((n = 4 \text{ at each time point})\).

**Experiment 3.** To investigate whether PT cells in rats recovered from AKI inhibited S phase progression in response to a proliferative stimulus, 72 rats in the vehicle group and AKI group were injected with 38 mg/kg of lead acetate \((a\text{ proliferative stimulus})\) \((15, 40)\) intravenously 14 days after the first treatment. Rats were euthanized with aortic perfusion (15, 19). Briefly, both kidneys were perfused via the aorta with EGTA-containing, Ca\(^{2+}\)-free HBSS and with HBSS containing 0.15% (wt/vol) collagenase (type II) and 2 mM CaCl\(_2\). Isolated renal tubular cells from the cortex and the outer strip of the outer medulla (OSOM) were separated into PT and DT cells by Percoll density-gradient centrifugation. Cells in the upper and lower quarters of the layer were considered PT and DT cells, respectively. Trypan blue exclusion was used to determine the number of viable cells present in the cell suspensions. Only isolated PT cells were used for cell cycle analysis by flow cytometry and for evaluation of p21 and p27 protein levels by Western blotting.

**Cell Cycle Analysis of Isolated PT Cells**

Detailed procedures for cell cycle analysis are described elsewhere \((15)\). Briefly, freshly isolated PT cells in Krebs-Henseleit buffer were permeabilized with Triton X-100 and incubated with propidium iodide solution. After incubation, DNA content was measured by using an Epics XL flow cytometer (Beckman Coulter, Brea, CA). To separate cells in the G1 phase from cells in the G0 phase, Hoechst/pyronin Y staining was performed \((5)\). Freshly isolated tubular cells fixed with ice-cold 70% ethanol were suspended at \(1 \times 10^6\) cells/ml in HBSS. A solution \((1 \text{ ml})\) containing Hoechst 33342/pyronin Y was added to the cell suspension. Cell cycle status was measured by using a FACSAria cell sorter (BD Biosciences, San Jose, CA).

**Immunocytochemistry**

Immunofluorescence of megalin (a brush-border protein in PT cells) in isolated tubule cells was performed as described \((15)\). Briefly, isolated PT and DT cells were centrifuged and each pellet smeared on a glass slide. PT and DT cells fixed with 2% paraformaldehyde were incubated with 10% donkey serum and exposed to goat anti-megalin IgG with Can Get Signal solution B at 4°C overnight. The primary antibody was detected with Alexa Fluor 633-conjugated donkey anti-goat IgG. The cells were observed with a confocal fluorescence microscope \((FV1000, \text{ Olympus, Tokyo, Japan})\). To discriminate G1 phase cells from G0 phase cells, immunocytochemistry for Cdt1, which is specifically expressed during G1 phase \((27, 33)\), was also performed. After PT cells were permeabilized with 0.5% Triton X-100, the cells were incubated with rabbit anti-Cdt1 IgG and then with Alexa Fluor 546-conjugated goat anti-rabbit IgG. For nuclear staining, cells were incubated with 4',6-diamidino-2-phenylindole. The cells were observed with a BX50 fluorescence microscope \((\text{Olympus, Tokyo, Japan})\).

**Histological Examinations**

Both kidneys were dissected after a brief flush with phosphate-buffered saline. Kidneys were bisected along their longitudinal axis, fixed with 4% paraformaldehyde, and embedded in paraffin. Sections of 3-μm thickness were examined. Immunohistochemical analysis was performed according to standard protocols described in detail elsewhere \((15)\). Apoptosis was assessed by the terminal deoxynucleotidyl transferase (TUNEL) technique by using the ApopTag Plus In Situ Apoptosis Detection Kit \((36)\).

**Western Blotting of p21 and p27**

Immunoblot analysis of p21 and p27 in isolated PT cells was conducted by using a standard method \((15)\). Briefly, blots were probed

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Values are means ± SD. PT, proximal tubule; DT, distal tubule; control, normal control rats; acute kidney injury (AKI), rats 14 days after 1 mg/kg of uranyl acetate (UA); V, rats 14 days after vehicle treatment. *P < 0.05 vs. rats before 4 mg/kg of UA administration. **P < 0.05 vs. rats recovered from AKI at same time after 4 mg/kg of UA administration.

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<th>Table 3. Purity (megalin positivity) of isolated tubular cells</th>
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Values are means ± SD. *P < 0.05 vs. rats before 4 mg/kg of UA administration. **P < 0.05 vs. rats recovered from AKI at same time after 4 mg/kg of UA administration.
with a primary antibody against p21 or p27 at 4°C overnight. GAPDH was used as an internal control. The band intensity was quantified by using ImageJ software.

Morphometric Analysis

Assessment of the purity of the isolated PT cells, the percentage of Cdt1+ cells among the PT cells and morphometric analysis in the renal sections were performed as previously described (15). The number of cyclin D1, Ki67, p21, p27, kidney injury molecule-1 (Kim-1), and TUNEL+ PT cells were counted in 20 randomly selected fields of the cortex and OSOM at a magnification of ×400. The percentage of megalin+ cells, vimentin+ cells, and paired box gene 2 (Pax2)+ cells among the PT cells was calculated in 20 randomly selected fields of the cortex and OSOM at a magnification of ×400. The mean score in each rat represented the average number or percentage of PT cells per field.

Statistical Analysis

All values were expressed as means ± SD. Differences between three or more groups were examined for statistical significance by using ANOVA, followed by Tukey’s post hoc test. Differences between the AKI group and vehicle group at the same time point were assessed using an unpaired t-test (Prism 6; GraphPad Software, San Diego, CA). A P value <0.05 was accepted as statistically significant.

RESULTS

Isolation of PT Cells and DT Cells

Viability data, as determined by trypan blue staining, for the PT and DT cells in experiments 1, 2, and 3 are shown in Table 2. The percentage of megalin+ cells with polarity in the PT and DT cells indicating the purity of mature PT cells is shown in Table 3. The findings indicated effective separation of PT and DT cells in experiments 1 and 3. In experiment 2, PT cell viability was significantly higher in the AKI group than in the vehicle group at 36 h, indicating that PT cells are resistant to subsequent UA treatment. However, the purity of mature PT cells in the AKI group was significantly lower than that in the vehicle group at 36 h, possibly reflecting the early phenotypic change in PT cells in the AKI group. The findings supporting vimentin and Pax2 expression, a dedifferentiation marker in PT cells, were described later.

Cell Cycle Status of PT Cells in Rats Recovering From AKI

A simplified schematic diagram of the cell cycle phases with respective CDKIs and G1 phase markers is depicted in Fig. 2.

![Fig. 2. Schematic drawing of the cell cycle phases with respective cyclin-dependent kinase inhibitors and G1 phase markers. Cdt1, whole G1 phase marker; cyclin D1, mid-late G1 phase marker; Ki67, late G1 to G2/M phase marker; p21, associates with G1-S and G2/M phase transition; p27, associates with G0–G1, G1–S, and G2/M phase transition.](http://ajprenal.physiology.org/)

| Table 4. Cell cycle status in isolated PT cells before and 14 days after UA administration |
|----------------------------------|--------|--------|--------|
| G0/G1, %                         | S, %   | G2/M, % |
| PT in normal rats                | 98.3 ± 0.4 | 0.4 ± 0.1 | 1.3 ± 0.3 |
| PT in rats recovering from AKI   | 97.8 ± 0.3 | 0.5 ± 0.2 | 1.7 ± 0.2 |
| P value                          | 0.08   | 0.43   | 0.04   |

The percentage of G0/G1 and S phase PT cells 14 days after UA administration was comparable to that in normal PT cells. However, the percentage of G2/M phase cells was higher in PT cells 14 days after UA administration than that in normal PT cells (Table 4). By using the Hoechst/pyronin Y method, the percentage of G1 phase cells among PT cells from control rats and rats 14 days after UA administration were 36.8 ± 5.7 and 36.9 ± 3.0%, respectively (Fig. 3A). This result suggests that the proportion of G1 phase cells among PT cells 14 days after UA administration was comparable to that in control rats. The percentage of Cdt1+ cells among the isolated PT cells from control rats and rats 14 days after UA administration were 31.4 ± 4.5 and 33.0 ± 4.2%, respectively (Fig. 3B), confirming the results obtained with the Hoechst/pyronin Y method.

In histological examinations, no morphologically injured tubular cells were visible in the kidney sections in control rats and rats 14 days after UA administration (data not shown). Immunohistochemically, the number of cyclin D1+ cells or Ki67+ cells was significantly lower in the PT of rats 14 days after UA administration than in control rats (Fig. 3, C–H), suggesting that the percentage of early G1 phase cells among total G1 phase cells was higher in the PT of rats that have recovered from AKI than in the PT of untreated rats. There were significantly more p21+ cells or p27+ cells in the PT of rats 14 days after UA administration than in the PT of control rats (Fig. 4, A–F). Western blotting showed that the protein levels of p21 and p27 were also higher in the PT cells of rats 14 days after UA administration than in PT cells from control rats (Fig. 4, G–I).

Cell Cycle Status and Cell Fate of PT cells in Rats Recovering From AKI in Response to a Nephrotoxic Stimulus

The ratio of G1 to G0 phase cells in the PT of rats from the vehicle group increased as early as 12 h, peaked at 24 h, and decreased from 36 h. In contrast, the ratio of G1 to G0 phase cells in the PT of rats from the AKI group also increased as early as 12 h, but this increase was sustained until 36 h (Fig. 5, A and B), suggesting that G1 arrest occurred in response to a subsequent nephrotoxic stimulus among these cells. Detection of G1 phase cells, according to the presence of Cdt1 (Fig. 5E), yielded results similar to those obtained with flow cytometry (Fig. 5B). Although the percentage of S phase cells in the PT of the rats in the vehicle group transiently increased at 24 h, it did not increase significantly until 36 h in the PT of rats in the AKI group, at which point it was higher than that of the vehicle group (Fig. 5C). These data suggest that G1 arrest occurred and S phase progression was delayed in PT cells of the AKI group rats in response to a subsequent nephrotoxic stimulus. Similarly, the percentage of G2/M phase cells among the PT cells of the vehicle group rats did not increase until 36 h, whereas it
A subsequent nephrotic stimulus. The number of Ki67+ PT cells increased as early as 48 h after the nephrotic dose of UA in the vehicle group (Fig. 6, D and F), whereas those of the AKI group did not change until 48 h (Fig. 6, E and F). At each time point, there were significantly fewer Ki67+ PT cells in the AKI group compared with the vehicle group (Fig. 6F). These results suggested that PT cells in the AKI group first arrest at an early G1 phase in response to a nephrotic stimulus.

Almost all of the PT cells in both groups expressed megalin until 24 h after UA administration. Almost all of PT cells in the vehicle group expressed megalin until 36 h after UA administration, and 11.4 ± 4.8% of PT cells in the vehicle group lost megalin expression as early as 48 h (Fig. 7, A–C and S). On the other hand, 24.4 ± 1.5% and 46.6 ± 5.2% of PT cells in the AKI group lost megalin expression at 36 and 48 h, respectively (Fig. 7, D–F and S). Vimentin expression was found as early as 48 h after treatment (6.3 ± 2.1% of PT cells) (Fig. 7, G–I and T) in PT cells in the vehicle group, and as early as 36 h (16.0 ± 3.8%) (Fig. 7, J–L and T) in PT cells in the AKI group. Although Pax2 expression was not found in PT cells in the vehicle group until 48 h (Fig. 7, M–O and U), its expression in the AKI group significantly increased as early as 48 h (Fig. 7, P–R and U). These data indicated that PT cells that have recovered from AKI dedifferentiated earlier than those of the vehicle group in response to a subsequent nephrotic stimulus.

We also examined Kim-1 as a biomarker for renal proximal tubular injury (14). Kim-1 expression increased significantly in the vehicle group as early as 48 h after UA administration (Fig. 8, A–C and M). However, some PT cells expressed Kim-1 in

increased significantly at 36 h for rats of the AKI group (Fig. 5D), suggesting that G2/M arrest occurred among PT cells in the AKI group rats in response to a subsequent nephrotic stimulus.

In histological examinations, a small number of cells detached from the tubular basement membrane were found scattered in the PT in both groups as early as 36 h (data not shown).

Immunohistochemically, the number of cyclin D1+ PT cells after UA administration decreased as early as 12 h, reached a trough at 36 h, and had a slight tendency to increase again at 48 h in the vehicle group PT cells (Fig. 6, A and C). Those of the AKI group, however, started to decrease as early as 24 h (Fig. 6C) and did not increase again until 48 h (Fig. 6, B and C), suggesting a delayed G0-to-G1 phase transition and a repression of entering a mid-late G1 phase in PT cells in response to
the AKI group at baseline (0 h) and sustained the expression until 48 h (Fig. 8, D–F and M). The number of Kim-1+ PT cells in the AKI group was significantly higher than that in the vehicle group at each time point. Values are means ± SD of 6 rats. *P < 0.05 vs. rats before UA administration. #P < 0.05.

The number of TUNEL+ PT cells increased as early as 36 and 48 h after UA administration in the vehicle and AKI groups, respectively. The number of TUNEL+ PT cells in the AKI group was significantly higher from 0 to 24 h after treatment compared with the vehicle group. However, there were significantly fewer TUNEL+ PT cells in the AKI group at 36 h (Fig. 8, J–L and N). Although the number of TUNEL+ PT cells in the AKI group at 48 h did not significantly differ from that in the vehicle group at 48 h, the increase in TUNEL+ PT cells from baseline was significantly lower in the AKI group. (Fig. 8O). These results suggest that PT cells that have recovered from AKI have repressed apoptosis.

There were significantly more p21+ cells in the AKI group than in the vehicle group from 0 to 36 h (see Fig. 11C). The number of p21+ PT cells increased as early as 48 h in both groups, and there was no difference between groups at 48 h (see Fig. 11C). Values are means ± SD of 4–6 rats. *P < 0.05 vs. rats before UA administration. #P < 0.05.

Fig. 6. Histological changes in PT cells from rats pretreated with vehicle and rats pretreated with 1 mg/kg of UA after administration of 4 mg/kg of UA. Shown are photomicrographs of cyclin D1-immunostained (arrows) renal sections from rats pretreated with vehicle (A) and rats pretreated with 1 mg/kg of UA (B) 48 h after 4 mg/kg of UA administration. C: temporal changes in the number of cyclin D1+ PT cells per field. Also shown are photomicrographs of Ki67-immunostained (arrows) renal sections from rats pretreated with vehicle (D) and rats pretreated with 1 mg/kg of UA (E) 48 h after 4 mg/kg of UA administration. F: temporal changes in the number of Ki67+ PT cells per field. Original magnification ×400. Values are means ± SD of 4–6 rats. *P < 0.05 vs. rats before UA administration. #P < 0.05.
Fig. 11, A–C). The number of p27+ PT cells in both groups drastically decreased as early as 12 h (see Fig. 12C). In addition, there were significantly more p27+ PT cells in the AKI group than in the vehicle group at 36 and 48 h (Fig. 12, A–C).

Cell Cycle Status and Cell Fate in PT Cells in Rats Recovering From AKI in Response to a Proliferative Stimulus

The ratio of G1 to G0 phase cells increased as early as 12 h after lead acetate and 24 h after UA administration in the PT of the vehicle group and AKI group, respectively (Fig. 9, A and B), suggesting that the G0-to-G1 transition was delayed in PT cells in the AKI group in response to a proliferative stimulus. Analysis of G1 phase cells according to Cdt1 expression yielded results similar to those obtained with flow cytometry (Fig. 9E). S phase progression in PT cells started to increase as early as 24 h after lead acetate administration in both groups. The number of S phase cells peaked at 30 h in the vehicle group, whereas there was no peak among the AKI group PT cells. In addition, there were fewer S phase cells among the
AKI group PT cells than in the vehicle group at 30 and 36 h (Fig. 9C), suggesting that S phase progression was repressed in PT cells in the AKI group in response to the proliferative stimulus.

There was a higher percentage of G2/M phase cells in the PT at 0 to 24 h in the AKI group than in the vehicle group (Fig. 9D), suggesting that G2/M arrest occurred in PT cells of the AKI group in response to the proliferative stimulus.

In histological examinations, all PT cells expressed megalin and none expressed vimentin or Pax2 until 36 h after lead acetate administration in both groups (data not shown). The number of cyclin D1/H11001 PT cells increased as early as 12 h and 24 h after lead acetate administration in the vehicle group (Fig. 10, A and C) and AKI group (Fig. 10C), respectively. This suggests that the mid-late G1 phase transition was delayed in PT cells that have recovered from AKI in response to the proliferative stimulus. The number of Ki67/H11001 PT cells started to increase as early as 24 h in both groups (Fig. 10F), although there were significantly fewer Ki67+ PT cells in the AKI group than in the vehicle group at 36 h (Fig. 10D–F). These results suggest that progression to the late G1 phase was repressed among PT cells in the AKI group in response to the proliferative stimulus. These data indicate that early G1 arrest was induced in PT cells in AKI group in response to the proliferative stimulus.

There were significantly more p21/H11001 PT cells in the AKI group than in the vehicle group from 0 to 24 h after treatment (Fig. 11F). The number of p21+ PT cells increased as early as 36 h in both groups, and there was no difference of the number of p21+ PT cells between groups at 36 h (Fig. 11D–F). In the vehicle group, the number of p27+ PT cells increased as early as 12 h, and then decreased from 24 h. In the AKI group, however, the number of p27+ PT cells did not change at 12 h and decreased as early as 24 h (Fig. 12F). There were signif-
significantly more \( p27^+ \) PT cells in the AKI group than in the vehicle group at 36 h (Fig. 12, D–F).

**DISCUSSION**

We hypothesized that stimulating G1 arrest in PT cells that have recovered from UA-induced AKI could contribute to the acquired resistance to rechallenge injury by subsequent UA treatment. In this study, we analyzed cell cycle status in isolated PT cells that have recovered from AKI and the factors related to G1 arrest before and after the rechallenge injury using UA or proliferative stimuli. A summary of the sequence of changes in the cell cycle status and expression of marker proteins after treatments is shown in Fig. 13.

Recently, we reported that a high ratio of G1 to G0 phase cells and a rapid accumulation of G1 phase cells before S phase progression in the normal PT of rats may be involved in explosive cell proliferation in response to toxic stimuli (15). In the present study, cell cycle status (G0/G1/S/G2/M) in isolated PT cells in the AKI group was comparable to that in the vehicle group. However, more early G1 phase cells (cyclin D1- and Ki67-) and p21- and p27+ cells, which may be related to G1 arrest, were found in the PT of the AKI group than the vehicle group.
The synthesis of cyclin D, a member of the cyclin protein family, is initiated during the G1 phase and promotes the G1/S phase transition. It has been reported that cyclin D can reach a peak level of concentration at the late G1 phase (near the G1-to-S boundary), and cyclin D can degrade very rapidly soon after mitogens are withdrawn. If cyclin D degradation occurs during the G1 phase, cells will not enter the S phase (7). Thus, in the present study, degradation of cyclin D1 could be involved in inhibiting the cell cycle of excess hyperplastic PT cells after completion of PT cell relining, and this might reflect the increased number of early G1 phase cells (cyclin D1-) in the PT of the AKI group.

In general, when p21/p27 is localized within the nucleus, it binds to cyclin-CDK complexes, thereby inhibiting their function in cell cycle progression, resulting in cell cycle arrest (2, 4, 35). In the cisplatin-induced AKI model in rats, we reported that the number of p21+ nuclei increased in the PT after the peak of cell proliferation, contributing to cell cycle inhibition and cell redifferentiation (22). This must also be true for the UA-induced AKI model. In the present study, the PT cells of the AKI group showed an increasing number of p21+ or p27+ cells with a nuclear pattern, also suggesting that the PT cells may carry over inhibitory conditions of S phase progression even after redifferentiation of PT cells as determined by megalin-/vimentin-/Pax2-.

Analysis of the cell cycle kinetics of PT cells in response to the rechallenge injury of the subsequent UA treatment showed that UA induced G1 arrest potently and inhibited S phase
progression with less apoptosis among PT cells in the AKI group compared with the vehicle group. Sustained p21 and increased p27 expression in PT cells were also found in the AKI group in response to UA treatment. We used lead acetate to examine whether a proliferative stimulus could enhance G1 arrest as well as a toxic stimulus. Interestingly, lead acetate also induced delayed G0/G1 transition and reduced S phase progression in PT cells of the AKI group compared with the vehicle group. Sustained p21 and increased p27 expression in PT cells were also found in the AKI group. These findings suggest that PT cells in the AKI group can primarily inhibit cell cycle progression with G1 arrest, probably via modulation of p21, p27, or both, in response to either toxic or proliferative stimuli (UA or lead acetate, respectively), resulting in cytotolerance to rechallenge injury by UA treatment.

It has been reported that upregulation of p21 is associated with PT resistance to injury by ischemic or nephrotoxic insults (26, 32, 43). Experimental overexpression of p21 in PT cells could induce G1 arrest during the development of ischemic or nephrotoxic AKI (21). In addition, DNA damage may be repaired during this period, resulting in the cells evading apoptotic cell death (31). Recently, it was also reported that small-molecule cell cycle inhibitors, besides p21, can ameliorate ischemia-reperfusion-AKI or cisplatin-induced AKI (6, 30). Thus it is conceivable that upregulation of p21 in the PT cells of the AKI group contributes to the ability of these cells to resist rechallenge injury by subsequent UA treatment. However, a UA-induced AKI model in rabbits did not demonstrate acquired resistance to ischemia-reperfusion injury (42). The induction level of p21 may not have been high enough to prevent ischemia-reperfusion injury in the PT cells. Alternatively, the mechanisms of acquired resistance in the UA-induced AKI model could be different from that reported in p21 overexpression models.

In the present study, administration of UA increased the percentage of G2/M phase cells at 36 h in the AKI group, suggesting stimulation of G2/M arrest. p21 was also expressed in the G2-M phases and plays a role in G2/M arrest (1). On the
other hand, degradation of p27 is required for entry into mitosis (24). Thus both sustaining p21 upregulation and reexpression of p27 might be associated with G2/M arrest in the AKI group. However, since the percentage of G2/M phase cells was low compared with the G1 phase cells, the contribution of G2/M phase cells to cytoresistance, if any, might not be important.

Although the PT cells in the AKI group showed a mature PT phenotype as determined by megalin+/vimentin−/Pax2−, the PT cells in the AKI group showed an early phenotypic change to megalin+/vimentin+/Pax2+, which is assumed to be a dedifferentiation in response to UA. Since the PT cells in the AKI group also enhanced G1 arrest without phenotypic change in response to proliferative stimuli, it seems that the phenotypic change per se is not directly associated with G1 arrest. However, it remains unclear whether the early dedifferentiation could contribute to cytoprotection of the PT cells. Kim-1 expression was found in the PT cells in the AKI group at baseline and did not change until 48 h, whereas the expression of Kim-1, although small, increased significantly in the vehicle group as early as 48 h. It is possible that Kim-1 positivity in the AKI group at baseline indicates the existence of subclinical tubular injury or the accumulation of inactive Kim-1 in the recovered PT cells. Interestingly, the protective role for Kim-1 in response to AKI has recently been reported (41). Although there was no upregulation of Kim-1 in the AKI group, the sustained expression of Kim-1 might also contribute to the acquired resistance.

It is known that this acquired resistance to rechallenge injury can last for limited periods, for example, for <6 mo in the gentamicin model (8). With regard to the UA model, we found that acquired resistance was attenuated 2 mo after UA administration, and was lost by 6 mo (Iwakura T et al., unpublished observations). This property of PT cells might only persist during the period before true maturity of the PT cells occurs, when expression of p21 and p27 and the composition of the cell cycle status in G1 are both normalized.

In conclusion, although normal PT cells have a natural ability of G1 arrest before S phase progression in response to toxic or proliferative stimuli, PT cells that have recovered from AKI primarily inhibit cell cycle progression with enhanced G1 arrest, probably by modulation of p21, p27, or both, in response to toxic or proliferative stimuli, resulting in cytoresistance to rechallenge injury. Our findings in the natural model of the acquired resistance to rechallenge injury support previous reports that the inhibition of cell cycle progression contributes to amelioration of kidney injury and suggest the inhibition of cell cycle progression could lead to the development of safe therapeutic applications to prevent AKI.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: T.I. provided conception and design of research; T.I. performed experiments; T.I. analyzed data; T.I. and Y.F. interpreted results of experiments; T.I. prepared figures; T.I. drafted manuscript; T.I., Y.F., and H.Y. approved final version of manuscript.

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