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Abstract

We investigated the proliferative capacity of renal proximal tubular cells in healthy rats. Previously, we observed that tubular cells originate from differentiated cells. We now found 1) by application of bromo-deoxyuridine (BrdU) for 14 days and costaining for BrdU, and the G(1)-phase marker cyclin D1 that the bulk of cells in the S3 segment of juvenile rats were involved in proliferation; 2) that although the proliferation rate was about 10-fold higher in juvenile rats compared with adult rats, roughly 40% of S3 cells were in G(1) in both groups; 3) that after a strong mitotic stimulus (lead acetate), proliferation was similar in juveniles and adults; 4) that there was a high incidence of cyclin D1-positive cells also in the healthy human kidney; and 5) by labeling dividing cells with BrdU for 2 days before the application of lead acetate and subsequent costaining for BrdU and cell cycle markers, that, although a strong mitotic stimulus does not abolish the period of quiescence following division, it shortens it markedly. Thus the capacity of the proximal tubule to rapidly recruit cells into division relies on a large reserve pool of cells in G(1) and on the shortening of the obligatory period of quiescence that follows division.

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Vogetseder A, Picard N, Gaspert A, Walch M, Kaissling B, Le Hir M. Proliferation capacity of the renal proximal tubule involves the bulk of differentiated epithelial cells. *Am J Physiol Cell Physiol* 294: C22–C28, 2008. First published October 3, 2007; doi:10.1152/ajpcell.00227.2007.—We investigated the proliferative capacity of renal proximal tubular cells in healthy rats. Previously, we observed that tubular cells originate from differentiated cells. We now found *1*) by application of bromo-deoxyuridine (BrdU) for 14 days and costaining for BrdU, and the G_1-phase marker cyclin D1 that the bulk of cells in the S3 segment of juvenile rats were involved in proliferation; *2*) that although the proliferation rate was about 10-fold higher in juvenile rats compared with adult rats, roughly 40% of S3 cells were in G_1 in both groups; *3*) that after a strong mitotic stimulus (lead acetate), proliferation was similar in juveniles and adults; *4*) that there was a high incidence of cyclin D1-positive cells also in the healthy human kidney; and *5*) by labeling dividing cells with BrdU for 2 days before the application of lead acetate and subsequent costaining for BrdU and cell cycle markers, that, although a strong mitotic stimulus does not abolish the period of quiescence following division, it shortens it markedly. Thus the capacity of the proximal tubule to rapidly recruit cells into division relies on a large reserve pool of cells in G_1 and on the shortening of the obligatory period of quiescence that follows division.

kidney; cyclin D1; bromodeoxyuridine; stem cells; regeneration

HOW THE KIDNEY maintains the homeostasis of tubular epithelial cell number has been a fundamental question for years. Since differentiated cells in most tissues, including renal tubules, have only a limited life span, it is crucial for these cells to be replaced to maintain organ function. Also a variation in tissue mass as seen during physiological adaptation encompasses the production of specialized cells. Furthermore, after injury, the generation of new cells is a prerequisite to restore the normal function of the injured organ.

The source of newly formed renal epithelial cells is debated. Some studies show that novel cells are derived from divisions of differentiated cells (1, 10, 27), and others claim that a subpopulation in the renal tubular epithelial cells function as progenitor cells (5, 9, 16, 19). In addition, hematopoietic stem cells (12, 15, 21, 22) and mesenchymal stem cells (18) have been shown to be able to repopulate the renal tubular system; however, under physiological conditions tubular cells originating from extrarenal stem cells are not detectable in the kidney, and even after severe organ injury these cells constitute only a small population (7).

Our previous studies revealed that tubular cell proliferation in healthy rats rely on the division of differentiated cells (25). Furthermore, we were unable to detect any slow-cycling stem cells or rapidly cycling transit amplifying (TA) cells to support a "classic" stem cell system (26).

In the present study we address the question whether the capacity to divide is restricted to a small subpopulation of the differentiated cells in S3 or whether it is broadly distributed among the cells in that segment. To that aim we investigated the cycling behavior of tubular cells in S3 in juvenile rats and in adult rats, both under physiological conditions and after stimulation of proliferation. The data showed that the bulk of cells in S3 participate in cell proliferation.

MATERIALS AND METHODS

Juvenile 4–5 wk old (110–140 g body wt) and adult 16–20 wk old (>400 g body wt) male Wistar rats were used. They were fed a standard laboratory diet and drank tap water ad libitum. The experimental protocol was approved by the Cantonal Veterinary Office of Zurich.

Paraffin-embedded human kidney biopsies were supplied by the University Hospital of Zurich. These biopsies were made to monitor organ rejection in kidney-transplanted patients. Only those biopsies showing no signs of rejection were used.

Experimental Protocol

Experiment 1: Continuous administration of BrdU for 2 wk. 5′-Bromo-2′-deoxyuridine (BrdU) (Sigma, St. Louis, MO) was added to tap water in a concentration of 0.5 g/l. Three juvenile rats received a 300-µl (10 mg BrdU/ml isotonic saline) loading dose by subcutaneous injection before receiving the BrdU drinking solution over a period of 2 wk up to perfusion fixation.

Experiment 2: Proliferation induction by lead acetate. Six juvenile and six 16-wk-old rats were used. To produce a potent proliferation stimulus, 190 mg of $C_4H_6O_4Pb\cdot3H_2O$ (Merck, Darmstadt, Germany) was dissolved in 20 ml distilled H_2O and administered intravenously at 3.8 mg/100 g body wt to three animals of each group (2). Three control animals of each group received the corresponding volume of isotonic saline. The rats were perfusion fixed between 9 AM and 11 AM, 36 h after injection.

Experiment 3: Proliferation induction by lead acetate after 2 days of BrdU labeling. Six juvenile rats were injected subcutaneously with 10 mg/kg body wt of BrdU for 2 consecutive days at 7 AM and 7 PM. Twenty four hours after the last BrdU injection, three animals received 3.8 mg/100 g body wt $C_4H_6O_4Pb\cdot3H_2O$ and the other three received a corresponding volume of isotonic saline solution. Thirty six hours later the animals were perfusion fixed.

Fixation and Tissue Treatment

The rats were anesthetized by an intraperitoneal injection of pentobarbital (100 mg/kg body wt) and fixed by vascular perfusion (4). The fixative contained 3% paraformaldehyde, 0.01% glutardialdehyde, and 0.5% picric acid, dissolved in a 3:2 mixture of 0.1 M cacodylate buffer (pH 7.4, added with sucrose, final osmolality 300 mosmol/kg) and 4% hydroxyl ethyl starch (Fresenius Kabi, Bad Homburg, Germany) in 0.9% NaCl. The kidneys were fixed for 5 min and then rinsed by vascular perfusion with 0.1 M cacodylate buffer for 5 min.

Antibodies

The antibody used was rabbit polyclonal anti-Ki-67 (Novocastra Laboratories, Newcastle, UK) and anti-p27^{kip-1} (Abcam, Cambridge, UK). Anti-cyclin D1 was a rabbit monoclonal antibody (clone SP4) (NeoMarkers).

The following antibodies were mouse monoclonals: anti-BrdU (clone B44; BD Biosciences), anti-Ki-67 (clone MIB-5; DakoCytomation, Glostrup, Denmark), and anti-retinoblastoma (phosphor-specific serine-608, clone 51B7; Thermo Scientific, Fremont, CA).

Immunofluorescence

Two-millimeter thick slices of fixed kidney were frozen in liquid propane, cooled down to the temperature of liquid nitrogen, and cut into 4-μm thick cryostat sections. Sections were thawed on slides and microwaved for 10 min in 0.01 M citrate buffer at pH 6.0. After pretreatment for 1 h in 5% normal goat serum in phosphate-buffered saline (PBS), the sections were incubated overnight in a humidified chamber at 4°C with the primary antibodies and diluted in PBS supplemented with 1% bovine serum albumin.

Binding sites of the primary antibodies were revealed with Cy3-conjugated goat-anti-rabbit IgG (red) and a fluorescein isothyocyanate (FITC)-conjugated goat-anti-mouse IgG. Antibodies with minimal cross-reactivity to other secondary antibody species were employed (Jackson ImmunoResearch Laboratories, West Grove, PA). For nuclear staining, 4′,6-diamidino-2-phenylindole (DAPI; Sigma) was added to the working dilution of the secondary antibodies. Multiple labeling was performed using cocktails of primary antibodies and of the respective secondary antibodies. Controls without primary antibodies were negative. The sections were coverslipped, using DAKO-Glycergel (Dakopatts), to which 2.5% 1,4-diazabicyclo[2,2,2]octane (DABCO; Sigma) was added as fading retardant.

Fluorescent-labeled specimens were examined using a confocal laser scanning microscope (CLSM SP2, Leica, Mannheim, Germany) or a Polyvar microscope (Reichert Jung, Vienna, Austria).

Immunoperoxidase Labeling of Cyclin D1

Three human kidney biopsies were removed and immediately fixed by immersion in phosphate-buffered freshly depolymerized 4% paraformaldehyde for 45 min at room temperature, dehydrated in alcohol series, and embedded in paraffin. Three-micrometer sections were deparaffinized and microwaved for 10 min in 0.0 1 M sodium citrate buffer, pH 6.0. After being blocked with 5% normal goat serum for 30 min at room temperature, the sections were washed in PBS and incubated overnight at 4°C with anti-cyclin D1 1:200 in PBS. The binding sites were revealed by immunoperoxidase using the Vectastain ABC kit (Vector Laboratories, Burlinghame, CA), with 3,3′-diaminobenzidine as the substrate. Nuclei were visualized by counterstaining with hematoxylin.

Quantitative Evaluation of Nuclear Labeling

With the use of the nuclear DNA labeling of DAPI, the outer stripe was located, and micrographs were made randomly using the ×40 magnification of the laser scanning microscope. The surface area of tissue on each micrograph was 0.38 mm^2. The proximal tubule was identified by the brush border, detectable in background fluorescence.

Nuclei of proximal epithelial cells positive for BrdU, Cyclin D1, or Ki-67 were counted on the whole micrographs. Because of the high numbers of DAPI and of cyclin D1-positive cells, these were evaluated in a uniform random systematic sample (10% sampling fraction) of the area of the micrograph.

Statistical analysis of the data was performed with the ANOVA software. Differences were considered significant if P was <0.05 in Student's t-test for unpaired samples. All data are given as means $\pm$ SD.

RESULTS

Bulk of Proximal Epithelial Cells in S3 Undergo Cell Cycle (Expt. 1)

To discern quiescent cells from cycling cells, BrdU was continuously administered to 4-wk-old rats for 2 wk up to perfusion-fixation of the kidneys. We chose 2 wk because the fraction of cells that divide twice during that period of time is small (26). Immunofluorescence detection was employed to visualize BrdU and cyclin D1, a protein that accumulates during early to mid G$_1$ phase and which is essential for cell cycle progression (6, 20) (Fig. 1). BrdU-positive cells are cells that divided during the BrdU application period. Colocalization of both markers represents the population of cells that divided during the 2-wk BrdU application period and reentered the cycle. Of the cells in S3, 50.9 $\pm$ 7.3% were positive for BrdU only, 27.5 $\pm$ 5.2% positive for cyclin D1 only, and 10.5 $\pm$ 1.0% were positive for both BrdU and cyclin D1. The remaining 11.1 $\pm$ 2.1% were negative for both markers. Together we have, in the investigated 14 days, a population of 88.9 $\pm$ 2.1% positive for at least one of the employed markers, indicating that the majority of cells participate in cycling.

Cyclin D1 was detected in 71.1 $\pm$ 3.6% of the BrdU-negative population but in only 17.3 $\pm$ 3.0% of the BrdU-positive population. Thus the cells that divided during the 2 wk
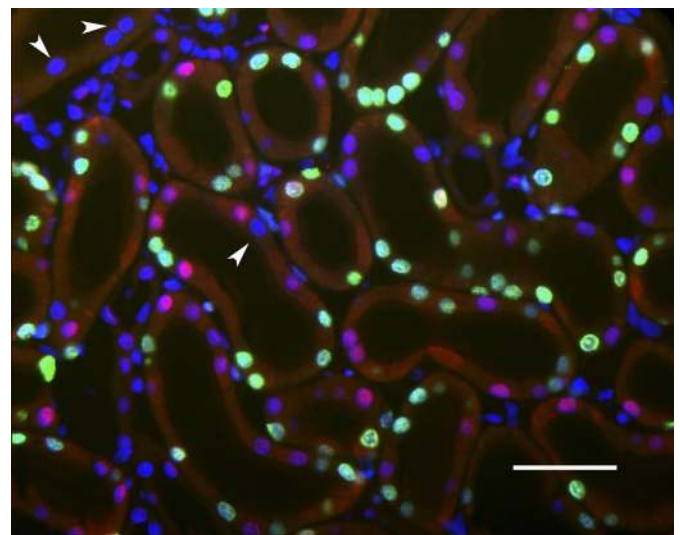
Fig. 1. Immunolabeling of bromodeoxyuridine (BrdU) and cyclin D1 in the outer stripe after 2 wk of BrdU administration. Green, BrdU; red, cyclin D1; blue, DAPI. BrdU labels cells that divided during the 2-wk application period. Cyclin D1 identifies cells in the G1-phase of cell cycle. Only very few cells in the proximal tubule are negative for BrdU and cyclin D1 (arrowheads indicate nuclei solely positive for DAPI, blue). Bar: 50 μm.

preceding euthanasia of the rats are less prone to enter cell cycle than cells that did not divide for a longer period of time, which is in agreement with the existence of a period of quiescence following cell division (26).

We found a much lower proliferation rate in S1 and S2 when compared with S3. Here $11.6 \pm 2.8\%$ of the tubular epithelial cells were positive for BrdU only, $17.9 \pm 4.4\%$ for cyclin D1 only, and $1.4 \pm 0.5\%$ double positive for BrdU and cyclin D1, and $69.1 \pm 6.0\%$ were negative for both markers. Thus, during the investigated time period, 4.5 times more novel epithelial cells (BrdU+) originated in S3 compared with that with S1 and S2. With only twice as many cells positive for cyclin D1 in S3, the time for a cell to go through G1 appears to be more than twice as long in S1 and S2 compared with S3.

Capacity to React to a Proliferative Stimulus is Age Independent (Expt. 2)

The cycling marker Ki-67 (labeling phases S, G_2, and M) revealed a much higher proliferation rate in the S3 segment in growing rats (Fig. 2B, $4.8 \pm 0.3\%$) compared with adult rats (Fig. 2D, $0.4 \pm 0.1\%$, $P < 0.05$). The incidence of cyclin D1-positive cells in S3 on the other hand was similar in both growing (Fig. 2B) and adult (Fig. 2D) rats: $41.7 \pm 8.1\%$ and $42.9 \pm 1.5\%$, respectively.

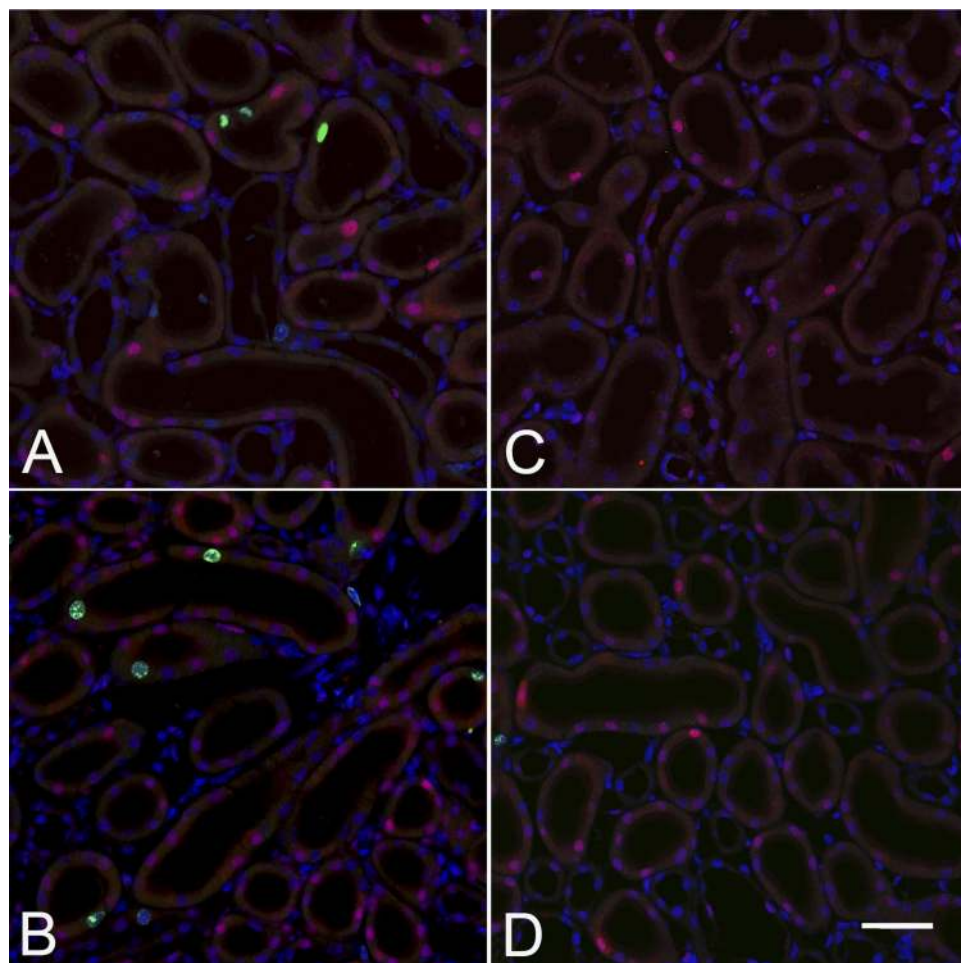Thirty six hours after proliferation induction by lead acetate, the proportion of Ki-67-positive cells was $26.1 \pm 5.3\%$ in juvenile (Fig. 3B) and $31.9 \pm 1.3\%$ in adult (Fig. 3D) rats, indicating that the capacity to react to a proliferative stimulus is age independent. At the same time, cyclin D1-positive cells were reduced to $19.3 \pm 5.5\%$ in growing (Fig. 3B) and $21.3 \pm 2.0\%$ in adult (Fig. 3D) rats ($P < 0.05$, compared with untreated). This suggests that the cyclin D1-positive cells can rapidly progress in the cell cycle when needed.

There was a comparable picture in proximal epithelial cells in the cortical labyrinth (containing segments S1 and S2). Also there is a similar amount of cyclin D1-positive cells in growing (Fig. 2A) and adult (Fig. 2C) rats ($23.8 \pm 1.9\%$ and $25.3 \pm 2.4\%$, respectively) but strongly diverging numbers of Ki-67 positive cells [$1.8 \pm 0.5\%$ in growing (Fig. 2A) and $0.17 \pm 0.04\%$ in adult (Fig. 2C) rats ($P < 0.05$)]. Thirty six hours after lead acetate treatment, a similar amount of Ki-67-positive cells in growing (Fig. 3A) and adult (Fig. 3C) rats was observed [$3.8 \pm 0.7\%$ and $2.8 \pm 0.7\%$, respectively ($P < 0.05$, compared with untreated)]. The incidence of cyclin D1-positive cells was reduced to $20.5 \pm 1.8\%$ in growing (Fig. 3A) and $21.9 \pm 1.5\%$ in adult (Fig. 3C) rats.

There was no obvious induction of cell proliferation by lead acetate in distal segments of the nephron or in nontubular structures of the kidney.

Since the cycling rate, as indicated by Ki-67, is more than 10 times higher in the whole proximal tubule of growing compared with adult animals, whereas the amount of cyclin D1-

Fig. 2. Immunolabeling of Ki-67 and cyclin D1 in the outer stripe and cortical labyrinth of growing (B and A, respectively) and adult (D and C, respectively) rats under control conditions. Green, Ki-67; red, cyclin D1; blue, DAPI. Ki-67 expression is higher in growing compared with adult rats in both the outer stripe and cortical labyrinth. Cyclin D1 expression is similar in both growing and adult rats. Bar: 50 µm.
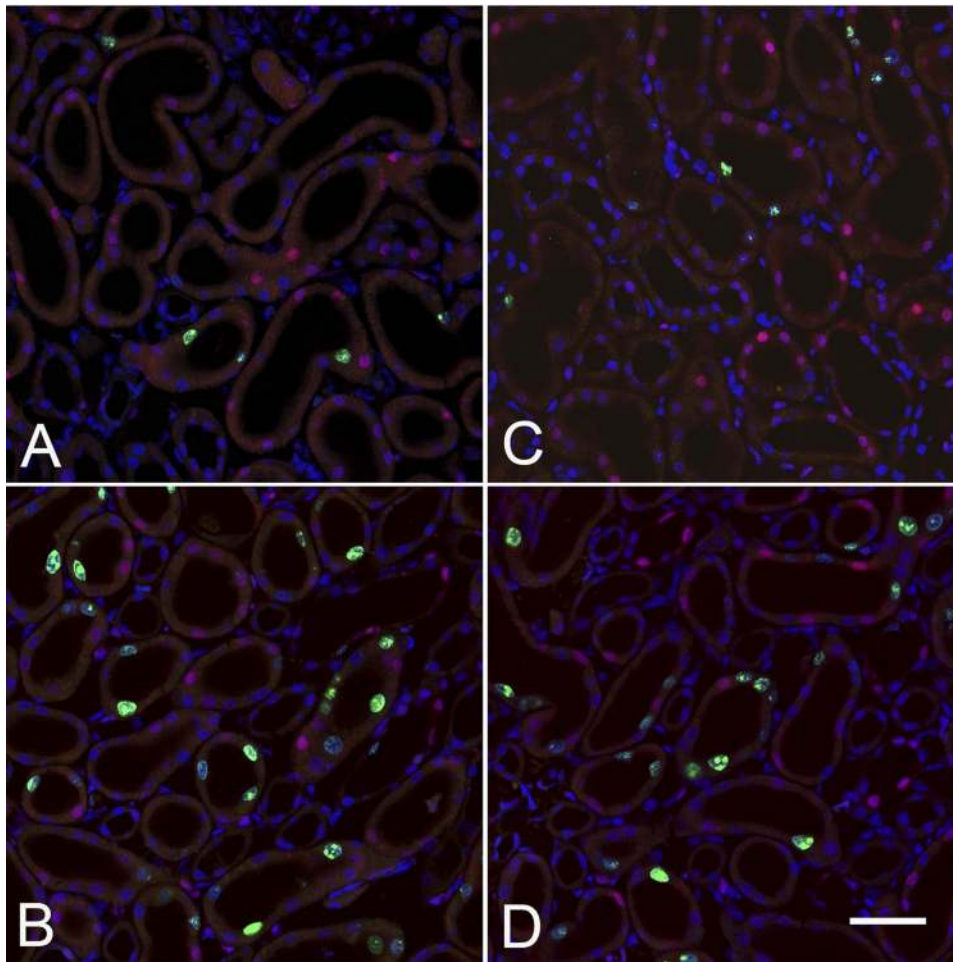
Fig. 3. Immunolabeling of Ki-67 and cyclin D1 in the outer stripe and cortical labyrinth of growing (*B* and *A*, respectively) and adult (*D* and *C*, respectively) rats after proliferation induction with lead acetate. Green, Ki-67; red, cyclin D1; blue, DAPI. Bar: 50 μm.

positive cells on the other hand is similar, the time span a cell resides in the G_1 phase is much longer in grownup than in growing rats.

Cycle Progression in Proximal Tubular Epithelial Cells is Regulated by p27^{kip-1} (Expt. 2)

The immunoreactivity of p27^{kip-1} and phosphorylation state of retinoblastoma (Rb) was investigated in growing and adult rats that received either lead acetate or isotonic saline solution. The employed antibody against hyperphosphorylated Rb specifically detects the cyclin-dependent kinase 4 (CDK4)-dependent phosphorylation at serine-608. CDK4 forms a complex with cyclin D1, which inactivates Rb by hyperphosphorylation leading to a degradation of cycling inhibitors like p27^{kip-1} and thus progression in the cell cycle (3, 6). In the control adult rat kidney, which displays a very low cycling rate, practically all epithelial cells strongly expressed p27^{kip-1} (Fig. 4*A*). After proliferation induction by lead acetate, levels of p27^{kip-1} were strongly reduced in a large fraction of tubular epithelial cells with a simultaneous hyperphosphorylation of Rb (Fig. 4*B*).

In untreated growing rats, hyperphosphorylated Rb-positive, p27^{kip-1}-negative nuclei were more abundant than in untreated adult rats (not shown), reflecting the higher rate of cell proliferation (26). After treatment with lead acetate, a comparable picture was observed in growing rats as in adult rats.

After Division, Proximal Epithelial Cells go Through a Period of Quiescence, Which can be Shortened by a Mitotic Stimulus (Expt. 3)

After division proximal tubular cells of growing rats are quiescent for more than 1 wk under physiological conditions (26). To test whether a strong mitotic stimulus could induce a cycle reentry shortly after division, we administered BrdU twice daily on *day 1* and *2*. Twenty-four hours after the last BrdU application, one group received lead acetate and the other received a corresponding volume of isotonic saline solution. Animals of both groups were perfusion fixed 36 h later on *day 5*. In S3 $6.7 \pm 0.8\%$ and $5.6 \pm 0.1\%$ of the cells were positive for BrdU in the control and lead acetate-treated group, respectively. In the control group, $4.4 \pm 1.4\%$ of nuclei were positive for Ki-67. In the lead acetate-treated animals $28.4 \pm 1.9\%$ of the overall cell population but only $1.7 \pm 1.1\%$ of the BrdU-positive cells were positive for Ki-67 (Fig. 5). Thus a notable level of resistance to reenter the cell cycle after division is evident even after application of a strong mitotic stimulus.

Because cyclin D1 is an earlier indicator of cycling than Ki-67, we examined the colocalization of BrdU with cyclin D1 in both control and lead acetate-treated groups. The incidence of cyclin D_1 in the overall cell population was 43.3 ± 5.8 in

Fig. 4. Immunolabeling of p27kip-1 and phosphorylated retinoblastoma (Rb) in the outer stripe of adult rats under control conditions (*A*) and after proliferation induction with lead acetate (*B*). Green, phosphorylated Rb; red, p27kip-1; blue, DAPI. p27kip-1 expression is decreased and phosphorylated Rb increased after proliferation induction. Arrow indicating a cell at the transition from G_1 to the S-phase, positive for p27kip-1, and phosphorylated Rb. Bar: 50 μm.
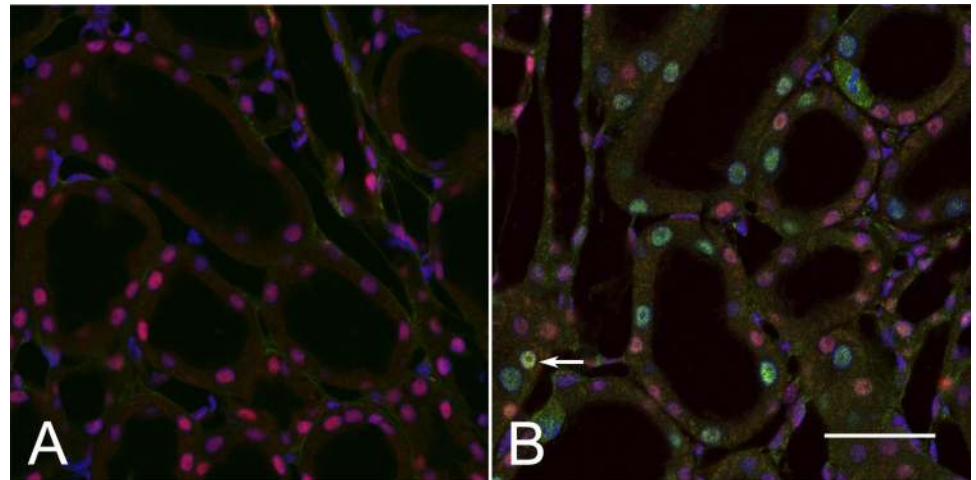
controls and 22.4 $\pm$ 4.2 in lead acetate-treated rats. In the BrdU-positive population 4.9 $\pm$ 3.2% and 14.4 $\pm$ 2.2% ($P <$ 0.05) of the cells were positive for cyclin D1 in the control and lead acetate-treated animals, respectively, indicating that the time span to reenter the G_1-phase after division can be reduced by a strong mitotic stimulus.

Expression of Cyclin D1 in Tubular Cells of Adult Human Kidney

In healthy human adult kidneys, cells were frequently positive for cyclin D1 in varying intensities (Fig. 6), which indicates that a reserve pool for rapid division could also be of relevance in humans. However, a word of caution needs to be added. Even though the selected biopsies had been classified as healthy by the pathologist, undetected pathological processes might have induced an abnormal rate of proliferation.

DISCUSSION

The segment S3 of the proximal tubule shows a high susceptibility to acute tubular necrosis (13, 14). This appears to be closely linked to some of the functions of this segment that include the transport and metabolization of xenobiotics (8, 11, 23, 24). Injury to this region is followed by a high proliferative activity to replace lost cells (8, 17). Also during renal growth in juvenile rats this segment displays the highest proliferative capacity of all nephron segments (26). The ability to proliferate is thus retained in S3 throughout life, making this segment a good candidate to investigate the origin of novel tubular cells.
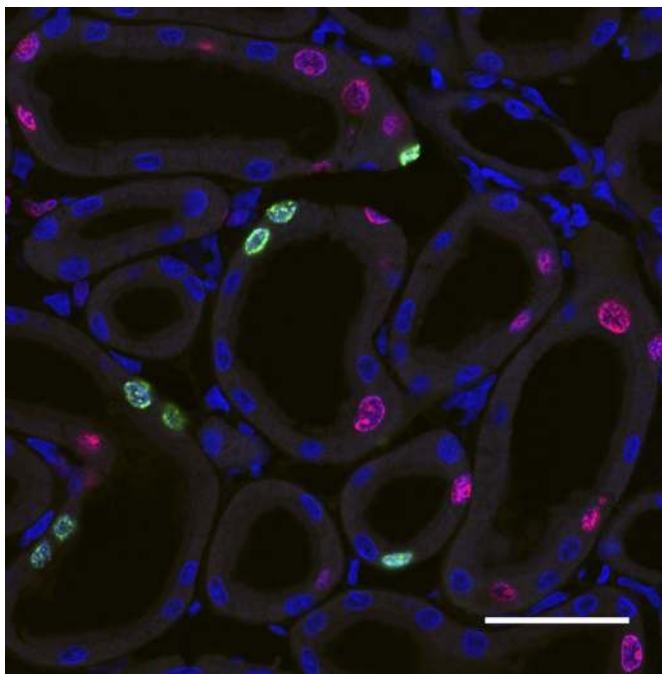
Fig. 5. Immunolabeling of BrdU and Ki-67 in the outer stripe after 2 days of BrdU application followed by lead acetate injection. Green, BrdU, red, Ki-67 and blue DAPI. No cells double positive for BrdU and Ki-67 (cells reentering cell cycle after BrdU incorporation) are seen on this micrograph. Bar: 50 μm.

Fig. 6. Detection of cyclinD1-positive cells in human control biopsies of kidney transplanted patients. Immunoperoxidase labeling of cyclin D1, counterstained with haematoxylin. Arrowheads indicating some nuclei positive for cyclin D1. Bar: 50 μm.

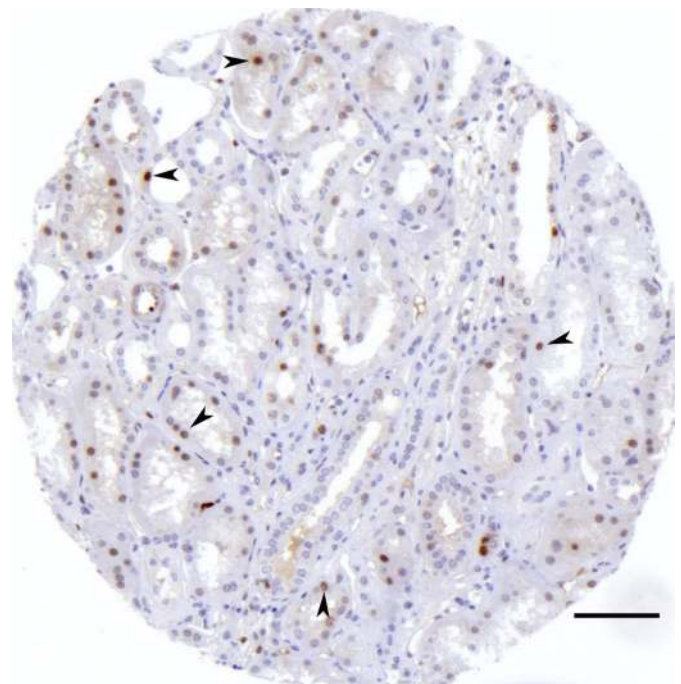In *experiment 1* a majority of cells in the S3 segment of growing rats either divided (BrdU+) or entered the cycle (cyclin D1) within 2 wk and only 11% of cells remained negative for BrdU and cyclin D1. Therefore, the proliferation potential is distributed in a large proportion, possibly the total of cells in S3. In the cell population that did not divide during 2 wk (BrdU negative) 71% of the cells were cyclin D1 positive, compared with 17% in the BrdU-positive population, indicating that the tendency to enter G_1 increased with time following division. This likely reflects the obligatory period of quiescence (G_0) following division, which was found with a different experimental protocol in a previous study (26).

The cycling behavior is quantitatively different in the cortical labyrinth, comprising the segments S1 and S2 of the proximal tubule, compared with S3. When compared with *experiment 1*, the fraction of cells in G_1 (cyclin D1 positive) and the fraction of cells that divided within 2 wk (BrdU positive), it appeared that the time period to pass through the G_1-phase of cycling is more than twice as long in S1 and S2 than in S3. The data above show that a very high proportion of proximal tubular cells are in G_1 and that there is no constant relationship between the actual rate of cell division and the fraction of cells in G_1. Thus entry in G1 does not seem to serve immediate progression through the cell cycle. This notion is further supported by comparison of growing and adult rats. Cyclin D1 expression was detected in roughly 40% of tubular cells in S3 in both groups, whereas the cycling rate in adult rats was less than a tenth of that during growth. It thus appears that, independently of the actual proliferation rate, a large pool of cells in G_1 represents a reserve that stays ready to undergo division if proliferation is urgently required. To test this hypothesis, in *experiment 2* we subjected both adult and growing rats to a strong proliferative stimulus by injecting lead acetate (2). This lead to a similar proliferative response in both groups, with about 30% of cells progressing in the cell cycle and becoming Ki-67 positive, a marker of the S, G_2, and M-phase. Concurrently, we saw a depletion of the cyclin D1-positive cell pool in both groups, suggesting that the cells progressed from that pool through the cycle with a delayed replenishment of the G_1 reserve.

If a large reserve pool of cells in G_1 is a vital protective mechanism, one would expect to see an acceleration of the replenishment of that pool after a period of stimulated mitosis. That hypothesis was tested in *experiment 3*. We applied BrdU on *days 1* and *2* and lead acetate on *day 3*. On *day 5* the incidence of cyclin D1 in the BrdU-positive population was roughly three times higher in the lead acetate group than that in the control group. This suggests that the refractory period, in which cells do not reenter G_1 after division, can be shortened by a strong proliferative stimulus.

The presence of roughly 40% of cells of the segment S3 are in G_1 implicates a potent control of the G_1-S progression. The cyclin-dependent kinase inhibitor p27, which blocks cycle progression at that site, was actually abundant in virtually all nuclei of S3 in adult rats. After induction of proliferation with lead acetate, a large fraction of nuclei displayed no abundance or only low abundance of p27. Those nuclei were positive for hyperphosphorylated Rb, which was hardly detectable in untreated adult rats. Thus it is likely that under normal conditions in adult rats hypophoshorylated Rb inhibits G_1-S progression

by stabilizing p27. The blockade can be released by hyperphosphorylation of Rb, leading to degradation of p27 (3, 6).

We propose the following model for cell proliferation in the S3 segment. All tubular cells can enter the cell cycle. After division the daughter cells are quiescent for at least a few days under physiological conditions, but they eventually reenter the cell cycle. The reentry into the cell cycle can be accelerated by a strong mitotic stimulus. A large fraction of cells is in the G_1-phase of cell cycle, even in adult rats, ensuring a rapid proliferative response when needed.

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