Glomerular visceral epithelial cells or podocytes are located on the outer surface of the glomerular basement membrane and play an indispensable role as a filtration barrier. The core cytoskeleton of the foot processes is actin filaments, which play an important role in maintaining the unique structure of podocytes. We previously established a transgenic mouse line (NEP25), which expresses human (h)CD25 selectively on podocytes. By injecting an hCD25-targeted recombinant immunotoxin (LMB2), podocyte injury can be induced on demand. After LMB2 injection, NEP25 mice develop nephrotic syndrome with downregulation of podocyte-specific proteins. In the present study, we genetically labeled podocytes with lacZ linked with β-actin-based CAG promoter. Utilizing the Cre-loxP system, this labeling was confined to the podocyte lineage. Without LMB2, all podocytes were positive for lacZ. After LMB2 injection, lacZ expression was rapidly downregulated, before podocytes showed any discernible morphological changes. Confocal imaging of filamentous (F)-actin-binding Alexa 488-phalloidin revealed that the normal continuous pattern of F-actin distribution in podocytes was punctuated after LMB2 injection. These collectively suggest that disturbance of actin filaments may be one of the key initial events leading to subsequent podocyte damage.

Key words: actin, foot processes, Cre-loxP system, transgenic mouse, glomerular sclerosis

Original Article

Rapid Downregulation of β-actin-based CAG Promoter and Filamentous Actin in Injured Podocytes

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Glomerular visceral epithelial cells or podocytes are located on the outer surface of the glomerular basement membrane and play an indispensable role as a filtration barrier. The core cytoskeleton of the foot processes is actin filaments, which play an important role in maintaining the unique structure of podocytes. We previously established a transgenic mouse line (NEP25), which expresses human (h)CD25 selectively on podocytes. By injecting an hCD25-targeted recombinant immunotoxin (LMB2), podocyte injury can be induced on demand. After LMB2 injection, NEP25 mice develop nephrotic syndrome with downregulation of podocyte-specific proteins. In the present study, we genetically labeled podocytes with lacZ linked with β-actin-based CAG promoter. Utilizing the Cre-loxP system, this labeling was confined to the podocyte lineage. Without LMB2, all podocytes were positive for lacZ. After LMB2 injection, lacZ expression was rapidly downregulated, before podocytes showed any discernible morphological changes. Confocal imaging of filamentous (F)-actin-binding Alexa 488-phalloidin revealed that the normal continuous pattern of F-actin distribution in podocytes was punctuated after LMB2 injection. These collectively suggest that disturbance of actin filaments may be one of the key initial events leading to subsequent podocyte damage.

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Introduction

Glomerular visceral epithelial cells or podocytes are located on the outer surface of the glomerular basement membrane and play an indispensable role as a filtration barrier1,2. Podocytes are highly and terminally differentiated cells that consist of three compartments; the cell body, the major processes, and the foot processes2. The foot processes are interdigitated with those of neighboring podocytes and support the glomerular capillary wall3. The core cytoskeleton of the foot processes is actin filaments, while both microtubules and vimentin intermediate filaments are distributed in the cell body and the primary processes, not in the foot processes4-6. Therefore, the actin cytoskeleton plays an important role in maintaining the unique structure of podocytes4-6. Foot process effacement and underlying cytoskeletal alterations are associated with podocyte dysfunction that leads to proteinuria in many forms of human and experimental glomerular diseases1,5-9.

We previously established a transgenic mouse line
(NEP25), which expresses human (h) CD25 selectively on podocytes. By injecting an hCD25-targeted recombinant immunotoxin, LMB2 or anti-Tac(Fv)-PE38, podocyte injury can be induced on demand. A few days after the injection of LMB2, NEP25 mice developed nephrotic syndrome and subsequently renal failure. Podocytes and other glomerular cells became progressively damaged, finally leading to glomerular sclerosis. Shortly after the LMB2 injection, podocyte-specific proteins, including synaptopodin, nephrin, WT-1 and podocalyxin, were downregulated.

In the present study, we genetically labeled podocytes with lacZ driven by β-actin-based CAG promoter, a promoter highly active in a wide range of cell types. We found that lacZ was rapidly downregulated after the injection of LMB2, while podocytes still maintained normal structure.

Materials and Methods

All experimental procedures were performed in accordance with the guidelines of both the Animal Experimentation Committee of Tokai University and the Institutional Review Board of the Tokyo Medical and Dental University.

Generation of Nphs1-Cre/CAG-CAT-Z/NEP25 Mice

We mated Nphs1-Cre mice (Fig. 1A) with CAG-CAT-Z transgenic mice (Fig. 1B). To confer the double transgenic mice (Fig. 1C) with the podocyte-selective hCD25 expression, we mated them with NEP25 mice (Fig. 1D), obtaining triple transgenic mice (Fig. 1E).

Transgenic mice were identified by PCR on tail DNA as previously described. The primers used were 5′-AGTTGCTTCTGCTCAGGA-3′ and 5′-TCGACCAGTTAGTTACC-3′ for the Nphs1-Cre transgene, 5′-TTTTATTATCATGATCGTCGAG-3′ and 5′-CGAAATCTCCTTCTGTTGTA-3′ for the NEP25 transgene, 5′-CGTCACTCGGCTGATTTGATT-3′ and 5′-CGCAATTCGCTGATTTGATTAC-3′ for the CAG-CAT-Z transgene.

LMB2 was intravenously injected under the anesthesia with diethyl ether. After the injection of LMB2, Nphs1-Cre/CAG-CAT-Z/NEP25 mice (n=10) and NEP25 transgenic mice (n=8) were analyzed.

Histological Analyses

For lacZ staining, frozen sections (4 μm) were fixed in 2% glutaraldehyde and stained with X-gal as previously described. For synaptopodin immunostaining, 4% buffered paraformaldehyde-fixed frozen sections were stained with monoclonal anti-rat synaptopodin antibody (Progen, Heidelberg, Germany). For filamentous (F)-actin staining, frozen sections (8 μm) were fixed in 2% paraformaldehyde, stained with Alexa Fluor 488-phalloidin (Molecular Probes, Eugene, USA), and observed by an inverted confocal microscope (LSM510, Zeiss).

Glomeruli were graded using scores of 0 to 4 for 0%, <25%, 25% to 50%, 50% to 75%, and >75%, respectively, of the tuft area that was positive for lacZ.

Results

Establishment of Nphs1-Cre/CAG-CAT-Z/NEP25 Mice Expressing hCD25 and LacZ in Podocytes

First, we mated Nphs1-Cre mice expressing Cre recombinase selectively in podocytes with CAG-CAT-Z mice. CAG-CAT-Z mice carry the β-actin-based
CAG promoter and the lacZ gene, which are separated by the loxP-flanked chloramphenicol acetyltransferase (CAT) gene. Without Cre-mediated recombination, the lacZ gene is not expressed due to an interruption by the CAT gene. In Nphs1-Cre/CAG-CAT-Z double transgenic mice, Cre recombinase is expressed only in podocytes. Since Cre-mediated recombination deletes the CAT gene, the lacZ gene is irreversibly activated, driven by the CAG promoter.

To equip the mice with the hCD25-mediated podocyte injury device, we mated Nphs1-Cre/CAG-CAT-Z double transgenic mice with NEP25 mice, obtaining Nphs1-Cre/CAG-CAT-Z/NEP25 triple transgenic mice. The triple transgenic mice showed no

![Histological analyses](image)

Fig. 2. Histological analyses.

(A, B) Specificity of the lacZ labeling of podocytes. Without LMB2, the staining patterns of lacZ (blue) (A; with counterstaining by Nuclear Fast Red, NFR) and synaptopodin (brown) (B; with counterstaining by Methyl Green, MG) in the adjacent sections of a normal glomerulus coincided with each other, demonstrating that all podocytes are specifically labeled with lacZ.

(C-H) Progressive downregulation of the lacZ expression. A representative glomerulus with or without the LMB2 injection (25 ng/g BW) stained for lacZ with counterstaining by PAS is presented at each time point. Without LMB2 (0 hour), all podocytes were positive for lacZ (C). Twelve hours after the injection, lacZ expression was preserved (D). Eighteen and twenty-four hours after the injection, the lacZ expression decreased segmentally with no apparent morphological change (E, F). Forty-eight and ninety-six hours after the injection, all podocytes were negative for lacZ (G, H).

(I, J) LacZ expression with lower doses of LMB2 (5, 15 ng/g BW). Twenty-four hours after the injection, lacZ was stained in a segmental pattern in spite of low LMB2 doses.

(K) Retention of synaptopodin expression after disappearance of lacZ. A representative glomerulus stained for synaptopodin with counterstaining by MG is presented. Ninety-six hours after the injection of LMB2 (25 ng/g BW), all podocytes were positive for synaptopodin.

(Magnification X400)
apparent abnormalities in growth pattern and behavior. The mice showed functionally and morphologically normal renal phenotype. We stained the adjacent sections of the kidney for lacZ and synaptopodin, a podocyte-specific marker. The staining pattern of lacZ coincided with that of synaptopodin, indicating that all podocytes are specifically labeled with lacZ (Fig. 2A and 2B).

Rapid Suppression of the CAG Promoter Activity after LMB2 Injection

Without LMB2, all podocytes were positive for lacZ (Fig. 2C). After LMB2 injection, Nphs1-Cre/CAG-CAT-Z/NEP25 mice showed the pathological changes of the kidney essentially the same as the NEP25 single transgenic mice\(^9\). After the injection of LMB2, lacZ expression in the kidney of the triple transgenic mice was rapidly disappeared. We semi-quantified lacZ-positive areas using score of 0 to 4. First, we examined kidneys to 96 hours after the injection of LMB2 at 25 ng/g body weight (BW). Six hours after the injection, 97% of the glomeruli had score 4, indicating that lacZ expression was initially preserved. Twelve, 18 and 24 hours after the injection, 80, 0.2 and 0% of the glomeruli had a score of 3 or 4, while 0.5, 85 and 100% of the glomeruli showed a score of 0 or 1, respectively, demonstrating that lacZ expression was progressively downregulated (Fig. 2D, 2E and 2F). Forty-eight, 72 and 96 hours after the injection, all glomeruli had a score of 0 for lacZ, indicating that all podocytes were negative for lacZ (Fig. 2G and 2H).

We next tested lower doses of LMB2. Twenty-four hours after the injection of LMB2 (5 or 15 ng/g BW), lacZ score was 0 or 1 in more than 95% of the glomeruli (Fig. 2I and 2J). In all conditions tested, most glomeruli showed no morphological abnormality and normal synaptopodin expression pattern, as compared with those of control glomeruli (Fig. 2K).

Disruption of Filamentous Actin in Podocytes

We visualized F-actin with Alexa488-phalloidin and observed by confocal microscopy. To compare the kidneys with or without LMB2 exposure through identical tissue preparation, kidneys were embedded in one block and stained in the same sections. Without LMB2, F-actin was clearly visualized in podocytes along the capillary wall in a linear and continuous pattern (Fig. 3A and 3B). Forty-eight hours after the injection of LMB2 (25 ng/g BW), F-actin staining showed no discernible changes (data not shown). Seventy-two hours after the injection, the intensity of F-actin in podocytes partially decreased, with occasional interruptions of the linear continuity (Fig. 3C and 3D). On the other hand, F-actin level was partially increased in mesangial areas (F), compared to control glomerulus without LMB2 (E).

Discussion

We established Nphs1-Cre/CAG-CAT-Z/NEP25 triple
transgenic mice, equipped with the podocyte-selective injury device and expressing the lacZ gene selectively in podocytes. After the injection of LMB2, lacZ expression was rapidly downregulated, and then the F-actin in podocytes was disrupted.

LMB2 is a recombinant protein composed of a binding portion of anti-hCD25 antibody and PE38, a mutant form of Pseudomonas exotoxin. After LMB2 is internalized into hCD25-bearing podocytes, PE38 is released to cytosol, and ADP-ribosylates elongation factor 2, thus inhibiting protein synthesis. A few days after the injection of LMB2, NEP25 mice develop massive proteinuria. Thereafter, podocytes are progressively damaged and ultimately lost from the glomerulus. The key target molecule(s) of LMB2 have not been identified.

In Nphs1-Cre/CAG-CAT-Z/NEP25 mice, the lacZ gene is driven by the β-actin-based CAG promoter, not by Nphs1 promoter. Without LMB2, all podocytes were positive for lacZ, demonstrating that the CAG promoter is active in intact podocytes. We found that the lacZ expression rapidly decreased as early as 18 hours after the injection of LMB2. Downregulation of synaptopodin, nephrin, WT-1 or podocalyxin, foot process effacement, proteinuria, and podocyte loss occurred at later time points. We performed similar experiments utilizing ROSA26-loxP mice instead of CAG-CAT-Z mice. In Nphs1-Cre/ROSA26-loxP/NEP25 mice, podocytes were labeled with lacZ driven by ROSA26 promoter. When podocyte injury was induced by LMB2 injection, the ROSA26 promoter-driven lacZ expression was not downregulated. Thus, the observed rapid downregulation of lacZ is related to the intrinsic property of the CAG promoter.

The 1.7kb-CAG promoter is composed of 1.3kb-chicken β-actin promoter, rabbit β-globin gene, and cytomegalovirus (CMV) enhancer for even higher and more stable activities. β-actin promoter on its own is highly active in a wide range of cell types, as is expected from the fact that β-actin is most abundantly expressed as a housekeeping gene in all non-muscle cells. Studies of transgenic mice, which carry green fluorescent protein under the control of the same CAG promoter we used, showed that the CAG promoter is highly active in podocytes as well as in skeletal muscle, heart and pancreas, which are rich in actin filaments. Although the downregulation of the β-actin promoter-based CAG promoter-driven lacZ expression may simply reflect downregulation of the promoter activity due to a direct action of LMB2 or indirectly through suppression of its enhancer element, we found that the intensity of F-actin was decreased in a punctuated pattern in podocytes 3 days after the injection of LMB2. These changes were observed only in podocytes, not in other cells. In mesangial areas, F-actin was partially increased.

Foot process effacement is associated with development of proteinuria and, if not repaired, leads to permanent deterioration of the filtration barrier. The molecular framework of foot process consists of actin filaments and actin-associated proteins. Disturbance of actin filaments is tightly correlated with the onset of massive proteinuria in puromycin aminonucleoside nephrosis model. NEP25 mice also show the disruption of F-actin, coinciding with proteinuria at early stage, and develop finally glomerular sclerosis. These suggest that disturbance of actin filaments may be one of the key initial events leading to subsequent podocyte damage and sclerosis.

In summary, we found that lacZ expression under the regulation of the CAG promoter was dramatically downregulated soon after the injection of LMB2 in NEP25 mouse model. Moreover, F-actin was disrupted also at an early stage. These suggest that disturbance of actin filaments may be one of the key initial events leading to subsequent podocyte damage.

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