Cell division and phenotypic regression of proximal tubular cells in response to uranyl acetate insult in rats

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Abstract

Background. We examined whether dedifferentiation is necessary for cell division of proximal tubule (PT) cells after acute PT injury.

Methods. Rats were injected with a low (0.2 mg/kg) or high (4 mg/kg) dose of uranyl acetate (UA) to induce acute PT injury. Proliferating PT cells were labelled with bromodeoxyuridine (BrdU) before sacrifice. Renal tissues were examined by double labelling of BrdU and megalin, aquaporin 1 (AQP1), Na\(^+\)–K\(^+\)-ATPase or vimentin, and by immunoelectron microscopy for BrdU+ cells.

Results. Under normal conditions, BrdU+ PT cells were positive for the PT phenotype (megalin-, AQP1- and Na\(^+\)–K\(^+\)-ATPase positive and vimentin negative, a mesenchymal marker). Low-dose UA induced focal PT injury, and BrdU+ initially proliferating PT cells were found in the proximal three quarters of the S3 segment of nephron as early as 12 h, which maintained the PT phenotype and were vimentin negative. Proliferating PT cells showed low expression of the PT cell protein phenotype from Day 2 to Day 5 with vimentin expression from Day 2. High-dose UA induced severe PT injury in the proximal three quarters of the S3 segment from Day 5. BrdU+ initially proliferating PT cells, which were found in distal areas of the S3 segment as early as Day 2, showed low expression of the PT protein phenotype but were vimentin positive. Immunoelectron microscopy showed mature PT morphology for BrdU+ PT cells in control rats. BrdU+ initially proliferating PT cells showed a relatively mature phenotype after low-dose UA insult but an immature phenotype after high-dose UA insult.

Conclusions. PT cells can initiate cell division without de-differentiation after mild PT injury by low-dose UA insult.
Keywords: acute renal failure; cell division; phenotype; proliferation; proximal tubule

Introduction

Rapid regeneration of damaged proximal tubule (PT) cells in the S3 segment of the nephron occurs after nephrotoxin- or ischaemia-induced acute tubular injury [1]. In the process of PT regeneration, surviving PT cells, which are normally quiescent cells, undergo dedifferentiation after the insult and begin to proliferate [2,3]. The fact that PT cells dedifferentiate to an immature phenotype similar to those in the developing kidney has major implications for renal repair/regeneration after acute tubular injury [3]. However, it was reported recently that PT cells can also proliferate by division of differentiated PT cells in healthy rats [4,5], suggesting that PT cells are inherently capable of proliferation without dedifferentiation. This raises the question as to whether PT cells can undergo cell division without dedifferentiation and contribute to recovery from acute PT injury under certain conditions.

In a previous study, we demonstrated two modes of progression of PT cell recovery after uranyl acetate (UA)-induced acute PT injury in rats [6]. A low dose of UA (<0.5 mg/kg) resulted in focal PT depletion mainly in the proximal three-quarters of the S3 segment of the nephron without a significant increase in serum creatinine (Scr), and the depleted PT cells were replaced focally by divided cells mainly from surviving PT cells in the same area [6]. On the other hand, a high dose of UA (>2 mg/kg) induced a significant increase in Scr and almost complete PT depletion in the proximal three-quarters of the S3 segment, and the damaged tubules were replaced with newly divided PT cells, most of which were descendents of a distinct population of tubular cells in the distal part of the S3 segment [6–8]. We postulated that the phenotype of PT cells at the time of initial cell division also differed between the two modes of progression of PT cell recovery after UA-induced acute PT injury.

The present study is an extension of our previous studies [6–8] and was designed (by using various immunoreactive phenotype markers of PT cells) to elucidate whether dedifferentiation or phenotype regression of PT cells is necessary for PT cell division in normal rats and rats with acute PT injury induced by low and high doses of UA. The results showed that PT cells can initiate cell division without dedifferentiation after mild PT injury.

Subjects and methods

Induction of acute tubular injury

Male Sprague-Dawley rats weighing 180–230 g (SLC Co., Shizuoka, Japan) were used in the present study. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Hamamatsu University School of Medicine. To induce acute PT injury, rats received a single intravenous injection of either 0.2 or 4 mg/kg of UA, and three rats were sacrificed at 12 h, 1, 2, 3, 5 and 7 days after a UA injection. Three rats that were not injected with UA served as controls. To identify proliferating PT cells, the rats were injected intraperitoneally with 40 mg/kg of bromodeoxyuridine (BrdU, Sigma Chemicals Co., St Louis, MO, USA) 1 h before sacrifice. For a better assessment of labelling of proliferating cells, three control rats were injected intraperitoneally with BrdU at 24, 12 and 1 h before sacrifice. The rats were anaesthetized with diethyl ether, and then both kidneys were removed after flushing phosphate-buffered saline (PBS) for histological examination.

Immunohistochrometry and immunofluorescence studies

The kidneys were bisected through the longitudinal axis and fixed with either a 4% paraformaldehyde or a methacarn solution and embedded in paraffin. For the histological examination of renal tissues, 4 µm sections were stained with periodic acid–Schiff (PAS).

For immunohistochromistry, a standardized avidin–biotin-complex technique was applied to detect a mouse monoclonal antibody against BrdU (clone BU-1, Amersharn International, Poole, UK) or vimentin (clone V9, Sigma Chemicals Co.) by using the method previously reported [7]. The reaction product was visualized by the peroxidase–diaminobenzidine system for BrdU.

To examine the phenotypes of BrdU+ proliferating PT cells, the tissues were double-labelled with BrdU and vimentin, aquaporin 1 (AQP1), vimentin or Na+–K+ ATPase. For megalin, AQP1 and Na+–K+ ATPase, 4% paraformaldehyde-fixed sections were used, while for vimentin, methacarn-fixed sections were used. The sections were first incubated with a goat polyclonal antibody against megalin (P-20, Santa Cruz Biotechnology Inc., CA, USA), goat polyclonal antibody against Na+–K+ ATPase α1 (C-20, Santa Cruz Biotechnology Inc.), rabbit polyclonal antibody against AQP1 (Chemicon International Inc., Temecula, CA, USA) or mouse monoclonal antibody against vimentin, followed by biotinylated donkey anti-goat, donkey anti-rabbit or donkey anti-mouse IgGs (Chemicon International Inc.). Then, the reaction products were visualized by the peroxidase–diaminobenzidine system for megalin. A vectastain ABC-AP reagent (Vector Laboratories, Burlingame, CA, USA) was added and the Vector Red Alkaline Phosphatase Substrate Kit I was used as chromogen for Na+–K+ ATPase, AQP1 and vimentin. Next, the sections were heated in a microwave oven for 15 min in 0.01 M sodium citrate, pH 6.0, at 800 W, and then incubated with the mouse monoclonal antibody against BrdU, followed by biotinylated donkey anti-mouse IgG and the peroxidase–diaminobenzidine system.

Since the reaction product of the Vector Red Alkaline Phosphatase Substrate is visible as a red fluorescence using a 540–596 nm band filter system, which is more sensitive than light microscopic observation, the sections labelled with Na+–K+ ATPase were next incubated with the mouse monoclonal antibody against BrdU, followed by fluorescein isothiocyanate-conjugated donkey anti-mouse IgG (Chemicon International Inc.), before they were examined with an Olympus BX50 fluorescent microscope (Olympus, Tokyo, Japan). The merged images were prepared using a digital camera (D70, Olympus) and processed with the DP manager software (Olympus).

For control histochemical sections, the first antibodies were omitted or replaced by normal serum of the corresponding animals. The signals in both control and experimental sections were negative or showed negligible signals.

Morphometric analysis

For morphometric analysis, the layer of the outer stripe of the outer medulla (OSOM) was arbitrarily divided into four zones of equal thicknesses (zones 1–4) (Figure 2A) under light microscopy by using the method previously reported [6]. The number of BrdU+ PT cells was counted in 20 randomly selected fields of each zone in each kidney at a ×400 magnification. The number of BrdU+ PT cells and the number of PT cells that were double labelled with BrdU and vimentin, AQP1, vimentin or Na+–K+ ATPase were counted in 40 randomly selected fields of the entire S3 segment in each kidney at a ×400 magnification.

To examine what percentage of BrdU+ PT cells express each phenotype marker, the number of BrdU+ PT cells and the number of PT cells that were double labelled with BrdU and megalin, AQP1, vimentin or Na+–K+ ATPase were counted in 40 randomly selected fields of the entire S3 segment in each kidney at a ×400 magnification.

To examine what percentage of PT cells with a mitotic figure have brush-border or vimentin positivity in the early phase after induction of PT injury, the number of PT cells with a mitotic figure and the number of PT cells that have both mitotic figure and brush-border or vimentin positivity were counted in 40 randomly selected fields of the entire S3 segment in each kidney at a ×400 magnification.
were stained with lead citrate, and examined with a JEM-1220 electron microscope (JEOL, Tokyo, Japan).

Pre-embedding immunoelectron microscopy was applied by using the method previously reported [9], to examine proliferating PT cells labelled with BrdU under normal conditions and after 0.2 or 4 mg/kg of UA insult. Briefly, 4% paraformaldehyde-fixed renal tissues were cryoprotected in sucrose and snap-frozen in cooled N-µ-hexane. Then, 6–8 µm cryostat sections were incubated with 10% normal donkey serum, followed by incubation with biotinylated donkey anti-mouse IgG. After fixation in 2% glutaraldehyde for 10 min, the reaction products were visualized by incubation with diaminobenzidine; then the sections were post-fixed in 1% osmium tetroxide for 1 h and embedded in Epon 812. Ultrathin sections were stained with lead citrate, and examined with a JEM-1220 electron microscope (JEOL, Tokyo, Japan).

**Results**

**Proliferation and phenotype of PT cells**

After a single injection of BrdU, PT cells in the control rats were rarely found to be positive for BrdU (Figure 2B). BrdU+ PT cells were positive for megalin, AQP1 and Na⁺–K⁺ ATPase, but negative for vimentin (Figure 1A, Table 1).

In rats treated with low-dose UA, focal PT injury was induced in the proximal three quarters of the S3 segment (data not shown). BrdU+ proliferating PT cells were sporadically found in that segment as early as 12 h after UA insult, increased in number afterwards and then decreased by Day 7 when PT cell relining was completed (Figure 2C). BrdU+ initially proliferating PT cells at 12 h were positive for megalin, AQP1 and Na⁺–K⁺ ATPase, but negative for vimentin, similar to the normal PT cells (Figure 1B, Table 1). PT cells with a mitotic figure at 12 h and Day 1 had

**Table 1. Immunostaining for megalin, AQP1, Na⁺–K⁺ ATPase and vimentin in BrdU+ proximal tubular cells in the S3 segment of a normal nephron and after uranyl acetate-induced acute tubular injury**

<table>
<thead>
<tr>
<th>Time</th>
<th>Megalin</th>
<th>AQP1</th>
<th>Na⁺–K⁺ ATPase</th>
<th>Vimentin</th>
</tr>
</thead>
<tbody>
<tr>
<td>2h</td>
<td>+ (100)</td>
<td>+ (100)</td>
<td>+ (100)</td>
<td>–</td>
</tr>
<tr>
<td>12h</td>
<td>+ (100)</td>
<td>+ (100)</td>
<td>+ (100)</td>
<td>–</td>
</tr>
<tr>
<td>2 days</td>
<td>±/+ (+100)</td>
<td>±/+ (+100)</td>
<td>±/+ (+100)</td>
<td>–/± (67.0)</td>
</tr>
<tr>
<td>3 days</td>
<td>–/± (60.3)</td>
<td>–/± (47.6)</td>
<td>–/± (42.6)</td>
<td>±/+ (95.0)</td>
</tr>
<tr>
<td>5 days</td>
<td>–/± (1.9)</td>
<td>–/± (1.7)</td>
<td>–/± (2.2)</td>
<td>+4 (98.6)</td>
</tr>
<tr>
<td>7 days</td>
<td>±/+ (98.6)</td>
<td>±/+ (98.0)</td>
<td>±/+ (100)</td>
<td>± (80.3)</td>
</tr>
<tr>
<td>2h</td>
<td>+ (100)</td>
<td>+ (100)</td>
<td>+ (100)</td>
<td>–</td>
</tr>
<tr>
<td>12h</td>
<td>+ (100)</td>
<td>+ (100)</td>
<td>+ (100)</td>
<td>–</td>
</tr>
<tr>
<td>1 day</td>
<td>+ (100)</td>
<td>+ (100)</td>
<td>+ (100)</td>
<td>–</td>
</tr>
<tr>
<td>2 days</td>
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<td>–/± (1.6)</td>
<td>± (60.3)</td>
<td>+ (97.3)</td>
</tr>
<tr>
<td>3 days</td>
<td>–/± (1.0)</td>
<td>–/± (1.5)</td>
<td>–/± (10.2)</td>
<td>+ (100)</td>
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<tr>
<td>5 days</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+4 (100)</td>
</tr>
<tr>
<td>7 days</td>
<td>±/+ (34.6)</td>
<td>±/+ (27.0)</td>
<td>±/+ (71.6)</td>
<td>±/+ (100)</td>
</tr>
</tbody>
</table>

Staining intensity: –, negative; ±, trace; +, positive.

*Maximal staining intensity in the same group.

Numbers (mean) in parentheses are given as the percentage of BrdU+ cells that expressed each marker (both ± and +) cell in all BrdU+ cells in the S3 segment.
a PAS-positive brush border (Figure 3A, Table 2) but were negative for vimentin (Figure 3B, Table 2). A proportion of BrdU+ PT cells showed low expression of all three markers of the PT phenotype and expressed vimentin as early as Day 2 (Figure 1B). Almost all BrdU+ PT cells were positive for vimentin at Day 5 in association with negative expression of all three markers of the PT phenotype (Figure 1B, Table 1). All three markers of the PT phenotype were re-expressed in PT cells with or without BrdU+ by Day 7, while vimentin was still positive in most PT cells (Figure 1B, Table 1).

In rats treated with high-dose UA, severe PT injury was induced in the proximal three quarters of the S3 segment by Day 5 (data not shown), and BrdU+ proliferating PT cells were limited to the distal areas of S3 as early as Day 2 (Figure 2D), increased in number throughout the S3 segment at Day 5 but subsequently decreased in number by Day 7 (Figure 2D) when PT cell relining was almost completed. At Day 2, the BrdU+ PT cells were found to be negative for megalin and AQP1 and faintly positive for Na\(^+\)-K\(^+\)-ATPase, but positive for vimentin (Figure 1C, Table 1). PT cells with mitotic figures at Day 2 had almost no PAS-positive brush border (Figure 3C, Table 2) but were positive for vimentin (Figure 3D, Table 2). BrdU+ and BrdU− PT cells became negative for all three markers of the PT phenotype but positive for vimentin at Day 5 and slightly re-expressed all three markers of the PT phenotype at Day 7, while vimentin was still positive in almost all PT cells (Figure 1C, Table 1).

Ultrastructure of proliferating PT cells

Immunoelectron microscopy revealed that BrdU+ PT cells were located on the unbroken tubular basement membrane in both control and UA-treated rats (Figure 4). In control rats, PT cells labelled with single- or pulse-BrdU injection showed a mature PT feature with relatively large nuclei, including an intact brush border, abundant microorganella and basal infoldings (Figure 4A). Although mitotic figures could not be observed in this study, BrdU-labelled PT cells that laid next to each other also showed features of mature cells (Figure 4B).

After low-dose UA insult, most BrdU+ initially proliferating PT cells at 12 h and 1 Day showed a relatively intact brush border, abundant cytoplasmic organelles and some of them had a high nucleus-to-cytoplasm ratio (Figure 4C). However, the BrdU+ PT cells began to show features of immature cells such as scanty cytoplasm and a reduced or absent brush border at Day 2, which became the main feature at Day 5 (not shown).

After high-dose UA insult, almost all BrdU+ initially proliferating PT cells at Days 2 and 3 showed features of immature cells with a high nucleus to cytoplasm ratio (Figure 4D), being comparable to those at Day 5 in low-dose UA insult. Isolated BrdU+ PT cells with or without cell division were occasionally observed (Figure 4E).

Discussion

The main purpose of the present study was to determine whether dedifferentiation is necessary for PT cell division in normal rats and rats after acute PT injury.

Recently, Vogtseider et al. [4,5] reported that cycling cells in the S3 segment of normal rats are differentiated PT cells. In the present study, we also found that BrdU+ proliferating PT cells in the S3 segment of a normal nephron...
showed a mature PT phenotype such as staining for megalin, AQP1 and Na\(^+\)–K\(^+\)ATPase and also maintained a mature PT ultrastructure. These cells did not express vimentin, a maker of mesenchyme or dedifferentiated PT cells. These findings suggest that normal PT cells can undergo cell division without dedifferentiation, although it is not known whether all PT cells have this ability.

In the mild PT injury model, BrdU\(^+\) PT cells were found as early as 12 h after low-dose UA insult, which were confined in the proximal three quarters of the S3 segment. BrdU\(^+\) initially proliferating PT cells at 12 h and 1 day were positive for all three markers of the mature PT phenotype with a relatively mature PT ultrastructure, suggesting that PT cells can undergo cell division without dedifferentiation even after mild UA insult. In this study, we also found a transient phenotype regression in BrdU\(^+\) PT cells from Day 2 to Day 5. As we previously reported [6], BrdU\(^+\) proliferating PT cells were also positive for vimentin as early as Day 2 after low-dose UA insult. Since vimentin is a major intermediate filament protein and is associated with the development of migratory capacity [10,11], it is conceivable that proliferating PT cells acquire vimentin expression to undergo cell division more than once and to migrate to cover the denuded tubular basement membranes after Day 2. This may not be the case in proliferating PT cells under normal conditions.

<table>
<thead>
<tr>
<th></th>
<th>Brush border</th>
<th>Vimentin</th>
</tr>
</thead>
<tbody>
<tr>
<td>normal</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Low-dose UA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 h</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>12 h</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>1 day</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>2 days</td>
<td>93.0</td>
<td>55.6</td>
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<td>3 days</td>
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<td>High-dose UA</td>
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<td>1 day</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>2 days</td>
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<td>99.2</td>
</tr>
<tr>
<td>3 days</td>
<td>0</td>
<td>100</td>
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</tbody>
</table>
On the other hand, in severe PT injury model, BrdU+ PT cells were found as early as Day 2 after high-dose UA insult, and these cells were confined to the distal area of the S3. BrdU+ initially proliferating PT cells showed regression of staining for all three markers of the mature PT phenotype, but became positive for a mesenchymal marker, together with an immature PT ultrastructure, suggesting that initially proliferating PT cells first acquired dedifferentiation and then underwent cell division or they acquired dedifferentiation at the same time of cell division. Initially proliferating PT cells may need continuous cell division because of large areas of PT depletion. However, it is not known how they sense such needs. A reduction in cell density could be a possible signal for cell division, but this was not the case in this model as PT necrosis and sloughing occur at another site of regeneration in the S3 segment of PT [7]. It is possible that the initially proliferating PT cells under this environment are unique since we previously found that they are slow cycling, resistant to 5-fluorouracil and reproliferated after the second UA treatment, suggesting a distinct population of cells that can regenerate the S3 segment in UA-induced acute renal failure.

The present results documented for the first time that in addition to the normal condition, PT cells can enter the cell cycle without apparent dedifferentiation in response to mild acute PT injury, like hepatic cells. Cell marking studies indicate that during normal liver turnover and after partial hepatectomy, hepatocytes are replaced by compensatory

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**Fig. 4.** Immunoelectron micrographs of BrdU+ initially proliferating PT cells in normal rats (A and B), at 12 h in rats treated with 0.2 mg/kg of UA (C) and 2 days in rats treated with 4 mg/kg of UA (D and E). (E) BrdU+ PT cell under cell division. Arrows: BrdU+ nuclei; #: BrdU− nuclei; *: brush border; L: tubular lumen. Scale bars = 5.0 µm.
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Background. Cisplatin-induced nephropathy has been related to increased lipid peroxide formation and decreased activity of antioxidant enzymes in the kidney. The present study aimed to examine whether treatment with α-lipoic acid (α-LA) prevents the cisplatin-induced nephrotoxicity.

Methods. Two groups of rats were treated with cisplatin, one of which being cotreated with α-LA. The control group was treated with vehicle only. Four days later, the expression of 

α-Lipoic acid prevents cisplatin-induced acute kidney injury in rats

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Received for publication: 4.1.09; Accepted in revised form: 7.4.09

doi: 10.1093/ndt/gfp176
Advance Access publication 17 April 2009