Mice are unable to endogenously regenerate podocytes during the repair of immunotoxin-induced glomerular injury

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ABSTRACT

Background. Recent studies have reported that podocytes are postnatally generated from progenitor cells localized in Bowman’s capsule or in the bone marrow. In the present study, we investigated whether or not podocyte regeneration is important in the repair of injured glomeruli after mild podocyte injury in mice.

Methods. Mild podocyte injury was induced in NEP25 mice (n = 8) by injecting an immunotoxin, LMB2 (0.625 ng/g body weight). Control mice, not injured by LMB2 injection (n = 7) was used as a comparison. Proliferating cells were labeled by continuous infusion of bromodeoxyuridine (BrdU). Podocytes, identified by nephrin, WT1 or podocin staining, that had incorporated BrdU were enumerated 4 weeks later.

Results. A total of 742 corpuscles were inspected in serial sections stained for BrdU and nephrin; 19% showed sclerosis. BrdU+ cells were observed in both the glomeruli and Bowman’s capsules, averaging 2.5 ± 3.1 in non-sclerotic corpuscles and 7.0 ± 5.8 in sclerotic corpuscles. Only one BrdU+ cell was also positive for nephrin. Another cell, localized at a position consistent with its potential identification as a podocyte, was nephrin negative but had incorporated BrdU. WT1 staining similarly revealed that only two nuclei were doubly positive for BrdU and WT1. Additional 1676 corpuscles were inspected by double staining for BrdU and podocin; none were doubly positive.

Conclusions. Podocytes are not replenished by proliferation of endogenous progenitor cells in mice with glomerular injury.

Keywords: chronic renal failure, glomerulosclerosis, mouse model, podocyte, regeneration

INTRODUCTION

Podocytes play a pivotal role as barriers to glomerular filtration. These cells are terminally differentiated and do not proliferate after birth, under normal conditions. However, podocytes are lost as a result of various kidney diseases and as part of the normal aging process [1–4]. Moreover, urinary podocyte loss has also been reported in healthy humans [5, 6]. The irreversible injury and loss of podocytes is a direct cause of glomerulosclerosis [1–4]. Therefore, an important question remains as to whether or not podocytes can be replenished by some means, after birth.

Bone marrow-derived cells have been reported to contribute to the regeneration of podocytes in a mouse model of Alport syndrome [7, 8]. This notion is supported by several reports that have shown that female-derived renal grafts transplanted into male recipients contained a small number of Y chromosome-positive podocytes [9, 10]. However, the efficiency of bone marrow cells traversing the glomerular basement membrane, in the absence of the Alport mutation, has not been clarified.

Recent studies by Appel et al. suggested that podocyte progenitor cells exist in Bowman’s capsule, a site that is more accessible to the visceral glomerular capillary surface [11]. Thus, utilizing a transgenic mouse line with inducible genetic tagging of parietal epithelial cells (PECs), they demonstrated...
that PECs on postnatal day 5 or 10, but not at the age of 6 weeks, can migrate onto the glomerular tuft and differentiate into podocytes. In addition, Ronconi et al. [12] have shown that in the adult human kidney a subset of cells in Bowman’s capsule, near the urinary pole, express CD133 and CD24 (stem cell markers) and can be differentiated into podocytes in vitro. Excitingly, when these cells were injected into SCID mice with adriamycin nephropathy, the engrafted cells differentiated into podocytes and the renal injury was improved. Moreover, CD133+/CD24+ cells have been shown to be major constituents of hyperplastic lesions in podocytopathies that contain cells positive for CD133, CD24 and nestin (a podocyte marker) [13]. Participation of PECs in the formation of sclerotic lesions was further confirmed by cell lineage tracing using genetically tagged PECs [14]. However, the capacity of these cells to differentiate into normal podocytes was not demonstrated in this study.

Previously, we established a mouse model of selective and inducible podocyte injury (NEP25) [15]. NEP25 mice express hCD25 selectively on podocytes. After injection of the hCD25-targeted immunotoxin, LMB2, only hCD25-expressing podocytes are injured. High doses of LMB2 cause NEP25 mice to develop progressive nephrotic syndrome and renal failure; these animals succumb to renal failure within 2 weeks [15]. In these mice, PECs often proliferate, occasionally forming a monolayer of lesions that covers the outer surface of the glomerular basement membrane [16]. Most podocytes are severely injured or lost, without any signs of podocyte regeneration within 14 days of LMB2 injection. On the other hand, a low dose [0.625 ng/g body weight (BW)] of LMB2 results in NEP25 mice developing moderate proteinuria, which peaks 1–2 weeks after the injection before gradually decreasing. After 3–4 weeks, some glomeruli develop global or segmental sclerosis, while other glomeruli recover from the podocyte injury and show normal structure [15]. Thus, LMB2-induced injury in NEP25 mice is selective for differentiated podocytes and is severe enough to effectively eliminate a portion of the podocyte population, but is also sufficiently transient to allow the recovery of injured glomeruli. Therefore, the NEP25 model, when used in conjunction with low-dose LMB2, is ideal for investigating podocyte regeneration. In the present study, this model was used to investigate whether or not newly generated podocytes contribute to the glomeruli that recover from injury. To detect newly generated podocytes, we labeled newly generated podocytes with BrdU, following the injection of LMB2.

**MATERIALS AND METHODS**

**Animal experiments**

The Animal Experimentation Committee of Tokai University School of Medicine approved the protocol, in accordance with the principles and procedures outlined in the National Institute of Health’s Guide for the Care and Use of Laboratory Animals.

Eight NEP25 mice heterozygous for hCD25 [15], 7–9 weeks old, backcrossed with C57BL/6 strain more than eight times, were used in this study. These mice received water and food *ad libitum*. LMB2 (0.625 ng/g BW) was intravenously injected, under diethyl ether anesthesia. Seven mice, the control, received an injection of saline under the same anesthesia. At the same time, osmotic minipumps (model 2002 or 2004; Alza, Palo Alto, CA, USA) containing BrdU were subcutaneously implanted in each animal. Ten mice were infused with BrdU for 4 weeks at a rate of 1.25 ng/h/g body weight, and 5 mice were for 2 weeks at the same rate (Table 1). Metabolic cages were used to collect 24-h urine samples prior to and at 7, 14, 21 and 28 days after the injection from each of the 15 mice. The mice were euthanized 28 days after the injection and samples were processed for renal histology.

A pilot study was also conducted in which five female NEP25 mice, at the same age, were similarly injected with the same dose of LMB2. For these animals, 24-h urine samples were collected 7 days, post-injection; their kidneys were also histologically analyzed.

Pregnant mice were treated with continuous infusion of BrdU from embryonic day 13.5 to term. The small intestine of the mother and the kidney of the neonates were analyzed.

**Histology and immunostaining**

All histological and immunostaining was performed on 7-μm thick, paraffin sections. Guinea pig anti-nephrin (1:200; Progen, Heidelberg, Germany), rabbit anti-human WT-1 (1: 500; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-podocin antibody (1:500; Sigma Aldrich, St. Louis, MO, USA) and rat anti-human/mouse CD44 antibody (1: 200; Bioscience, San Diego, CA, USA) were used for immunohistochemistry at the indicated dilution. Two adjacent sections, both before and after the BrdU-staining, were examined. The sections were then stained for BrdU using a fluorescent secondary antibody (1:200; eBioscience, San Diego, CA, USA) and rat anti-human/mouse CD44 antibody (1: 200; Bioscience, San Diego, CA, USA), rabbit anti-podocin antibody (1:500; Sigma Aldrich, St. Louis, MO, USA) and mouse anti-human/CD133 (1:200; Progen, Heidelberg, Germany), rabbit anti-WT-1 (1: 500; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and rabbit anti-HLA class II antibody (1: 200; eBioscience, San Diego, CA, USA) were used for double immunostaining.

**Table 1. Characteristics of the NEP25 mice used in the experiment**

<table>
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<th>No.</th>
<th>LMB2 dose (ng/g BW)</th>
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ACR, albumin/creatinine ratio; BW, body weight; BrdU, bromodeoxyuridine.
BrdU-staining kit (BrdU-immunohistochemistry System, Calbiochem, Darmstadt, Germany). The signal was visualized with Ni-Co diaminobenzidine. Subsequently, the sections were treated with 0.03% hydrogen peroxide/methanol for 15 min and then with an avidin/biotin blocking kit (Vector Laboratories, Burlingame, CA, USA). The sections were then incubated with anti-podocin antibody, overnight. The podocin signal was visualized with biotin-anti-rabbit antibody (Vector Laboratories), ABC kit (Vector Laboratories) and diaminobenzidine. The numbers of BrdU-positive cells in each renal corpuscle were counted.

In periodic acid-Schiff (PAS)-stained sections, glomerulonephritis was semi-quantified in each glomerulus using a score of 0–4, as described previously [15]. The average score was calculated for each mouse.

**Urinalysis**

The urinary creatinine and albumin concentrations were determined using an enzymatic method and a turbidimetric immunoenzyme assay, respectively, by an outside contract laboratory (SRL, Tokyo, Japan).

**Statistical analyses**

Results are expressed as means ± SD. Data were analyzed using Mann–Whitney’s U-test, and the values were regarded as significant if P < 0.05.

**RESULTS**

Early-phase podocyte injury in NEP25 mice (n = 5) was characterized 1 week after the injection of the pilot group of mice with low-dose (0.625 ng/g BW) LMB2. Over this time, the urinary albumin/creatinine ratio (ACR) had moderately increased from 0.1 ± 0.0 to 44.5 ± 19.5 mg/mg. At this time point, the renal histology also appeared normal in PAS-stained tissue (Figure 1C). However, podocin staining was reduced and showed a diffuse, discontinuous pattern in all glomeruli (Figure 1D and E), in contrast to the continuous pattern observed in the mouse without LMB2-induced injury (Figure 1B).

Separately, eight NEP25 mice were injected with the same dose of LMB2 and also received mini osmotic pumps that were implanted to continuously infuse BrdU for 2 (n = 4) or 4 weeks (n = 4 in Table 1). All of the NEP25 mice again showed moderate albuminuria. The urinary ACR at 1 week was comparable to that observed in the pilot group of animals, averaging 41.1 ± 22.4 mg/mg, which was greater than that observed at baseline (0.2 ± 0.1). Thereafter, the urinary ACR gradually decreased to 7.8 ± 8.8 at 4 weeks, post-injection, suggesting the recovery of the injured glomeruli (Table 1). Seven mice were injected with saline, alone, and were similarly implanted with a BrdU pump (Table 1). These mice did not demonstrate abnormal albuminuria.

We tested the possibility that BrdU incorporation may disturb normal development of podocytes by continuously infusing BrdU into pregnant mice from embryonic day 13.5 to term. BrdU staining of the neonatal mouse kidney revealed

that the majority of kidney cells, including podocytes, were labeled with BrdU (Supplementary data, Figure S1B). The morphology of BrdU+ glomeruli was normal (Supplementary data, Figure S1A). All BrdU+ podocytes expressed nephrin normally (Supplementary data, Figure S1C), confirming that BrdU incorporation per se had no appreciable effect on the development of podocytes and expression of podocyte markers.

Four weeks after LMB2 injection, eight NEP25 mice developed focal segmental glomerulosclerosis (Figure 1F), with 5 (mouse 2, Table 2) to 54% (mouse 6, Table 2) of glomeruli containing segmental or global sclerosis. The sclerosis index, quantified by 0–4 scores ranged from 0.09 to 1.42 (Table 1). Podocin staining showed the normal, continuous pattern in most non-sclerotic glomeruli (Figure 1H). In contrast, podocin staining completely disappeared in sclerotic lesions. In addition, podocin staining was also reduced in podocytes in some non-sclerotic portions of the glomeruli with segmental sclerosis (Figure 1G). Thus, all of the glomeruli showed mild injury at 1 week, as evidenced by the discontinuous pattern of podocin staining in all of the glomeruli (Figure 1D and E); some later recovered from the injury, whereas others developed sclerosis (Figure 1F). Some investigators have proposed that PECs are activated in damaged glomeruli, express CD44 and participate in the development of glomerulosclerosis or repair of the glomerulus [13, 14]. We therefore examined the expression of CD44 in this NEP25 model. CD44+ cells were present only in a few glomeruli at 1 week after LMB2 injection (Supplementary data, Figure S2D). PECs with intense CD44 staining were frequently observed in glomeruli with established sclerosis at 4 weeks after the injection, but were never seen in glomeruli without sclerosis (Supplementary data, Figure S2F).

We then examined whether or not podocytes proliferate during this mild glomerular injury and recovery period. To do this, renal sections were stained for BrdU and counterstained with PAS. To accurately identify podocytes, three serial sections were used. The first and third sections were stained for nephrin, and the second was for BrdU. Data from individual mice are shown in Table 2. Cumulatively, 742 renal corpuscles, i.e. glomeruli (glomerular tufts) and Bowman’s capsules, from 8 NEP25 mice with LMB2-induced injury were examined. Of these, 601 (81%) corpuscles had no sclerosis and 141 (19%) had segmental or global sclerosis. On average, the corpuscles without sclerosis contained 1.7 ± 2.0 BrdU+ cells in the glomerulus and 0.8 ± 1.6 BrdU+ cells in Bowman’s capsule. Sclerotic corpuscles contained more BrdU+ cells in both the glomeruli (4.0 ± 3.8) and Bowman’s capsules (3.0 ± 3.0). Most cells that had incorporated BrdU within the glomerulus appeared to be mesangial cells, endothelial cells or circulating cells in the capillary lumen. We extensively searched adjacent sections for cells doubly positive for BrdU and nephrin (a marker for differentiated podocytes), but virtually all of the BrdU+ cells were negative for nephrin (Figure 2A–D). Out of 1588 BrdU+ cells in the glomeruli, only 1 was also positive for nephrin (Figure 2E and F). In addition, in one glomerulus with segmental sclerosis, 1 BrdU+ cell was localized at a position consistent with that for podocytes (Figure 2G and H). This cell was negative for nephrin, suggesting that it was an injured podocyte. We continuously infused BrdU into seven
control NEP25 mice without LMB2 injection for 4 weeks. The control mice had normal renal histology without sclerotic glomeruli (Table 1, Figure 1A). The corpuscles contained 1.1 ± 1.2 BrdU+ cells in the glomerulus and 0.5 ± 0.7 BrdU+ cells in Bowman’s capsule. All of the BrdU+ cells were negative for nephrin (Table 2).

Since nephrin is a cytoplasmic marker for podocytes, we next examined the positivity of WT1, a nuclear marker for podocytes, for BrdU-incorporated cells in a similar fashion. Out of 876 BrdU+ nuclei in 577 corpuscles from 8 LMB2-treated NEP25 mice, only 2 nuclei were also positive for WT1 (Figure 3A and B). These two nuclei appeared within a single podocyte, which was weakly positive for nephrin (Figure 3C). None of BrdU+ nuclei in seven control mice were positive for WT1.

We also searched podocytes that had incorporated BrdU by double immunostaining for BrdU and podocin. Cumulatively, 1676 renal corpuscles were examined, without finding any single cell that was positive for both BrdU and podocin (Figure 4). Outside the glomeruli, avid incorporation of BrdU was observed in atrophic or dilated tubules. Most of these lesions were remote from Bowman’s capsules.

Finally, to confirm that BrdU indeed efficiently labeled cells which underwent DNA synthesis during the infusion periods, we performed BrdU staining in the small intestine. As shown in Supplementary data, Figure S3, all of the intestinal epithelial cells in the microvilli were stained for BrdU, indicating that 100% of newly generated cells from the progenitor cells, at least in the small intestine, were labeled in this experiment.

**DISCUSSION**

Four weeks after the injection of LMB2 (0.625 ng/g BW), the NEP25 mice contained glomeruli with a wide variety of injuries, ranging from normally recovered glomeruli to globally sclerotic glomeruli. BrdU was administrated by continuous infusion. BrdU therefore labeled both cells undergoing DNA synthesis at the time of sacrifice, as well as cells that...
synthesized DNA during the period of BrdU infusion even if they exited the cell cycle, e.g. differentiated podocytes, at the time of sacrifice. Through an extensive search in >2000 glomeruli, with or without sclerosis, using three independent methods, only one definitive and two possible podocytes that had incorporated BrdU were found. These results caused some potential concerns that need to be considered when interpreting the results of this study. One potential concern was that the injuries induced in the present study may have been too mild to effectively activate podocyte regeneration. Indeed, although podocin staining was diffusely affected (Figure 1D), CD44+ cells (activated PECs) were rarely seen 1 week after LMB2 injection. In this regard, we also found that BrdU+ damages PECs that potentially contain the progenitor cells. Therefore, it is unlikely that LMB2 directly damages PECs that potentially contain the progenitor cells.

Another potential concern is that LMB2 directly injured the progenitor cell population, since PECs are also injured in NEP25 mice. Of note, PECs in NEP25 mice showed vacuolar degeneration, as well as proliferation and migration, after injection of LMB2, in particular at high dosage (≥1.25 ng/g BW) [15]. These changes of PECs were not caused directly by LMB2, but were a secondary response to podocyte injury for the following reasons. First, PECs were negative for hCD25 staining. Secondly, when chimeric mice made up with hCD25-carrying and wild-type cells were treated with LMB2, PECs not carrying hCD25 transgene were also injured [17]. Thirdly, inspection by EM revealed that damage is limited in the podocyte and not observed in PEC 12 h after LMB2 injection. Finally, these PEC lesions are commonly observed in other podocyte-specific injury models, including HIV-1 transgenic mice [18], podocin knockout mice [19] and Thy-1 transgenic mice [20]. Similar secondary lesions are also observed in proximal tubular cells. Therefore, it is unlikely that LMB2 directly damages PECs that potentially contain the progenitor cells.

The study by Ronconi et al. suggested that the progenitor cells in Bowman’s capsule, near the urinary pole, have the potential to differentiate into either podocytes or tubular cells [12]. We found avid, focal BrdU incorporation in the tubules, but all of these lesions were remote from the glomeruli. Moreover, sporadic PECs incorporated BrdU, but none were found to be continuous to BrdU+ tubular glomerular cells. These findings suggest that mice may not have progenitor cells in Bowman’s capsules.

However, several other issues should also be considered. First, BrdU does not label podocytes transdifferentiated from other types of cells without DNA synthesis. Studies using cell lineage tracing of presumptive progenitor cells are necessary to clarify the presence of transdifferentiated podocytes. Secondly, newly recruited podocytes may not be fully differentiated and may not express nephrin or podocin during the 4-week period after LMB2 injection. In this regard, we also found that BrdU+ cells were rarely positive for WT1, which is known to be expressed in immature prospective podocytes [21]. Thirdly, progenitor cells may exist, but podocyte depletion, alone, may not effectively activate their proliferation. Other factors, such as growth factors (e.g. Notch [22]), may be necessary to effectively stimulate the growth of progenitor cells. Fourthly, even though endogenous progenitor cells cannot replenish podocytes, the exogenous administration of progenitor cells, as Ronconi et al. showed [12], may replenish podocytes. Finally, the percentage of BrdU+ podocytes may be influenced by the
FIGURE 2: BrdU and nephrin staining in NEP25 mice, 4 weeks after LMB2 injection. The left and right panels represent adjacent sections (×400). In the non-sclerotic glomeruli (A and B), BrdU was observed in both the glomeruli and Bowman’s capsules (arrows). None of the cells are positive for nephrin. In glomeruli with segmental sclerosis (C and D), more BrdU+ cells are observed; they are all negative for nephrin. An extensive search of 742 glomeruli revealed only one cell that was positive for both BrdU and nephrin (E and F, long arrows). Another BrdU+ cell, although negative for nephrin, is speculated to be an injured podocyte, based on its location (G and H, long arrows).
labeling efficiency. In this regard, it was impossible to accurately assess the labeling efficiency of presumptive podocyte progenitor cells that cannot be definitively identified. We instead examined the intestine to monitor the BrdU labeling efficiency. We found that 100% of intestinal epithelial cells incorporated BrdU, indicating that the labeling efficiency of newly generated cells from the intestinal progenitor cells was 100%. There appears no reason to believe that labeling efficiency was low specifically in the presumptive podocyte progenitor cells.

There may be species differences associated with podocyte regeneration. Similar to this study, Appel et al. [11] also showed that PECs in normal, adult mice do not differentiate into podocytes. In another recent study, PECs were genetically tagged and traced in crescentic glomerulonephritis and collapsing glomerulopathy [23]. Differentiation from PECs to podocytes was not documented in the study. On the other hand, the progenitor cells found by Ronconi et al. [12] were of human origin. The markers that they used to identify progenitor cells, CD133 and CD24, cannot be used in mice due to interspecies differences. In addition, the shorter life spans and smaller glomeruli in mice may make the replenishment of podocytes less important than in humans.

Nonetheless, the present study showed that podocyte progenitor cells, even if they exist, have little impact on the capacity to replenish podocytes and do not contribute to the recovery of glomerular function in a mouse model of podocyte injury.
Antifibrotic, nephroprotective effects of paricalcitol versus calcitriol on top of ACE-inhibitor therapy in the COL4A3 knockout mouse model for progressive renal fibrosis

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ABSTRACT

Background. The COL4A3−/− mouse serves as animal model for progressive renal fibrosis. Using this animal model, the present study investigates the nephroprotective effects of Paricalcitol versus Calcitriol alone and on top of ACE-inhibitor therapy.

Methods. Eighty six mice were divided into six groups: (PC) with Paricalcitol 0.1 mcg/kg, (CA) Calcitriol 0.03 mcg/kg (dose equipotent), (PLAC) vehicle 0.1 mL i.p. five times per week.

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