Research Article

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Humanized mice efficiently engrafted with fetal hepatoblasts and syngeneic immune cells develop human monocytes and NK cells

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See Editorial, pages 245–248

Background & Aims: Human liver chimeric mice are useful models of human hepatitis virus infection, including hepatitis B and C virus infections. Independently, immunodeficient mice reconstituted with CD34$^+$ hematopoietic stem cells (HSC) derived from fetal liver reliably develop human T and B lymphocytes. Combining these systems has long been hampered by inefficient liver reconstitution of human fetal hepatoblasts. Our study aimed to enhance hepatoblast engraftment in order to create a mouse model with syngeneic human liver and immune cells.

Methods: The effects of human oncostatin-M administration on fetal hepatoblast engraftment into immunodeficient *fah*$^{-/-}$ mice was tested. Mice were then transplanted with syngeneic human hepatoblasts and HSC after which human leukocyte chimerism and functionality were analyzed by flow cytometry, and mice were challenged with HBV.

Results: Addition of human oncostatin-M enhanced human hepatoblast engraftment in immunodeficient *fah*$^{-/-}$ mice by 5–100 fold. In contrast to mice singly engrafted with HSC, which predominantly developed human T and B lymphocytes, mice co-transplanted with syngeneic hepatoblasts also contained physiological levels of human monocytes and natural killer cells. Upon infection with HBV, these mice displayed rapid and sustained viremia.

Conclusions: Our study provides a new mouse model with improved human fetal hepatoblast engraftment and an expanded human immune cell repertoire. With further improvements, this model may become useful for studying human immunity against viral hepatitis.

Lay summary: Important human pathogens such as hepatitis B virus, hepatitis C virus and human immunodeficiency virus only infect human cells which complicates the development of mouse models for the study of these pathogens. One way to make mice permissive for human pathogens is the transplantation of human cells into immune-compromised mice. For instance, the transplantation of human liver cells will allow the infection of these so-called "liver chimeric mice" with hepatitis B virus and hepatitis C virus. The co-transplantation of human immune cells into liver chimeric mice will further allow the study of human immune responses to hepatitis B virus or hepatitis C virus. However, for immunological studies it will be crucial that the transplanted human liver and immune cells are derived from the same human donor. In our study we describe the efficient engraftment of human fetal liver cells and immune cells derived from the same donor into mice. We show that liver co-engraftment resulted in an expanded human immune cell repertoire, including monocytes and natural killer cells in the liver. We further demonstrate that these mice could be infected with hepatitis B virus, which lead to an expansion of natural killer cells. In conclusion we have developed a new mouse model that could be useful to study human immune responses to human liver pathogens.

Keywords: Xenotransplantation; Liver chimeric mice; Human immune cells; Hepatitis B virus.

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Abbreviations: NK cell, natural killer cell; HBV, hepatitis B virus; HSC, hematopoietic stem cell; HIS, human immune system; HIS-Hep mice, mice with human immune system and liver xenograft; HCV, hepatitis C virus; fah, fumaryl acetoacetate hydrolase; hAlb, human serum albumin; NRG, NOD *rag1*$^{-/-}$ *il2rγnull* mouse; FRG, *fah*$^{-/-}$*rag2*$^{-/-}$*il2rgnull* mouse; FNRG, *fah*$^{-/-}$ NOD *rag1*$^{-/-}$*il2rgnull* mouse; NTBC, 2-(2-nitro-4-fluoromethylbenzoyl)-1,3-cyclohexanedione, OSM, oncostatin-M.

Introduction

Many human pathogens fail to infect small animals, limiting investigations to nonhuman primates or clinical observational studies. One strategy to study such pathogens *in vivo* is the engraftment of human tissues into mice. For example, human immune system (HIS) mice are widely used to study leukotropic

pathogens like human immunodeficiency virus (HIV) and Epstein-Barr virus [1]. Separately, human liver chimeric mice have proven relevant models for investigations of HBV, hepatitis C virus (HCV) and malaria species [2–4].

Humanization of mice generally follows two principles: a human graft most efficiently develops when the mouse equivalent is impaired, and recipient mice are immunodeficient in order to prevent xenorejection. In the case of HIS mice, transplantation of human CD34$^+$ HSC into immunodeficient recipients, e.g. NOD *rag1*$^{-/-}$ *il2r*γ^{null} (NRG) mice, reliably results in the development of human T and B cells [1]. However, the development of human monocytes and natural killer (NK) cells is impaired in HIS mice, likely due to limited cross-reactivity of mouse and human hematopoietic cytokines, e.g. granulocyte-macrophage colony stimulating factor (GM-CSF), macrophage colony stimulating factor (M-CSF), interleukin(IL)-3 and IL-15 [1]. In the case of human liver chimeric animals, a form of mouse liver injury is combined with a murine immunodeficiency, typically lymphocyte deficiency [3]. After transplantation of adult or pediatric human hepatocytes in such models, the murine liver injury creates a niche and drives the proliferation of human hepatocytes in the mouse liver, and this can result in high human chimerism [5,6]. However, the required murine immunodeficiency in these animals has precluded the study of cellular immune functions in human liver disease.

One solution to address this lack of cellular immunity in human liver chimeric mice is to combine transplantation of human hepatocytes with human HSC into the same recipients. This has recently been reported by transplanting adult human hepatocytes and mismatched HSC derived from fetal human liver tissue, which resulted in doubly engrafted animals [7,8]. However, to avoid allogeneic immune responses both grafts should be derived from the same human donor. Possible solutions include the generation of human hepatocytes and HSC from induced pluripotent stem cells (iPS cells). Human HSC can also be obtained from adults [9] but hepatocytes from these same individuals are rarely available. When fetal hepatoblasts are combined with syngeneic HSC the liver graft either was lost [7] or the engrafted hepatoblasts fail to expand to high levels [10,11]. Together, these findings suggest that allogeneic doubly reconstituted mice can be generated but that human fetal hepatoblasts insufficiently engraft human liver chimeric mice, which limits the generation of syngeneic doubly reconstituted mice.

Here we first set out to generate mice that were highly engrafted with fetal hepatoblasts, and then combined this model with HSC transplantation obtained from the same human donor. These doubly engrafted animals, which we term 'HIS-Hep' mice, display improved human monocyte and NK cell development compared to HIS mice and can support HBV infection.

Materials and methods

Generation of liver chimeric mice

Adult human hepatocytes were harvested from surgical resection specimens as described [12], shipped overnight and transduced with VSVg pseudotyped lentiviral particles encoding for firefly luciferase under the human albumin promoter (FLuc pp) [13] prior to transplantation. For human hepatoblast transplantation, human fetal livers were procured from Advanced Bioscience Resources, Inc. Fetal livers were cut with scissors into gel-like substance and digested with 0.05% collagenase (Roche) for 30 min at 37 °C. Cells were put over 100 μm cell strainer (BD Bioscience), washed with William's E media and after resuspension allowed to sediment at 1 g for 1 h [13]. The cell pellet, designated the large cell fraction, was freshly transplanted or after culture for 3 days to 2 weeks as described [13]. Cultured cells were resuspended using Accumax (eBioscience). Mouse hepatocytes from adult mice or unfractionated embryonic day 14 fetal liver cells were obtained from Cre recombinase transgenic mice crossed to FLuc transgenic mice preceded by a floxed stop codon, both obtained from Jackson Labs. Using isoflurane anesthesia human or mouse cell suspensions were injected intrasplenically into *fah*$^{-/-}$*rag2*$^{-/-}$*il2rgnull* (FRG) [14,15] of either gender or female *fah*$^{-/-}$ NOD *rag1*$^{-/-}$*il2rgnull* (FNRG) mice that were generated by 13 generation backcrossing of the *fah*$^{-/-}$ allele [16] to NOD *rag1*$^{-/-}$*il2rgnull* (NRG) animals obtained from Jackson Labs. Human fetal hepatoblasts were transplanted at 2.5–5 $\times$ 10^5 cells per mouse, adult human hepatocytes at 2.5 $\times$ 10^6 cells per mouse, and mouse hepatocytes or fetal liver cells at 5 $\times$ 10^5 cells per mouse. Starting on the day of transplantation mice were cycled off the drug NTBC/nitisinone (Yecuris) as described by others [5,14].

Generation of doubly reconstituted HIS-Hep animals

For concurrent transplantation, adult (>6 week old) female FNRG mice were irradiated with 100 cGy 12–24 h prior to intrasplenic transplantation of 2.5–5 $\times$ 10^5 fresh fetal hepatoblasts and 1.5–2 $\times$ 10^5 freshly isolated syngeneic HSC. hOSM treatment was started one day after transplantation for 3–4 weeks while mice were cycled off NTBC. For sequential transplantation mice received fresh hepatoblasts and, once median human albumin levels reached >10 μg/ml levels, mice were irradiated and received cryopreserved syngeneic HSC. After HSC transplantation NTBC cycling continued. As negative controls NRG mice were doubly transplanted or female FNRG mice were singly transplanted with HSC and cycled off the drug NTBC in parallel to doubly transplanted FNRG mice.

Ethics statement

All human materials were obtained after written informed consent was obtained from patients. The use of these materials was reviewed and approved by the institutional review boards of the Rockefeller University, Weill Cornell Medical College and Mayo Clinics. All procedures involving mice were in accord with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and approved by the Rockefeller University Institutional Animal Care and Use Committees (protocol 12536).

All other methods are described in the Supplementary materials and methods section.

Results

Hepatoblasts inefficiently reconstitute mouse livers

Since the large cell fraction of human fetal liver contains hepatoblasts, we set out to create human liver chimeric mice starting with this fraction, from here on referred to as hepatoblasts. After transplantation of fresh or *in vitro* cultured [13] hepatoblasts into fumaryl acetoacetate hydrolase deficient (*fah*$^{-/-}$)*rag2*$^{-/-}$ *il2r*γ^{null} (FRG) mice [5,14] no human albumin (hAlb) (Supplementary Fig. 1A) or human transferrin (hTF) could consistently be detected in mouse serum. In order to distinguish whether this was due to a failure to engraft or to proliferate after transplantation, we transduced fresh hepatoblasts with firefly luciferase (FLuc) lentiviral vectors. In some recipients, a luminescent signal could be detected (Supplementary Fig. 1B) for up to 4 months after transplantation. However, and in contrast to adult hepatocytes, the luminescent signal did not consistently increase over this time period (Supplementary Fig. 1C). Because this could be due to an inherent inability of hepatoblasts to proliferate in such models [7,17] or because certain factors cross-react poorly between mouse and human, we next attempted to reconstitute FRG livers with murine hepatoblasts expressing FLuc. Adult mouse hepatocytes engrafted faster and at higher frequency than embryonic day 14 mouse liver cells, but several fetal cell recipients showed