ABSTRACT

The development of humanized liver with *Rag1* knockout rats

Background: The animal model with humanized liver is useful for testing of drug metabolism and toxicity in pre-clinical studies. It has been reported in mouse, which the liver was repopulated more than 90% with human-hepatocytes, however, in rat the target is far from being reached. In this study, we attempt to develop a humanized liver model with an immunodeficient rat.

Methods: *Rag1* knockout rats were treated with neonatal thymectomy. At 3 and 4 weeks of age, they were injected with hepatotoxin retrorsine; 2 weeks after, the animals were subjected to 70% partial hepatectomy and transplanted with immature human hepatocytes via portal vein. The recipients were also treated with anti-asialo GM1 antibody weekly from the day before transplantation, and were injected with FK506 every 3 days after transplantation.

Results: In *Rag1* knockout rats, B lymphocytes were deleted almost completely in peripheral blood. However, T and NK lymphocytes were kept present. When they were treated additionally with neonatal thymectomy for T lymphocytes deletion, and suppressed neutralized NK lymphocytes with anti-asialo GM1, B, T and NK cells in lymphocytes down to very low levels as 0.75%, 1.58% and 0.26% respectively. After transplanted human donor hepatocytes into retrorsine-treated recipient livers, at week 3 the human cell-derived hepatic colonies were expanded in recipient liver and the liver repopulated rate with human hepatocytes reached to approximately 17%. The human

the recipient rat.

In conclusion, it is possible to generate a chimera animal with humanized liver by a novel severe immunodeficient rat model.

TITLE PAGE

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INTRODUCTION

Laboratory animal is essential for *in vivo* studies. However, it has limit to use as alternative to human. As a solution, to develop humanized animals such as humanized liver model has been expected. Humanized liver animals are useful for testing of drug metabolism and toxicity in pre-clinical studies. It is reported in mouse, which the liver is repopulated more than 90% with human-hepatocytes (1), but in rat the target is far from being reached (2-4). Since the rat has historically been the preferred animal model for biomedical research, with physiology closer to humans and with larger sample volumes, to develop a humanized liver model with rat has an increased need. Recently, established Zinc finger nuclease technology makes it possible for immunodeficient rats (4). In this study, we attempt to develop a humanized liver model with an immunodeficient rat.

MATERIALS AND METHODS

Rag1 knockout (KO) rats (SAGE Labs) were treated with neonatal thymectomy less than 24hours after birth (5). At 3 and 4 weeks of age, they were injected with hepatotoxin retrorsine (Sigma) intraperitoneally; after 2 weeks, transplanted with immature human hepatocytes (Cell Systems Kirkland) via portal vein after 70% partial hepatectomy (PH). The recipients were injected with anti-asialo GM1 (Wako, Japan) antibody weekly from the day before transplantation, and were injected with FK506 (Astellas, Japan) every 3 days after transplantation. Peripheral blood lymphocytes of rats were analyzed using fluorescence-activated cell sorter (MoFlo Astrios,

Beckman Coulter) with IOTest Anti-rat CD45-PE (Beckman Coulter), IOTest Anti-rat CD3-FITC, CD45RA-PC7, and CD161a-APC (Beckman Coulter). Liver repopulation rate with human hepatocyte was analyzed using immunohistochemical staining with anti-human CK8/18 (Progen) antibody and fluorescence microscope BZ-9000 (KEYENCE, Japan). Human derived hepatocyte function was detected with ABI TaqMan Gene Expression Assays (Applied Biosystems) for human hepatocyte-specific gene, albumin, alpha-fetoprotein, CYP3A4, CYP2C18, CYP2C9 and CYP2C19.

RESULTS

In *Rag1* KO rats, B lymphocytes were deleted almost completely in peripheral blood. However, T and NK lymphocytes were kept present. To further block the left lymphocytes, we combined with neonatal thymectomy for T lymphocytes suppression, and neutralized NK lymphocytes with anti-asialo GM1 antibody. The results showed as in Fig 1A that B, T and NK cells in all lymphocytes down to very low levels as 0.75%, 1.58% and 0.26% respectively. Compared with that in wild type animals, B, T and NK lymphocytes were inhibited to 1/9, 1/29, and 1/5 fold respectively. Those results showed that the model rat expressed extremely low-level of the three-immunocytes. After transplanted human donor hepatocytes into retrorsine-treated recipient livers, at week 3 the human cell-derived hepatic colonies were expanded in recipient liver and the repopulation rate with human hepatocytes reached to approximately 17% (Fig1B). Comparing with the non-transplant donor cells, the human hepatocyte-specific gene expressions, albumin, CYP3A4, CYP2C18 and

CYP2C9 could be enhanced in the recipient liver as much as 3.5-, 88.5-, 23.7- and 584.9-fold respectively (Fig1C), however, alpha-fetoprotein and CYP2C19 were kept at a low or non-detectable level.

DISCUSSION AND CONCLUSION

Why it is difficult to generate a humanized liver in rat? One of the key reasons, there was no immunodeficient rats by underdeveloped genome editing technology. Recently, the technology for rats was well established (4). By use of Zinc finger nuclease, *Rag1*KO rats have early marketed immunodeficient animals. *Rag1* is important for T and B cells differentiation. However, in this study, we found that knockout *Rag1* have no critical affect on the T cell development. As a solution to this problem, we treated thymectomy in *Rag1*KO rat before T cell maturation, to delete almost all of the T cells from peripheral blood. Combined with using anti-asialo GM1 antibody, it led to not only neutralize NK cells, but also suppress the mature T cells which developed out of thymus. The results clearly demonstrated that we succeeded to have developed a novel severe immunodeficient rat model with extremely low-level of the three-immunocytes. After subject to above multiple treatments, *Rag1*KO rats could be used as the recipient animals for humanized liver generation. The next limiting factor, as previous study showed that the transplanted cells, poor proliferative adult human hepatocytes led to repopulation rate of humanized rat no more than 2.5% (2). To overcome the problem, we transplanted with immature hepatocytes that have high proliferation capacity. As a result, at week 3 the human cell-derived hepatic colonies were expanded in immune

suppressed livers and the repopulation rate with human hepatocyte reached to approximately 17%.

In conclusion, it is possible to generate a chimera animal with humanized liver by developing the novel severe immunodeficient rat model in present study. However, the further efforts should be made to improve the chimeric rate and enhance the hepatic function in the future.

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REFERENCES

1. Hasegawa M, Kawai K, Mitsui T, et al: The reconstituted 'humanized liver' in TK-NOG mice is mature and functional. Biochem Biophys Res Commun 405(3):405-10, 2011.
2. Tachibana A, Tateno C, Yoshizato K: Repopulation of the immunosuppressed retrorsine-treated infant rat liver with human hepatocytes. Xenotransplantation 20(4):227-38, 2013.
3. Igarashi Y, Tateno C, Tanaka Y, et al: Engraftment of human hepatocytes in the livers of rats bearing bone marrow reconstructed with immunodeficient mouse bone marrow cells. Xenotransplantation 15(4):235-45, 2008.
4. Mashimo T, Takizawa A, Kobayashi J, et al: Generation and characterization of severe combined immunodeficiency rats. Cell Rep 2(3):685-94, 2012.
5. Gordon C Hard: Thymectomy in the neonatal rat. Lab Anim 9(2):105-10, 1975.

Figure Legend

Figure 1. A severe immunodeficient rat model and the humanized liver.

- A.** The frequency of T, B, and NK cell in CD45-positive lymphocytes of wild type (WT) SD rats and *Rag1*KO rats at 6 weeks of age treated with no, anti-asialo GM1 only, thymectomy only and both anti-asialo GM1 and thymectomy. The data is collected from 3-6 individual experiments and analyzed as following with flow cytometry: T cell = $CD3^+CD45^+$; B cell = $CD45RA^+CD3^-CD45^+$; NK cell = $CD161a^+CD3^-CD45^+$; **B.** The chimeric liver with human donor derived hepatic colonies at 3 weeks after transplantation in severe immunodeficient *Rag1* KO rat. The liver repopulation with human hepatocytes reached to 17%. Human CK8/18 positive immunostained cells means human derived hepatocytes. Scale bar = 1 mm. **C.**

Relative expression of human hepatocyte genes in rat recipient liver with TaqMan PCR and

normalized by Human ACTB (beta-actin). Filled bars represent non-transplant cell controls

and open bars represent donor derived human cells in rat livers after 3 wk transplantation.

Figure 1

A

C

B