Involvement of Renal Progenitor Tubular Cells in Epithelial-to-Mesenchymal Transition in Fibrotic Rat Kidneys

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Renal progenitor tubular cells (label-retaining cells [LRC]) were recently identified in normal kidneys by in vivo bromodeoxyxuridine (BrDU) labeling. This study was conducted to examine the behavior of LRC in renal fibrosis. BrDU was injected intraperitoneally into normal rats daily for 7 d. After a 2-wk chase period, unilateral ureteral obstruction (UUO) was induced in these rats. In normal and contralateral kidneys, LRC were observed scattering among tubular epithelial cells. After UUO, the number of the LRC significantly increased, and most of them were positive for proliferating cell nuclear antigen (PCNA). In contrast, PCNA⁺ cells lacking BrDU label were rarely observed. It is interesting that LRC were detected not only in tubules but also in the interstitium after UUO. Laminin staining showed that a number of the LRC were adjacent to the destroyed tubular basement membrane. Some tubules, including LRC, lost the expression of E-cadherin after UUO. A large number of cell populations expressed vimentin, heat shock protein 47, or α-smooth muscle actin in the UUO kidneys, and each population contained LRC. None of the LRC was positive for these fibroblastic markers in contralateral kidneys. When renal tubules from BrDU-treated rats were cultured in the gel, some cells protruded from the periphery of the tubules and migrated into the gel. Most of these cells were BrDU⁺. Neither the total content of BrDU in the kidneys nor the number of LRC in bone marrow significantly changed after UUO. Collectively, these results suggest that LRC is a cell population that proliferates, migrates, and transdifferentiates into fibroblast-like cells during renal fibrosis.

tively contribute to tubular regeneration, are also involved in fibrotic processes of the kidney.

Materials and Methods

In Vivo BrdU Labeling

Male Wistar rats that weighed 200 g each were obtained from Nihon SLC, Inc. (Hamamatsu, Japan). LRC were detected by BrdU labeling as described previously (13). Briefly, BrdU (100 mg/kg), an analogue of thymidine, was injected intraperitoneally into normal rats daily for 1 wk. After a 2-wk chase period, the rats were killed, and the kidneys were removed, fixed with 10% formaldehyde, and embedded in paraffin. LRC were detected with a cell proliferation kit (Amersham, Tokyo, Japan) according to the manufacturer’s instructions and counterstained with periodic acid-Schiff or hematoxylin.

UUO

UUO was performed as described previously (14). Briefly, after induction of general anesthesia by intraperitoneal injection of pentobarbital (50 mg/kg body wt), the abdominal cavity was exposed via a midline incision and the left ureter was ligated at two points with 4-0 silk. At the indicated times after UUO, the rats were killed and the kidneys were removed and used for histologic examination. Ureteral obstruction was confirmed by observation of dilation of the pelvis and proximal ureter and collapse of the distal ureter. Contralateral kidneys were used as controls. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Gunma University.

Quantification of BrdU- and/or Proliferating Cell Nuclear Antigen–Positive Cells

Quantitative analysis of BrdU+ cells was performed by counting the positive nuclei in the tubular area or in the interstitial area from five randomly selected fields of the outer medulla under a light microscope at ×400 magnification. The average of the five determinants was calculated. The number of BrdU+ tubular cells was quantified and expressed as a percentage of total tubular cells per field. The number of BrdU+ interstitial cells was also quantified and expressed as the number of BrdU+ cells per field.

Quantification of the number of BrdU+/proliferating cell nuclear antigen–positive (PCNA+), BrdU+/PCNA+, and BrdU+/PCNA+ cells was performed by counting single-positive or double-positive nuclei from five randomly selected fields of the kidneys under a fluorescence microscope at ×400. The average of five determinants was calculated and expressed as a percentage of total nuclei (4'-diamidino-2-phenylindole [DAPI]-positive nuclei) per field.

Indirect Fluorescence Immunohistochemistry

The paraffin-embedded sections (4 μm) were deparaffinized, rehydrated in a routine manner, and incubated at 4°C for overnight with primary antibodies. After washing with PBS, sections were incubated with fluorescein-labeled secondary antibodies and DAPI (Boehringer Mannheim, Mannheim, Germany) at room temperature for 1 h.

Frozen sections (8 μm) embedded with Tissue-Tek OCT compound (Miles Inc, Elkhart, IN) were also used. Sections were fixed with 4% paraformaldehyde and incubated at room temperature for 1 h with primary antibodies. After washing with PBS, sections were incubated with fluorescein-labeled secondary antibodies and DAPI at room temperature for 1 h. Immunofluorescent images were recorded as described previously (14). Antibodies used in this study were as follows: Rat monoclonal anti-BrdU antibody (Abcam, Cambridge, UK), goat anti-human PCNA antibody (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit polyclonal anti-laminin antibody (Progen, Biotecnik GmbH, Heidelberg, Germany), mouse monoclonal anti-vimentin antibody (NeoMarkers, Fremont, CA), rabbit polyclonal anti-HSP-47 antibody (Stressgen, Victoria, Canada), mouse monoclonal anti-α-SMA antibody (Progen Biotecnik GmbH, Heidelberg, Germany), rabbit polyclonal anti-matrix metalloproteinase-2 (MMP-2) antibody (Chemicon, Temecula, CA), mouse monoclonal anti-MMP-9 antibody (DFK, Toyama, Japan), and mouse monoclonal anti-E-cadherin antibody (BD Transduction Laboratories, Franklin Lakes, NJ).

Isolation of Renal Tubules and Collagen Gel Culture

The kidneys were removed from the normal rats labeled with BrdU as described above. The cortex and outer medulla were separated, minced finely with scissors, and treated with 0.2% collagenase for 30 min at 37°C. Renal tubules were isolated manually under a stereomicroscope, suspended in a neutralized collagen solution (Koken, Tokyo, Japan) in 12-well plates, and incubated at 37°C. After the collagen solution gelled, DMEM supplemented with 5% FBS and antibiotics (penicillin and streptomycin) was added and gels were maintained under humidified conditions of 95% air and 5% CO2 at 37°C. The culture medium was changed every 2 to 3 d. Cultures were photographed at the indicated times under phase contrast using a Nikon Diaphot TMD inverted microscope (Tokyo, Japan).

Whole-Mount Immunostaining

BrdU+ cells in the collagen gel were detected by whole-mount immunostaining using a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA). Briefly, collagen gel cultures were fixed in 4% paraformaldehyde, washed in Tris-buffered saline (TBS) that contained 1 mM CaCl2 (pH 7.6; TBS-Ca), and then fixed in cold 100% methanol for 20 min at −20°C. After incubation in 2% BSA-TBS-Ca for 1 h at 4°C, cultures were reacted with mouse anti-BrdU antibody at 4°C overnight. After 8 h of washing in TBS-Ca, cultures were incubated with peroxidase-labeled anti-mouse IgG antibody at 4°C overnight. Cultures were washed in TBS-Ca and then reacted with Vectastain Elite ABC Reagent. Antibody was detected with diaminobenzidine in PBS and photographed under a phase-contrast microscope. Quantification of the number of migrating BrdU+ cells migrating in collagen gel was performed by counting the number of BrdU+ cells and total number of migrating cells from tubules into gels per each well.

Measurement of Total BrdU Content in the Whole Kidneys

Total BrdU content in the UUO kidneys was measured using Cell Proliferation Biotrak ELISA System, version 2 (Amersham Biosciences, Piscataway, NJ), according to the manufacturer’s instructions. Briefly, kidneys were removed from BrdU-treated rats with or without UUO, minced finely with scissors, and treated with 0.6% collagenase for 30 min at 37°C. After passing through a 90-μm mesh for removing contaminating glomeruli, digests were treated with trypsin for 30 min at 37°C to obtain single cell suspensions. Cells (2 × 105 cells/well) were attached to 96-well ELISA plate (Immulon 4 HBX, ThermoLabsystems, Franklin, MA) by centrifugation at 300 × g for 10 min. After supernatant was removed, cells were fixed, blocked with containing reagents, and incubated with peroxidase-labeled anti-BrdU antibody for 90 min at room temperature, followed by reaction with 3,3',5,5'-tetramethylbenzene substrate. Optical density was measured by Wallac 1420 ARVO SX (Wallac, Turku, Finland) at 450 nm. As negative controls, we also measured total BrdU content in the whole kidneys of BrdU-untreated rats. Values were shown as adjusted absorbance, in which absorbance of negative control was subtracted.
Detection of LRC in Bone Marrow

Normal rats were labeled with BrdU as described above and subjected to UUO. Femurs and tibias were removed and split in two by pliers, and bone marrow cells were gathered. Bone marrow cells were put on a filter paper, fixed in 10% formaldehyde, and embedded in paraffin. LRC were detected as described above. Quantification of the number of LRC in bone marrow was performed by counting BrdU+ nuclei from five randomly selected fields under a light microscope at ×400 magnification. The average of five determinants was calculated and was recorded as a percentage of total cells.

Statistical Analyses

The differences between means were compared by t test, with \( P < 0.05 \) considered significant.

Results

Existence of LRC in Interstitium in Kidneys after UUO

We examined the localization of LRC in fibrotic kidneys with UUO. BrdU was injected into normal rats daily for 7 d. After a 2-wk chase, UUO was generated in these rats. In normal kidneys, LRC were scattered among the tubular epithelial cells (Figure 1A). Consistent with previous study (13), none of the LRC was present in the glomeruli, capillary endothelial cells, and interstitium (data not shown). At 1 d after UUO, most of the LRC were still localized in the tubules (Figure 1B). It is interesting that some LRC were detected in the interstitium, and many pairs of LRC were observed in the UUO kidneys at 3 d and thereafter (Figure 1, C through E). None of the LRC was detected in the interstitium in contralateral kidneys (Figure 1F).

Increase in Number of LRC in Kidneys after UUO

We next examined the changes in the number of LRC in the kidneys after UUO. A large number of LRC were detected after the UUO operation (Figure 2A, b and c) compared with those in normal (Figure 2A, a) and contralateral kidneys (Figure 2A, d). Quantitative analysis showed that the number of tubular LRC was significantly increased at 3, 5, and 7 d after UUO (Figure 2B). In contrast, the number of tubular LRC in contralateral kidneys was not changed.

However, the number of LRC detected in the interstitium was significantly increased in the UUO kidneys (Figure 2C).

Co-localization of BrdU and PCNA in Kidneys after UUO

To examine further the localization of proliferating cells in the kidneys after UUO, we performed immunostaining for PCNA, which specifically recognizes the early G1 and S phases of the cell cycle. Some LRC were co-localized with PCNA+ cells in the kidneys at 5 d after UUO (Figure 3A). Quantitative analysis showed that approximately half of the LRC were PCNA+. In contrast, PCNA+ cells lacking BrdU label were rarely observed (Figure 3B). PCNA+ cells were not detected in normal and contralateral kidneys (data not shown), raising the possibility that most of the proliferating cells in the UUO kidneys were essentially derived from LRC.

Destruction of TBM in Kidneys after UUO

We next performed immunostaining for laminin, which is one of the components of TBM. In contralateral kidneys, the TBM was preserved throughout the experiments (Figure 4, A and B). In contrast, loss of laminin expression was observed in some tubules of the kidneys after UUO, indicating the destruction of TBM, and some of the LRC were adjacent to the destroyed TBM (Figure 4, C and D).

The expression of E-cadherin, one of the adhesion molecules that play the essential role in maintaining the structural integrity of renal epithelia and its polarization (4), was investigated. As shown in Figure 5, A and B, renal tubular epithelial cells including LRC express E-cadherin in normal kidney. In contrast, the expression of E-cadherin was decreased in several tubules including LRC in the UUO kidneys (Figure 5, C and D).

We also examined the expressions of MMP in the UUO kidneys. Neither MMP-2 nor MMP-9 was expressed in normal and contralateral kidneys. In contrast, MMP-2 was detected in tubular epithelial cells after UUO; MMP-9 expression was up-regulated in the interstitial cells in the UUO kidneys. However, none of the LRC expressed MMP-2 or MMP-9 after UUO (data not shown).
Expressions of Myofibroblast-Related Markers in Kidneys after UUO

We examined the expressions of myofibroblast-related markers in the kidneys after UUO by immunostaining. Some of the tubular cells (Figure 6, A and B) and interstitial cells (Figure 6, C and D) expressed a mesenchymal marker, vimentin, in the UUO kidneys. These vimentin-expressing cells contained LRC. The expression of HSP-47, a molecular chaperone bound to type I collagen, was detected in both the tubules (Figure 6, E and F) and the interstitium (Figure 6, G and H) in the UUO kidneys. Most of these HSP-47–positive cells also contained LRC. Some interstitial cells, not tubular cells, expressed α-SMA, a marker of myofibroblasts, in the kidneys after UUO, and a number of them were LRC (Figure 6, I and J). In normal and contralateral kidneys, none of the tubular cells, including LRC, expressed vimentin, HSP-47, or α-SMA (data not shown).

Potential of Migration in LRC

For examining the nature of LRC, renal tubules that were derived from normal rats that were labeled with BrdU for 7 d followed by a 2-wk chase were cultured in collagen gel. Five days later, collagen gel cultures were fixed in 4% paraformaldehyde and the localization of LRC was examined by whole-mount immunostaining. Most of the BrdU+ cells were localized within the renal tubules (data not shown). However, there is a number of cells protruded from the edge of the renal tubules (Figure 7A, a through d) or migrated into the collagen gel (Figure 7A, e and f). Importantly, most of these cells were BrdU+ (Figure 7B).
Total BrdU Content in Whole Kidney and Bone Marrow of Normal and UUO Kidneys

It is reported that a part of interstitial fibroblasts comes from bone marrow in fibrotic kidneys (5), raising the possibility that BrdU$^+$ interstitial cells are derived from bone marrow. To test this idea, we examined total BrdU content in the whole kidneys by ELISA. As shown in Figure 8A, there was no significant difference in the BrdU content between normal and UUO-operated kidneys. The BrdU content in bone marrow was also investigated by immunohistochemistry. As shown in Figure 8B, after a 2-wk chase period, BrdU$^+$ cells were detected in bone marrow of rats that were treated with BrdU for 7 d, indicating the presence of LRC in bone marrow. However, there was no significant difference in the number of BrdU$^+$ cells in bone marrow between normal and UUO-operated rats. Therefore, it is unlikely that bone marrow LRC migrated into the interstitium of the kidneys after UUO.

Discussion

In this study, we demonstrated that LRC, which were normally present in renal tubules of normal kidneys, were detected in the interstitium of the UUO kidneys (Figures 1 and 2). Approximately half of the LRC became positive for PCNA after UUO (Figure 3). Some LRC were localized adjacent to the destroyed TBM (Figure 4). After UUO, LRC lost the expression of E-cadherin (Figure 5) and expressed myofibroblast-related markers such as vimentin, HSP-47, and α-SMA (Figure 6). In vitro collagen gel culture demonstrated the potential of LRC to migrate into the gel (Figure 7). There was no significant difference in total BrdU content in the whole kidneys or in bone marrow between normal and UUO-operated rats (Figure 8), supporting the idea that interstitial BrdU$^+$ cells in the UUO kidneys are derived from tubular LRC. Taken together, these results strongly suggest that LRC are a certain tubular population that proliferate, migrate, and transdifferentiate into fibroblast-like cells during fibrosis.

The origin of interstitial fibroblasts in fibrotic kidneys is considered to be heterogeneous (6). They are composed of resident fibroblasts, tubular epithelial cells undergoing EMT, and bone marrow–derived cells (3–5,15). It is reported that 15% of interstitial fibroblasts after UUO are derived from bone marrow (5), which raised the possibility that interstitial BrdU$^+$ cells were derived from bone marrow in this study. To address this issue, we quantified the amount of BrdU in the whole kidney as well as in bone marrow before and after UUO (Figure 8). These results demonstrated that LRC exist not only in the

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**Figure 4.** Destruction of tubular basement membrane (TBM) in the kidneys after UUO. Localization of BrdU and laminin in the kidneys at 7 d after UUO was examined by indirect fluorescence staining. (A and B) Contralateral kidney 7 d. (C and D) UUO kidneys 7 d. (A and C) BrdU (red), nuclei (blue), laminin (green). (B and D) Nomarski images. Arrowheads indicate BrdU$^+$ cells. Magnification, ×1000.

**Figure 5.** Expression of E-cadherin in normal and UUO kidneys. Expression of E-cadherin in normal kidney (A and B) and the UUO kidneys at 7 d (C and D) was examined by indirect fluorescence staining. BrdU (red), nuclei (blue), E-cadherin (green). (B and D) Nomarski images. Magnification, ×1000. Arrowheads indicate BrdU$^+$ cells.
kidney but also in bone marrow. However, the number of bone marrow LRC did not decrease after UUO (Figure 8). Furthermore, no significant increase in the BrdU content of the whole kidney was observed after UUO (Figure 8), suggesting no infiltration of BrdU⁺ cells from other tissues (including bone marrow) into the kidney after UUO. Therefore, it is likely that interstitial BrdU⁺ cells in the UUO kidneys are intrinsic renal cells. Considering that there were no BrdU⁺ cells in the interstitium of normal kidneys, interstitial BrdU⁺ cells in the UUO kidneys should be different from resident fibroblasts. Collectively, it is possible that interstitial BrdU⁺ cells are derived from tubular LRC undergoing EMT.

A previous study reported that 36% of interstitial fibroblasts were EMT derived during fibrogenesis (5). We counted the

Figure 6. Expressions of myofibroblast-related markers in LRC in the kidneys after UUO. Localizations of BrdU and vimentin (A and C), heat shock protein (HSP-47; E and G), and α-smooth muscle actin (α-SMA; I) in the kidneys at 7 d after UUO were examined by indirect fluorescence immunostaining. (A, B, E, and F) Tubules. (C, D, G, H, I, and J) Interstitium. (B, D, F, H, and J) Nomarski images. BrdU (red), nuclei (blue), markers (green). Arrowheads indicate BrdU⁺ cells expressing myofibroblast-related markers. Magnification, ×1000.
number of BrdU-positive cells in the interstitium of the UUO kidneys and found that approximately 2% of interstitial vimentin-positive cells were positive for BrdU (Yamashita et al., unpublished observation). This difference might be due to the difference of fibroblast marker used (vimentin versus FSP1) or species (rat versus mouse) or might be caused by some limitations of this BrdU labeling method. One week might be insufficient to label all tubular LRC. In addition, it is difficult to trace all descendents from LRC, because incorporated BrdU label should be diluted out and become undetectable after multiple cell divisions. It is possible that the number of LRC is underestimated in this study. Nonetheless, considering that the number of LRC transiently increased in the kidneys after UUO (Figure 2) and that most of the PCNA-positive cells were co-localized with LRC at the early phase after UUO (Figure 3), it is possible that neighboring cells around the LRC are descendents of the LRC. Most of the interstitial LRC and their neighboring cells expressed vimentin, HSP-47, or -SMA in the UUO kidneys (Figure 6). In contrast, no LRC expressed these fibroblastic markers in normal and contralateral kidneys. Therefore, it is likely that LRC acquired a fibroblastic phenotype and produced a large number of fibroblastic descendents after UUO, although it cannot be denied that tubular cells other than LRC migrated into the interstitium after UUO.

TBM prevents contact between tubular epithelial cells and interstitial matrix components and plays a pivotal role in preserving the tubular epithelial phenotype. Previous ultrastructural analysis clearly demonstrated that destruction of TBM occurred in the developing process of renal fibrosis (16). In the early stage of EMT, tubular epithelial cells were localized parallel to the partially disrupted TBM and migrated into the

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**Figure 7.** Potential of LRC to migrate into collagen gel. (A) BrdU was injected intraperitoneally into normal rats once a day for 7 d. After a 2-wk chase period, kidneys were removed. Isolated tubules were cultured in collagen gel with DMEM that contained 5% FBS. Five days later, cultures were fixed in 4% paraformaldehyde and BrdU-positive cells were detected by whole-mount immunostaining as described in the Materials and Methods section. (a through c) Identical sample images with different focus. Arrowheads indicate BrdU-positive cells. (B) Quantitative analysis of the number of migrating BrdU-positive cells. The numbers of migrating cells as well as BrdU-positive cells per each well (containing 30 to 40 tubules) were counted (n = 3). Magnification, ×600 in A.

**Figure 8.** Total BrdU content in the whole kidneys and bone marrow of normal and UUO-treated rats. After BrdU labeling for 7 d followed by a 2-wk chase period, rats were subjected to UUO, and both kidneys and bone marrow were removed at the indicated periods. (A) Total BrdU content in normal and UUO kidneys were measured by ELISA. Values are means ± SE (n = 4). (B) BrdU-positive cells in bone marrow at days 0 (a) and 5 (b) after UUO were examined by immunohistochemistry. (C) Quantitative analysis of the number of BrdU-positive cells in bone marrow. Values are means ± SE (n = 4) and were shown as a percentage of total cells. Magnification, ×1000 in B.
peritubular interstitium through the extensively damaged TBM in the late stage of EMT (16). In this UUO model, the loss of laminin expression, a component of basement membrane, was observed, indicating the presence of TBM destruction in the UUO kidneys. Some of the LRC were adjacent to the destroyed TBM (Figure 4), raising the possibility that LRC produce proteases involved in TBM degradation, such as MMP-2 or MMP-9. These proteases specifically break down type IV collagen and laminin, the principal component of TBM. Consistent with previous studies (4,6), the expressions of MMP-2 and MMP-9 were upregulated in this UUO model. However, LRC expressed neither MMP-2 nor MMP-9 (data not shown). Proteases other than MMP-2 and MMP-9 might be involved in this drastic change of the microenvironment around the LRC.

In general, in vitro collagen gel culture system using renal epithelial cell line is used as a tubulogenesis assay. In this study, renal tubules from BrdU-treated rats were cultured in collagen gel. Given that EMT is influenced by changes in the underlying ECM substrate and type I collagen is one of the extrinsic factors inducing EMT (17), collagen gel culture system using isolated renal tubules may mimic, at least partially, in vivo fibrotic events. Some cells constituting renal tubules migrated into gels, and most of them were BrdU+ (Figure 7). When renal tubules were isolated, there were some interstitial fibroblasts attached to the tubules. However, it is unlikely that migrating BrdU+ cells were attached fibroblasts, because there were no BrdU+ cells in the interstitium of normal rat kidneys (Figure 1). In addition, although renal fibroblasts show spindle shape when cultured in collagen gel (Yamashita et al., unpublished observation), migrating BrdU+ cells did not show such morphology (Figure 7). Taken together, these findings suggest that tubular LRC have a higher potential to migrate compared with other tubular cells. Contact with type I collagen might be an important external cue for activation of LRC or initiation of their migration.

Selective blockade of EMT will be required to protect the kidney from fibrosis. LRC, which are localized in renal tubules of the normal kidneys, proliferated, migrated into the interstitium, and transdifferentiated into fibroblast-like cells in this UUO model, suggesting that LRC might be a main population undergoing EMT in diseased kidneys. Therefore, identification of factors or the signaling pathway that is involved in the mechanism of LRC transdifferentiation will potentially be of considerable importance for the treatment of fibrotic kidneys.

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