Age-related decline in label-retaining tubular cells: implication for reduced regenerative capacity after injury in the aging kidney

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The kidney has the capacity for nearly complete regeneration after ischemia-reperfusion or toxic injury (40). After renal ischemia, there is a marked increase in proliferation of tubular cells. Differentiated tubular cells are thought to dedifferentiate and proliferate in response to injury. Following a surge of cell proliferation, undifferentiated regenerating cells are considered to repopulate the damaged area and then redifferentiate into mature epithelial cells and reconstruct the functional integrity of the nephron. Through these steps, it is believed that most damaged tubules regain their essential functions and recover from damage (3).

It has been shown that a specific tubular cell population plays an important role in tubular regeneration after ischemic injury (19). Using the bromodeoxyuridine (Brdu)-labeling method, one of the most common approaches for identification of stem cells in adult tissues (9, 10), slow-cycling cells were detected. Slow-cycling cells are thought to go through the cell cycle infrequently to maintain a pool of cells for tissue turnover and repair. After 1 wk of BrdU labeling followed by a 2-wk chase period, slow-cycling cells were identified as label-retaining cells (LRCs) in renal tubules of normal rat kidneys. In the recovery phase after ischemic injury, LRCs divided into many daughter cells, which actively proliferated and appeared to differentiate into tubular epithelial cells, suggesting that LRCs were the origin of the regenerating cells after renal ischemia (19). In vitro analysis demonstrated that LRCs possess a phenotypic plasticity, tubulogenic capacity, and integration capability into the developing kidney (18). Collectively, LRCs appear to be renal progenitor tubular cells (16).

Aging causes structural and functional changes in human tissues. The systemic impact of the aging process often results in exposure to renal stressors such as nephrotoxic drugs, invasive interactions, and systemic diseases (43). The diminished ability of the aged kidney to repair coincides with a significantly increased susceptibility of the aged kidney to develop acute kidney injury (AKI) (6, 29). Experimental studies in rats indicated that the aged kidney is more likely to develop AKI after ischemic injury (22, 42). In humans, it is considered that kidneys from older organ donors have a higher risk of delayed graft function, and delayed or unsuccessful repair after transplantation-induced AKI (11, 26). According to recently published data, it is estimated that patients who are older than 65 yr carry a 28% higher risk for incomplete recovery of renal function after surviving an episode of AKI (30). Aging is thus strongly associated with both increased incidence and severity of acute renal failure. The current means of treating AKI is limited, and there are no specific strategies to adjust treatment options for the aging patient. A better understanding of how the aged kidney changes with regard to its susceptibility and response to injury is an indispensable step toward preventative strategies for AKI in the elderly population.

In the present study, the role of LRCs in the age-related decrease in the regeneration capacity of the kidney after renal ischemia was investigated. A significant decline in the proliferation burst of tubular cells after injury was found in addition to a decline in the number of LRCs with age. An age-related reduction of peritubular capillary area and a significant increase in the number of LRCs in renal tubules by endothelial cell-derived factor(s) were seen in vitro. The present data suggest that the peritubular capillary endothelium is a target for therapies that are aimed at increasing the LRC number in aged kidneys, decreasing susceptibility to injury.
METHODS

Ischemia-reperfusion injury. Male Wistar rats aged 7 wk, 7 mo, and 12 mo were obtained from Charles River Japan (Tokyo, Japan). Ischemia-reperfusion injury was performed as described previously (20). Briefly, under anesthesia with pentobarbital sodium (30 mg/kg body wt), renal ischemia was induced by clamping unilateral (left) renal arteries for indicated periods using a nontraumatic vascular clamp. At 24 h after removal of the clamp to allow reperfusion, rats were euthanized and the kidneys were removed for histological analysis. Sham operations were performed in a similar manner, except without clamping of the renal arteries. The care and use of animals described in this study conformed to the procedures of a protocol approved by the Ethics Review Committee for Animal Experimentation of Gunma University Graduate School of Medicine.

Detection of LRCs. LRCs were detected by BrdU labeling with a method described previously (19) with slight modification. Using ALZET osmotic pumps (DURECT, Cupertino, CA), BrdU (50 mg/kg·day⁻¹), an analog of thymidine, was intraperitoneally administered to rats for 7 days. For exclusion of rapidly cycling cells, kidneys were removed after a 2-wk chase period, fixed in 4% formaldehyde, and embedded in paraffin. At this dose, the animals appeared healthy with normal kidney histology during the entire course of the experiment. Four-micrometer sections were immunostained using a cell proliferation kit (GE Healthcare UK, Buckinghamshire, UK) or rat anti-BrdU antibody (ab6326, Abcam, Cambridge, MA) according to the manufacturer’s instructions. BrdU-positive cells were considered to be LRCs.

Measurement of injured area. Injured areas of the kidneys were microscopically examined using periodic acid-Schiff (PAS)-stained sections. The area of tubular dilation, flattened tubular epithelial cells, and cast formation in the outer medulla (where tubular damage was most obvious) were measured and expressed as the percentage of total area per selected fields. Five sections per rat were used for analysis. The data are presented as means ± SE (n = 6).

Serum analyses. Serum creatinine and blood urea nitrogen levels were assessed by a Hitachi 7180 autoanalyzer (Hitachi High-Technologies, Tokyo, Japan).

Measurement of DNA synthesis. DNA synthesis in renal tubular cells was assessed by BrdU incorporation as described previously (20).

Indirect fluorescence immunohistochemistry. For immunofluorescence analysis, 4-μm sections were deparaffinized, rehydrated, and autoclaved at 120°C for 15 min in 10 mM citric acid buffer to retrieve antigens. Sections were blocked for 15 min with a protein block (Dako Cytomation, Carpenteria, CA) and then incubated overnight at 4°C with primary antibodies as follows: goat anti-proliferating cell nuclear antigen (PCNA) antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and mouse anti-vimentin antibody (Lab Vision, Fremont, CA). After washing with PBS, sections were then incubated with secondary antibody. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI).

For rat endothelial cell antigen (RECA)-1 staining, kidneys were removed and embedded in a Tissue-Tek OCT compound (Miles, Elkhart, IN) and frozen in liquid nitrogen. Frozen sections were cut, mounted on poly-L-lysine-coated slides, and fixed in 4% PFA for 15 min at room temperature (RT). Sections were then washed in PBS, fixed with cold methanol for 30 min at −30°C, blocked for 1 h with 3% BSA-PBS, and covered with mouse anti-RECA-1 antibody (AbD Serotec, Oxford, UK) at RT for 1 h. After washing in PBS, the sections were covered with secondary antibody and DAPI at RT for 1 h. Images were recorded as described previously (17). For immunohistochemical controls, primary antibody was replaced with normal goat serum and did not show positive staining, thus confirming specificity.

Quantification of morphological data. Quantitative analysis of PCNA-positive cells was performed by counting positive nuclei in tubular cells from five randomly selected fields of the outer medulla at ×400 magnification. The average of the five counts was calculated and recorded as the number of PCNA-positive cells per square micrometer. The percent area positive for peritubular capillary staining by RECA-1 was determined using computer image analysis, and is expressed as the percentage of the total area per selected field. Data are expressed as the means ± SE.

Cell culture. Primary human umbilical vein endothelial cells (HUVEC; Clonetics, Walkersville, MD) were cultured in endothelial basal medium (HuMedia-EB2, Kurabo, Osaka, Japan) supplemented with HuMedia-EG (1 μg/ml hydrocortisone, 10 ng/ml hEGF, 5 ng/ml hFGF-B, 10 μg/ml heparin, 50 g/ml gentamycin, 50 ng/ml amphotericin B, and 2% FCS) in an atmosphere of 5% CO₂ and 100% humidity at 37°C. The culture medium was changed every 2 days.

Gel culture of isolated renal tubules. After BrdU labeling for 1 wk and a 2-wk chase period, kidneys were removed, minced with a razor, and incubated for 30 min at 37°C in DMEM containing 2 mg/ml collagenase (Sigma-Aldrich Japan, Tokyo, Japan). The resulting suspension was centrifuged and washed several times in PBS. The final pellet was then suspended in a 200-μl mixture of Matrigel (BD Biosciences, San Jose, CA)/collagen I (Koken, Tokyo, Japan) on Transwell filters (Corning, Corning, NY), under which HUVEC were cultured at 37°C. In some experiments, instead of HUVEC, HUVEC-derived conditioned medium (HUVEC-CM) collected from HUVEC cultured in endothelial basal medium supplemented with HuMedia-EG for 48 h, were used. Endothelial basal medium supplemented with HuMedia-EG alone was used as a control. After the indicated periods, the gels containing isolated renal tubules were fixed in 4% formaldehyde, removed from the inserts of Transwell culture plates, dehydrated, and embedded in paraffin. LRCs were detected as BrdU-positive cells.

Statistical analysis. The differences between means were compared by Student’s t-test, with P values of <0.05 considered significant.

RESULTS

Histological changes in the kidney after renal ischemia. To examine age-related differences in the potential for tubular recovery after acute kidney injury, various degrees of ischemia-reperfusion injury were induced in 7-wk- and 12-mo-old rats by clamping the left renal artery for 30, 45, and 60 min. There were no significant differences in the baseline levels of serum creatinine and blood urea nitrogen (BUN) between 7-wk- and 12-mo-old rats (Fig. 1A). Body weight of 12-mo-old rats was significantly heavier than that of 7-wk-old rats (Fig. 1A). Histological changes in the kidney after renal ischemia were assessed by PAS staining. At baseline, renal histology was almost normal at any ages of rats tested (data not shown). Tubular expansion, flattened tubular epithelial cells, and cast formation were observed in the outer medulla of the ischemic kidneys 24 h after reperfusion (Fig. 1B), but were not observed in sham-operated kidneys (data not shown). Unilateral renal ischemia did not affect renal function (data not shown). Quantitative analysis demonstrated that the size of the injured area of the kidney was positively correlated with ischemic periods at all ages of rats tested. After renal ischemia for 60 min, the size of the injured area of 12-mo-old rat kidneys was significantly larger than that of 7-wk- and 7-mo-old rat kidneys (Fig. 1C).

Decrease in DNA synthesis in tubular cells after injury with age. Next, DNA synthesis was examined in the kidney after renal ischemia. Various degrees of ischemic injury was induced in 7-wk-, 7-mo-, and 12-mo-old rats as described above, and BrdU was intraperitoneally administered into these rats 1...
h before euthanasia. At 24 h after reperfusion, rats were killed and the number of BrdU-positive cells was assessed. BrdU-positive cells were mainly observed in the outer medulla of the ischemic kidneys of all rats tested (Fig. 2A). Quantitative analysis demonstrated that the number of BrdU-positive cells decreased with age independently of the length of the ischemic period (Fig. 2B).

**Age-related decrease in the number of proliferating cells after renal ischemia.** Cell proliferation was also examined in the kidneys after renal ischemia by PCNA staining. Most PCNA-positive cells were present in tubular cells of the outer medulla of the kidney after renal ischemia, but not in sham-operated kidneys (Fig. 3A). Consistent with the data above, quantitative analysis demonstrated that the number of PCNA-positive cells decreased with age independently of the length of the ischemic period (Fig. 2B).

**Age-related decrease in the number of LRCs in normal rat kidneys.** Next, whether slow-cycling cells are present in normal rat kidneys was examined by BrdU-labeling methods as described previously (19) with slight modification. Normal rats were labeled with BrdU using an osmotic pump for 7 days, and kidneys were removed after a 2-wk chase period. By using this protocol, slow-cycling cells were identified as LRCs, since rapidly cycling cells would have divided many times during the chase period and incorporated BrdU should have been diluted out. Consistent with previous studies (18, 19), most LRCs were detectable in proximal tubules and some were also detected in distal tubules and collecting ducts in the outer medulla of the kidneys (Fig. 4, A and B). LRCs were not detected in glomeruli and capillary vessels (data not shown). The phenotype of LRCs after injury was also examined. After BrdU labeling for 1 wk followed by a 2-wk chase period, ischemia-reperfusion injury was induced in normal 7-wk-old rats and the kidneys were removed at 24 h after reperfusion. Expression of a mesenchymal marker, vimentin, was not observed in normal kidneys (data not shown) and in sham-operated kidneys (Fig. 4C). In contrast, reexpression of vimentin was observed in dividing LRCs in the kidney after renal ischemia (Fig. 4D).

Next, whether tubular LRCs are present in the aged kidneys was examined. Using an osmotic pump, normal 7-wk-, 7-mo-, and 12-mo-old rats were labeled with BrdU for 7 days, and their kidneys were removed after a 2-wk chase period. Most LRCs were detectable in proximal tubules in the outer medulla of the kidneys at all ages of rats tested (Fig. 5A). Quantitative analysis showed that the number of LRCs in 7- and 12-mo-old...
rats were significantly decreased compared with that in 7-wk-old rats (Fig. 5B).

No significant difference in the ratio of LRC division among 7-wk-, 7-mo-, and 12-mo-old rats. The number of tubular LRCs after renal ischemia were examined. Normal 7-wk-, 7-mo-, and 12-mo-old rats were labeled with BrdU for 7 days, and ischemia-reperfusion injuries were induced in these rats after a 2-wk chase period. Kidneys were removed at 24 h after reperfusion. Single LRCs were scattered in renal tubules of sham-operated kidneys. In contrast, many side-by-side labeled cells of tubular LRCs were observed in the kidney of these rats after renal ischemia (Fig. 6A). Quantitative analysis showed that the number of LRCs in the ischemic kidneys was significantly increased compared with that in sham-operated kidneys at any age of rats tested (Fig. 6B). Importantly, there was no significant difference in the LRC proliferation index (the number of LRCs at 24 h after reperfusion divided by the basal number of LRCs) among these rats (Fig. 6C). These data suggest that a potential of each LRC to undergo cell division after injury is preserved throughout life, while the number of tubular LRCs declines with age.

Reduction of peritubular capillary area with age. Stem cells reside within the context of a complex microenvironment of different cell types and extracellular matrix molecules that control stem cell self-renewal and progeny production in vivo (23), suggesting the possibility that microenvironments surrounding renal tubules play a role in tubular repair after injury. To address this issue, the peritubular capillary area of 7-wk-, 7-mo-, and 12-mo-old rat kidneys was examined by RECA-1 staining. RECA-1-positive peritubular capillary endothelial cells were observed in the interstitial area of the kidneys of all rats tested (Fig. 7, A–C). Quantitative analysis demonstrated...
that the peritubular capillary area significantly decreased with age (Fig. 7D).

Increase in the number of LRCs in renal tubules when cocultured with HUVEC in vitro. The data above suggest the possibility that certain factor(s) produced by peritubular capillary endothelium are involved in LRC growth and differentiation. To test this hypothesis, coculture experiments were performed in vitro. After BrdU labeling and chase periods as described above, renal tubules that contained LRCs were isolated from 7-wk-old rats, and cultured in gels on Transwell filters under which HUVEC were cocultured (Fig. 8A). As shown in Fig. 8B, LRCs were detectable in renal tubules...
cultured in gels. Quantitative analysis showed that the number of LRCs significantly increased when cocultured with HUVEC compared with those without HUVEC (Fig. 8C). To further examine the involvement of HUVEC-derived factor(s) on LRC proliferation, HUVEC-CM were also cocultured with renal tubules isolated from 7-wk-old rats treated with BrdU labeling followed by chase periods. There was a significant increase in the number of LRCs treated with HUVEC-CM compared with those treated with control (endothelial basal medium supplemented with HuMedia-EG) (Fig. 8D). When isolated tubules from 12-mo-old rats were cocultured with HUVEC, the number of LRCs did not significantly increase compared with those without HUVEC (Fig. 8E).

**DISCUSSION**

The process of normal aging affects organ homeostasis as well as responses to acute and chronic injury. It is considered that the susceptibility and response to injurious stimuli in the aged kidney are caused by various factors, such as diminished proliferative reserves, increased tendency for apoptosis, alterations in growth factor profiles, and changes in potential...
progenitor and immune cell functions (29). The present study demonstrated that a burst of epithelial cell proliferation, an integral part of the renal repair process after ischemia-reperfusion injury (40), declined with age (Figs. 2 and 3). In an ischemia-reperfusion injury model, ischemic conditions significantly influenced the severity of tissue damage as well as the proliferative response after injury. The peritubular capillary area that supplies renal tubules with oxygen differs with age (Fig. 7). This raises the possibility that the degree of kidney injury is not equal among the tested rats of different ages, even if ischemic injuries were induced in a similar manner. As expected, the severity of tissue damage was found to become more severe as the time of renal ischemia increased (Fig. 1). However, the degree of DNA synthesis in renal tubules after injury significantly decreased in aged kidneys compared with that in young kidneys independently of ischemic periods (Figs. 2 and 3). Collectively, it is possible that older kidneys have a decreased proliferative reserve.

Emerging evidence suggests that diverse tissue-specific stem cell reserves decline with age and lead to pathophysiological consequences for tissue aging (27). Consistent with this notion, the number of LRCs was found to significantly decline with age (Fig. 5). Given that LRCs are the source of regenerating cells after injury, and actively contribute to reconstruction of renal tubules after renal ischemia (18, 19), the impairment of tubular repair after renal ischemia in aged kidneys might be partly explained by the shortage of LRCs. Several groups have also identified slow-cycling cells in the kidney (12, 25, 36). Consistent with previous data (19), it has been shown that LRCs were present in renal tubules of normal kidneys (12, 36). On the other hand, papillary LRCs have also been identified (24, 25). The variation in LRC location, properties, and behavior after injury seems to be due to differences in timing and duration of the pulse, and length of the chase. In the context of adult tissue stem cells, renal tubular LRCs are morphologically mature and differentiated epithelial cells (36–38), which is inconsistent with an undifferentiated stem cell phenotype. Whether these LRCs with the capacity to divide after injury should be referred to as unipotent stem/progenitor cells or simply as cells that have retained proliferative potential remains to be clarified.

It also remains unknown by which mechanism the number of LRCs is regulated. It has been proposed that aging is associated with reduced regenerative capacity, which is caused by the senescence of stem/progenitors with age (5). The increased susceptibility of older kidneys toward acute injury and inability of an appropriate repair is the result of tubular cell senescence due to telomere shortening (39). In the present study, it was demonstrated that the number of LRCs significantly declined with age (Fig. 5). However, there was no significant difference in the ratio of LRC division after injury among the tested rats of different ages (Fig. 6), suggesting that the potential of LRCs
to undergo cell division for tissue repair is preserved throughout life. In the case of renal tubules, the age-related decrease in the number of LRCs appears to be unrelated to senescence. It has been recently reported that increased zinc-α(2)-glycoprotein (Zag) expression in the aged kidney acts to suppress the proliferative response to injury (31). On the other hand, the expression of the NAD-dependent deacetylase sirtuin 1 (Sirt1), an important modulator of renal cytotoxic responses to aging, has been demonstrated to be decreased in aged kidneys (15). This suggests a possible linkage between LRCs and Sirt1 function.

Stem cells from a variety of tissues reside in close proximity to specialized support cells that extrinsically regulate stem cell self-renewal, differentiation, and aging (27). This microenvironment, known as a niche, influences stem cell behavior to govern tissue homeostasis under diverse physiological and pathophysiological conditions. Vasculature has been implicated as contributing to the stem cell niche in the hematopoietic system (13), intestines (1), hair follicle stem cells (2), testis (11), and brain (32–34), indicating that vascular systems may serve a conserved supportive role for stem cells throughout the body. Given that the kidney is a highly vascular organ, it is quite possible that an age-related reduction in the capacity of tubular recovery after injury will be associated with alterations in the integrity of the renal vasculature. Consistent with previous studies (14, 35), the present study demonstrated an age-related reduction of peritubular capillary area surrounding renal tubules (Fig. 7). In addition, the number of LRCs in the isolated tubules cultured in gels significantly increased when cocultured with HUVEC or HUVEC-CM (Fig. 8), suggesting the presence of endothelial cell-derived factor(s) that support LRC division. When isolated tubules from 12-mo-old rats were cocultured with HUVEC, the number of LRCs did not significantly increase compared with that without HUVEC (Fig. 8E). The expression level of receptors for such factor(s) in LRCs might be reduced in aging kidneys. Considering that LRCs were still present in 5⁄6 nephrectomized renal fibrosis model (unpublished observations), such activator(s) of LRCs may be involved in the induction of LRC division remain to be determined. In any case, the peritubular capillary endothelium could be a niche for LRCs.

Recent studies have demonstrated that a decline in niche function, including decreased production of local self-renewal factors, contributes to reduced tissue homeostasis and repair in a number of systems (4, 7, 28). In addition, changes in circulating systemic factors lead to decreased stem cell activity (7, 21). In contrast, restoration of niche function can counter age-related changes in stem cell behavior (4, 8, 21). Together, strategies to rejuvenate or expand the peritubular capillaries may enhance LRC-based therapies for acute kidney injury in the elderly population.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

Author contributions: M.M. performed experiments; M.M. and A.M. analyzed data; M.M. and A.M. prepared figures; M.M., A.M., K.M., N.S., H.I., T.K., K.H., and Y.N. approved final version of manuscript; A.M. provided conception and design of research; A.M. interpreted results of experiments; A.M. drafted manuscript; A.M. edited and revised manuscript.

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