学 位 論 文 の 要 旨

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学 位 論 文 名　Optimal Route of Diphtheria Toxin Administration to Eliminate Native Nephron Progenitor Cells in vivo for Kidney Regeneration

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論 文 内 容 の 要 旨

INTRODUCTION

Owing to the increase in patients with chronic kidney disease and those needing dialysis, the lack of organs for transplantation is a serious problem. There are many approaches to address this lack of organs. Research in regenerative medicine has made remarkable progress; for example, iPS cells can differentiate into nephron progenitor cells (NPCs) and ureteric bud cells in vitro. NPCs have attracted attention as cell sources for kidney regeneration.

We previously reported kidney regeneration from NPCs ex vivo and developed a host NPC elimination strategy using the iDTR system. The diphtheria toxin receptor (DTR) is expressed in Six2-positive NPCs; we eliminated host NPCs by diphtheria toxin (DT) administration. Using this method, 100% replacement of host NPCs with donor NPCs was possible by simultaneously eliminating host NPCs and transplanting donor NPCs. This kidney regeneration strategy has two important features: (1) the nephron genesis area of a xenogeneic host is used and (2) donor NPCs form host NPCs using the progenitor replacement system.

In our previous study, we evaluated the method ex vivo; in the progenitor replacement system, DT was added to fetal kidney culture medium and NPCs were replaced. We have also used an in vivo strategy to generate a functional kidney (Neo Kidney) de novo using an organogenic niche method that uses the inherent developmental system of an immunocompromised xenogeneic host. However, the natural kidney environment is considered important for kidney regeneration. Hence, it is necessary to investigate the progenitor replacement system using living fetuses in the womb, but such analyses using the iDTR system

are lacking. DT is a very large molecule; therefore, it likely cannot pass through the blood-placental barrier by intra-peritoneal DT administration, a common route.

Establishment of a method for elimination of NPCs in fetuses in the uterus, without embryonic lethality, is necessary for the success of our kidney regeneration strategy. Therefore, we investigated (1) the optimal route of DT administration and (2) optimal DT dose for NPC elimination. We successfully established a living NPC elimination model by optimized intra-amniotic DT injection, without embryonic lethality.

MATERIALS AND METHODS

All experiments with animals in this study were approved by the Ethics Committee for Animal Experimentation of Jikei University School of Medicine. C57BL6/NCrSlc mice were purchased from SLC Japan (Shizuoka, Japan). C57BL/6-Gt (ROSA) 26Sor [tm1(HBEGF)Awai]/J mice (iDTR) were purchased from Jackson Laboratory (Bar Harbor, ME, USA). Six2-GFP-Cre transgenic mice (Six2-mice) were gifted by McMahon. Six2-mice were crossed with iDTR mice to obtain bigenic offspring (Six2-GFP +/+ iDTR-mice).

Two pregnant female mice were injected intraperitoneally (IP) at E13.5 with 300 ng DT in 300 mL PBS. Additionally, for intra-amniotic injection, four pregnant female mice were anesthetized, a laparotomy was performed, and the uterus was delivered through the incision. Each embryo was microinjected with 25, 5, 0.5, or 0.05 ng/fetus-body DT diluted in 50 μL PBS. The uterus was placed back into the abdominal cavity, and the incision was closed. Embryos were allowed to develop in situ until the indicated stages. The fetus was delivered by caesarean section at E18.5, and the fetal mouse kidney and RNA expression were evaluated.

Kidneys were approximated to spheroids and the kidney volume was calculated using the spheroid calculation method (Kidney volume (mL) = $\pi/6 \times L \times W^2$).

Glomeruli were counted in the hematoxylin-eosin-stained E18.5 fetal mouse kidney and the sectioned area was determined. Next, the glomerular number was divided by the area of the section to obtain the glomerular number per unit area (pieces/mm^2). The number of glomeruli were counted by two different researchers.

RESULT AND DISCUSSION

We investigated the route of DT administration for fetal NPC elimination in the uterus. In the IP group, the fetal kidney exhibited a slightly reduced volume; however, the number of glomeruli per unit area did not differ between groups, and sufficient NPC elimination was not observed. For intra-amniotic injection, the fetal kidney exhibited a reduced volume, number of glomeruli per unit area decreased by 94%, and we obtained sufficient NPC elimination.

We performed a histological analysis of the fetal kidneys of Six2-GFP +/+ iDTR

transgenic mice (Six2-GFP +/+ iDTR) and Six2-GFP −/+ iDTR transgenic mice (Six2-GFP −/+ iDTR). The Six2-GFP +/+ iDTR fetal kidney was defective and the glomeruli were almost entirely eliminated. In Six2-GFP +/+ iDTR mice, Six2-positive cells and cells positive for podocin, a marker of mature glomeruli, decreased compared to those of Six2-GFP −/+ iDTR mice. The ureteric bud had complex branching in Six2-GFP −/+ iDTR, but less branching in Six2-GFP +/+ iDTR.

We compared mRNA expression levels between Six2-GFP +/+ iDTR and Six2-GFP −/+ iDTR fetal kidneys. The relative expression level of Six2 in Six2-GFP +/+ iDTR fetal kidneys was lower than that in Six2-GFP −/+ iDTR fetal kidneys. In addition, podocin, a marker of mature glomeruli, and Calbindin levels were lower in a Six2-GFP +/+ iDTR fetus kidney than in a Six2-GFP −/+ iDTR fetal kidney

We investigated the appropriate dose of DT. We administered 25, 5, 0.5, and 0.05 ng/fetus-body DT by intra-amniotic delivery. At 5 ng/fetus-body DT or greater, fetal kidney volume and number of glomeruli per unit area decreased. NPC elimination was lower for 5 ng/fetus-body DT than 25 ng/fetus-body DT.

The intra-peritoneal route was not sufficient for NPC elimination. For intra-peritoneal route, DT must pass through the placenta to exert its effects, and it is assumed that complete NPC elimination is difficult. By establishing that intra-amniotic injection is the optimal administration route for DT, these results will facilitate studies of kidney regeneration in vivo. In addition, this method might be useful for analysis of kidney development at various time points by deleting NPCs during development.

Establishment of NPC elimination in the uterus using the iDTR system is very important for establishing a new kidney regeneration method using NPCs. Furthermore, this method can also be applied to eliminate cells expressing other genes of interest, thereby helping to analyze the mechanisms underlying the development of congenital anomalies of the kidney and urinary tract by the elimination of specific cells at an arbitrary developmental stage. In addition, this model, in which Six2-positive NPCs are eliminated, can be used to analyze the effects of nephron reductions in mice.

CONCLUSION

This study is the first to report that DT eliminates NPCs in the viable state using the iDTR mouse model. This method for NPC elimination in the mother's womb using the iDTR system can not only be applied to kidney regeneration, but also to various disease models and embryology research.