Development of murine models to study Hepatitis C virus induced liver pathogenesis

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Abstract Hepatitis C virus (HCV) is involved in different liver pathologies worldwide. In contemporary scenario, HCV treatment is lagging behind owing to absence of vaccines against virus. The only consideration for HCV treatment is pegylated interferon-alpha and ribavirin that results in sustained virological response in 50 % of patients. Two feasible hosts for HCV infection are chimpanzee and humans. For decades, chimpanzees are sole host to study HCV pathogenesis, but their use is limited due to ethical issues. The dilemma behind HCV therapy is the need of sustainable animal models that can help simulate in vivo conditions. We have assembled recent advances in animal models to study liver diseases for targeted therapy.

Keywords Murine models · Hepatitis C virus · Liver pathogenesis

Introduction

Hepatitis C virus (HCV) affects 180 million people worldwide chronically with a risk of progression to hepatic steatosis, insulin resistance, fibrosis, and cirrhosis and hepatocellular carcinoma [1]. There is no vaccine available for HCV and contemporary treatment choice offered is

combination therapy of interferon alpha (IFN- α) and ribavirin with subordinate responses [2]. The development of additional preventive measurements has been hampered due to lack of suitable animal model. The reason might be the limited species tropism of HCV [1].

Chimpanzees are the sole offered host to decipher the physiology of disease outside the human body. However their use is limited due to high cost, restricted accessibility and ethical issues. The natural history of HCV in chimpanzee differs from human in that they rarely evolve towards chronic disease conditions and once become chronically infected show no signs of liver damage precluding their use for the understanding of end stage liver disease. A tiny animal model with exogenously introduced virus that mimics actual disease conditions have always been a need of time. These models are necessary for preclinical testing whereas designing antiviral compounds and opening new horizons to untangle the mechanisms involved in viral pathogenesis [1].

Chimeric mouse models carrying human liver-uPA–SCID had been made for the analysis of antivirals against HCV and HBV [3]. Unfortunately, chimeric mice although can replicate HCV in appropriate environment however phenomenon of virus assembly and unleash still remains unknown [1]. To better understand the phenomenon, humanized mice model with human immune system has been made that mimics the human immune system with successful induction of liver diseases. This model proved to be an excellent success to elucidate the HCV pathogenesis with effective human immune system [4]. Another innovation towards humanized mouse model was development of xenograft model of Hepatocellular carcinoma (HCC). In this xenograft mouse model, human tumor cells were implanted in the liver of an immune deficient mouse. This model is relatively simple compared to the human liver chimeric mouse model [5]. Besides successful efforts on

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humanized mice, a lot of work has been done on genetically modified mouse models using knock down strategies to understand role of various genes in liver diseases.

Recent advances in the murine model have helped to understand the liver diseases as well immune responses, mimicking human systems. Engraftment of human liver as well hematopoietic system allowed scientists to focus on studying human liver infections and resolution at the same time. This review radically focuses that how these different models can be useful for scavenging fatal disease condition in chronic liver disease. But these areas need more investigation for rational designing of models that can further help to sequester disease at early stage and find new targeted therapies, designing of antivirals or vaccines for clearance of disease.

Humanized mouse models

Humanized mouse models have recently taken place as powerful tools in the investigation of human pathologies [6–8]. These small animal models have been adapted with human cell or tissue engraftments that supposed to be ideal for studying different disease conditions [9].

Liver-uPA–SCID

Enormous efforts have been ended to make humanized mouse models to study the role of HCV for emerging antiviral strategies [10]. An efficacious mice model with capability of engrafting human hepatocytes into the diseased livers of albumin-urokinase (Alb-uPA) transgenic mice was made [11]. The Alb-uPA transgenic mouse carried a tandem array of four murine urokinase genes controlled by an albumin promoter that targets urokinase over-production to the liver resulting in augmented hepatic cell death [12]. Mice homozygous for SCID mutation lacks mature T and B lymphocytes and are unable to reject xenografts [13]. Hence Alb-uPA and C.b-17/SCID/bg mouse were crossed to get homozygous SCID mice. These mice livers were repopulated with human hepatocytes. After successful engraftment homozygous SCID/Alb-uPA mice with chimeric human livers were infected de novo with HCV-positive human serum. HCV replication was well supported in human liver portions. A number of infections with different genotypes were successfully established [12].

Humanized mouse model with immune system

Alb-uPA/SCID was an efficacious model for HCV replication but lacks functional immune system to study HCV immune-pathogenesis. A mouse engrafted with human liver cells possessing a functional human immune system

represented itself an excellent model to study viral pathogenesis in natural host. This animal model involved the engraftment of a fusion protein FK506 binding protein (FKBP) and caspase 8 under control of the albumin promoter (AFC8) which depleted liver cells in mice. Latter, transgenic mice was co-transplanted with human CD34⁺ hematopoietic stem cells (HSC) and hepatocytes progenitors that led to efficient engraftment of human leukocytes and hepatocytes. The manipulated infection in this model induced hepatic inflammation, hepatitis fibrosis and immune responses similar to humans (Fig. 1).

There was an increase in the multiple human leukocyte subsets including CD3⁺CD4⁺ and CD3⁺CD8⁺ T cells, CD3⁺CD56⁺ natural killer cells, and plasmacytoid dendritic cells (CD123⁺CD4⁺CD3⁺). AFC8-hu HSC/Hep mice developed liver fibrosis determined by expression of Collagen 1A1 and tissue inhibitor of metalloproteinase TIMP1 in activated hepatic stellate cells signifying successful infection of HCV. Hence, this model was an innovation to uPA/SCID transgenic mice that only supported HCV infection however no substantial liver fibrosis [4].

Xenograft model for hepatocellular carcinoma

Development of a novel antiviral to target HCV is a real goal. There had been a lot of work done to target multiple

Fig. 1 Liver mouse models categorized on the basis of their construction. Humanized mouse model made by engraftment of human hepatocytes and hematopoietic cells in mouse. Xenograft model made by mouse adapted replicon cell-line with luciferase reporter. Genetically humanized models made by intravenous delivery of recombinant adenoviruses encoding human CD81, Scavenger receptor class B member 1 (SCARB1), claudin (CLDN1) and/or occludin (OCLN) to mice. Transgenic mice were made by random integration of transgenes or at encoded sites by homologous recombination in embryonic stem (ES) cells

steps in HCV life cycle, virus assembly, genome replication, and release, HCV-full length genome availability in cell culture system [5]. A number of strategies used before were anti-sense oligonucleotides, siRNA, ribozymes and recombinant antibodies to inhibit HCV replication in cell culture models [14, 15]. There was a need to cultivate a small animal model to test all these experimental therapies that had been developed using cell culture systems. A number of models had been urbanized by transfecting HCV RNA into the mouse liver using transgenic technology [16]. But these transgenic mice models were not suitable for HCV infection because of stable integration of HCV cDNA into the mouse chromosomes, making it difficult to differentiate the viral RNA replication from the HCV RNA produced due to cellular transcription in the transgenic animal models [5]. To study HCV infection in vivo different mouse models had been developed: the immunotolerized rat model [17]; the Trimera mouse model [18]; the uPA/SCID mouse model; [12, 19, 20] and the Fah^{-/-} Rag^{-/-} IL-2^{-/-} mouse model [21]. However, these models required specialized surgical skills, mouse strains and human hepatocytes.

Recently, a xenograft mouse model had been developed encompassing a mouse adapted replicon cell-line with luciferase reporter [5]. Huh-7 cells were transfected with a transcribed sub-genomic HCV-RNA replicon. A stable Huh-7 cell line with replicating HCV GFP chimera RNA (S3-GFP replicon) was developed (Fig. 2). Stable cell line expressing sub-genomic RNA of JFH1 strain tagged with GFP was injected subcutaneously into γ -irradiated SCID mice. The replicon cell line was implanted in SCID mice for the development of tumor. And passaging of replicon assures the mouse-adapted HCV-GFP replicon cell line; tumor cells were collected with replicating HCV-GFP from SCID mice. HCV RNA titer measured in the tumor was comparable to chronically infected HCV patients. Further, inhibition with interferon- α ensures the presence of replicating RNA in tumors [5].

Genetically humanized mice

Chimpanzee model showed restricted tropism that might result from viral dependence on host factors present in only a few cell types. Murine cells have been found resistant to HCV entry and showed inefficient viral replication that might block at later life cycles [22]. Previously, this fact has been established that HCV uptake by human hepatocyte depends minimally on two factors CD81 and OCLN. This led to the hypothesis that expression of these human orthologous could render mice susceptible to HCV infection in vivo.

Recombinant adenoviruses encoding human CD81, Scavenger receptor class B member 1 (SCARB1), claudin

(CLDN1) and/or occludin (OCLN) were intravenous delivered to mice. These vectors resulted in 100 to 1,000-fold over-expression of the corresponding messenger RNA in the murine liver and strong expression of all four proteins with the expected sub-cellular distribution. As virus replication has been reported inefficient in murine liver in vitro and in vivo, so challenging mice expressing all four human factors with a firefly luciferase (Fluc) encoding HCV genome (Jc1FLAG2(p7Fluc2A) did not yield bioluminescent signal above background. This defect arose due to in-compatibility between the viral replication machinery and murine factors and/or from exacerbated murine innate antiviral responses. Furthermore, adenoviral gene delivery strongly induces interferon-stimulated mouse genes creating an environment that mimics recombinant IFN- α treatment and may antagonize HCV replication [23].

As an alternative approach, a bicistronic HCV genome expressing CRE recombinase (Bi-nlsCre-Jc1FLAG2, abbreviated HCV-CRE), which activates a lox P-flanked luciferase reporter in the (Rosa26-Fluc) mouse, was hydrodynamic delivered into Rosa26-Fluc mice. Rosa26-Fluc mice contain the firefly luciferase (luc) gene inserted into the Gt (ROSA) 26Sor locus. Expression of the luciferase gene is blocked by a loxP-flanked STOP fragment placed between the luc sequence and the Gt (ROSA) 26Sor promoter. CRE recombinase mediated excision of the transcriptional stop cassette results in luciferase expression in Cre-expressing tissues. That led to reporter signal in the liver, indicating that CRE recombinase is active in the context of the HCV genome [22]. For the estimation of number of HCV-infected liver cells, an indicator mouse was used in which CRE leads to activation of a nuclear-localized green fluorescent protein/ β -galactosidase (GNZ) reporter (B6.129-Gt(ROSA)26-Sortm1Joe/J, abbreviated Rosa26-GNZ) [24].

HCV infection in this mouse model was observed across a panel of chimaeras expressing the structural proteins of diverse HCV genotypes. HCV was evaluated in genetically humanized animals (Rosa26-Fluc) after immunizing intraperitoneally with plaque forming units (p.f.u.) of recombinant vaccinia virus expressing the HCV proteins C-E1-E2-p7-NS2 (strain HCV-1, genotype 1a) [25]. Robust titer of anti-E2 antibody and decreased susceptibility to heterologous challenge with HCV-CRE, expressing the structural proteins of genotypes 1b, 2a, was observed.

Transgenic mouse model

The mouse genome has greatly been modified by random integration of transgenes or at encoded sites by homologous recombination in embryonic stem (ES) cells. This provides us with a great advancement for understanding mammalian gene function in health and diseases [26, 27].

Fig. 2 Development of stable Huh-7 cell line with replicating HCV GFP chimera RNA (S3-GFP replicon). Cell line expressing RNA of JFH1 strain tagged with GFP injected subcutaneously to SCID/bg mice for the development of hepatocellular carcinoma

Site-specific recombination proved to be powerful tools that allow chromosomal manipulation in controlled manner in mice and allow establishment of human disease models through understanding genome functions.

Liver cancer mouse model

Hepatocellular carcinoma is the third major cause of deaths worldwide particularly in Africa and Asia. This end stage liver disease has revealed the engrossment of a number of genes. Some of them spontaneously induce cellular carcinoma and others augment the chemically induced HCC. Cellular-myelocytomatosis (c-Myc) and transcription factor (E2F1) have been found to involve in a wide variety of tumors, including HCC [28–30]. The importance of c-Myc and transcription factor (E2F1) in tumour development is outlined due to the role in DNA repair, proliferation and apoptosis. Both oncogenic proteins are capable of sensitizing cells to a wide range of pro-apoptotic stimuli, via both p53-dependent and -independent mechanisms. At the same time, it interacted with other signaling pathways to contribute towards malignant phenotype in HCC [31]. In c-Myc driven experimental hepatocarcinogenesis and in human HCC, activation of Wnt/ β -catenin signaling has been frequently reported.

A c-Myc/E2F1 transgenic mouse was generated by crossing homozygous c-Myc [32] with homozygous E2F1 mice [29] to understand the role of β -catenin in hepatocarcinogenesis. It is demonstrated that co-expression of c-Myc and transcription factor (E2F1) allows activation of wnt protein signaling pathway with more aggressive

damage to liver. There were over expression of Wnt-1, Frizzled 1 and 2 receptors, Dishevelled-1, GSK-3 β inactivation, microsatellite instability at the Axin locus as well as induction of β -catenin target genes, such as glutamine synthetase, glutamate transporter-1, and Wisp-1. Hepatocellular carcinomas (HCCs) with activated β -catenin level displayed significantly higher proliferation rate and larger tumor size [28].

Liver fibrosis mouse models

Hepatic fibrosis, pathological features of end stage liver disease, is a wound healing process that involves the replacement of type-IV collagen rich extracellular matrix with “scar” matrix composed of type I collagen. The main culprit of fibrosis is perivascular mesenchymal cells, the hepatic stellate cells. In hepatic injury, HSC undergo transition to myofibroblast like cells. In addition, HSC also produce matrix metalloproteinases which degrade extra scar material, so help in remodeling of the normal architecture of liver [33].

LIM homeobox gene has been reported to express in hepatic stellate cells HSCs. Lhx2 expression in HSC is important to maintain proper cellular architecture and differentiated liver symmetry. Lhx2 $^{-/-}$ livers have been shown with disrupted cellular architecture and altered gene expression of hepatic cells and augmentation of extra cellular matrix proteins leading to fibrosis. This ultimately triggers wound healing process leading to chronic liver injury [34].

To determine the role of Lhx2 $^{-/-}$ in fibrosis, Lhx2 gene was disrupted using homologous recombination in ES

cells. A replacement targeting vector, having neomycin resistance-gene PGK-neo, was used to delete the exons encoding the LIM domains [35]. Herpes simplex thymidine kinase, under control of the phosphoglycerate kinase promoter (PGK-tk), has been used to select against random insertion events [36]. The confirmed clones were injected into the blastocysts of C57BL/6 mice then heterozygotes were inter-crossed to obtain *lhx2*^{-/-} mice. Liver tissues obtained from the mutant mice showed an ongoing increase in the fibrogenic process with augmentation of various extra cellular matrix proteins collagens type I and III, fibronectin, collagen type IV and laminin [36].

CRP/TGF- β 1 transgenic mouse model

Transforming growth factor (TGF)- β 1 has a fundamental role in the cytokine signaling pathway involved in the hepatic fibrosis [37]. TGF- β 1 predominantly affects activated hepatic stellate cells (myofibroblasts) and causes deposition of extracellular matrix components like fibronectin, collagens type I, III, and IV, tenascin, elastin, osteonectin, biglycan, and decorin by fibroblasts [38, 39]. The basal level of hepatocellular TGF- β 1 was controlled by the highly inducible human CRP(C-reactive protein) gene promoter. CRP gene was used as a signature that TGF- β expression measured firmly belongs to liver and also to control the low basal levels of TGF- β . To understand the role of TGF- β 1 in collagen deposition in hepatic fibrosis, transgenic mice with fusion gene consisting of a modified cDNA for simian TGF- β 1 and modified portion of the human CRP gene was generated.

This transgenic construct of TGF- β 1 was made by site-directed mutagenesis replacing cysteines at positions 223 and 225 with serine to produce active form of TGF- β 1 without need for dimerization and cleavage of the pro-region [40]. Transgenic mice were generated by microinjection of CRP/TGF β 1 construct in fertilized eggs of FVB/NHSD mice. Offspring tail DNA was isolated 3 week after birth and was further confirmed for the presence of the CRP/TGF β 1 by Southern blotting. Hepatic expression of the transgene-derived TGF- β 1 was achieved by intraperitoneal injection of 100 μ g lipo-polysaccharide (LPS). There was transient up-regulation of pro-collagen I and pro-collagen III.

This transgenic mouse proved to be an important advancement for in vivo evidence that TGF- β 1 has role in myofibroblast activation and hepatic fibrogenesis. As TGF- β 1 expression was controlled in this model, this model allowed us the successful study of the regulation and kinetics of fibrogenesis and fibrolysis as well as the degree of reversibility of liver fibrosis. The CRP/TGF- β 1 transgenic mouse model might be used in future for the testing of antifibrotic agents [41].

Concluding remarks

To sum up the entire chronicle, animal models for liver disease explicated up till now, have provided us with rejuvenated concept of disease and host defenses against HCV. A u-PA/SCID mouse supports human hepatocytes grafts and chimeric animal supports HCV infection. But mice have poor health particularly neonatal death. There is no evidence of liver fibrosis, so this model can't be well suited for chronic disease study supported by HCV. Additionally, lack of functional human immune system failed to help understand the immune responses in HCV. AFC8 mice support liver apoptosis by caspase8 and allow studying immune response as well as helping understand liver disease at chronic stage as liver fibrosis. But it supports only 15 % of engraftment compared with u-PA/SCID mice that supports 85 % of engraftment. This model also shows very less viremia observed due to lesser engraftment. A xenograft mouse is a less expensive and non-infectious model to study tumours showing high replication supported by SCID mice. This cannot be used to study HCV related immuno-pathogenesis as SCID mouse lacks B and T lymphocytes. Genetically humanized mice help understand HCV entry receptors in hosts via genetic approach and targeting of these receptors by rVV expression HCV proteins in immunocompetent mice but hampers vaccination studies due to lack of functional immune system. Transgenic mice allow modification in ES cells targeting a particular gene to be modified using homologous recombination. But Integration of genes into host might result in an increased copy number with profound expressions.

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