



Animal models for the study of hepatitis C virus infection and replication

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Abstract

Hepatitis C virus (HCV) hepatitis, initially termed non-A, non-B hepatitis, has become one of the leading causes of cirrhosis and hepatocellular carcinoma worldwide. With the help of animal models, our understanding of the virus has grown substantially from the time of initial discovery. There is a paucity of available animal models for the study of HCV, mainly because of the selective susceptibility limited to humans and primates. Recent work has focused modification of animals to permit HCV entry, replication and transmission. In this review, we highlight the currently available models for the study of HCV including chimpanzees, tupaia, mouse and rat models. Discussion will include methods of model design as well as the advantages and disadvantages of each model. Particular focus is dedicated to knowledge of pathophysiologic mechanisms of HCV infection that have been elucidated through animal studies. Research within animal models is critically important to establish a complete understanding of HCV infection, which will ultimately form the basis for future treatments and prevention of disease.

INTRODUCTION

The hepatitis C virus (HCV) is a positive-sense, single stranded RNA virus of the family Flaviviridae. There are six distinct genotypes with several subgenotypes that have been identified. The World Health Organization estimates that the virus affects approximately 3% of people worldwide. Approximately 170 million people are chronic carriers of the virus. There is higher prevalence in the Far East, Mediterranean countries and in certain African and eastern European areas. HCV infects primarily liver cells causing an acute hepatitis. Of those acutely infected, roughly 15% will clear the virus without medical intervention while the remainder will develop chronic hepatitis C. Approximately 30% of chronic hepatitis C will progress to cirrhosis, of which 20% will develop hepatocellular carcinoma. Thus, HCV infection remains a global health problem requiring continued research efforts to understand viral infection and to develop improved drug therapies.

Efforts to obtain information regarding the intricacies of hepatitis C viral entry, lifecycle and replication have been limited by the lack of model organisms available to serve as experimental hosts to the virus. Hu-

mans and other primates are the only known organisms naturally permissive to HCV infection. Costs and ethical concerns, thus, limit the study of the disease in chimpanzees. Recently, novel animal systems have permitted detailed examination of HCV infection, replication, and host responses. In this review, the status of HCV animal models is summarized, and the advantages as well as disadvantages, are discussed in terms of the potential impact on the development of future novel anti-HCV therapies.

ANIMAL MODELS

Chimpanzees and immune responses to HCV infection

The path leading to the discovery of HCV began in 1975 when some patients with viral hepatitis were found to lack markers for hepatitis A virus or hepatitis B virus (HBV) in serum. This led to the recognition that a separate unrelated hepatitis virus was responsible. This entity was initially termed non-A non-B hepatitis (NANBH). Thus began a wide body of research to isolate and identify the virus using chimpanzees as hosts for infection with human NANBH-infected serum. In 1989, Choo *et al.*^[1] and Houghton *et al.*^[2] created a complementary DNA clone of NANBH (clone 5-1-1) derived from positive-strand RNA, and confirmed that it encoded an antigen specific to NANBH infections. The cDNA was used to create antigens (c100-3) subsequently used as substrates to detect circulating serum antibodies *via* enzyme immunoassay. Through these discoveries in chimpanzees, antibody testing enabled screening of blood for the presence of the agent, now named HCV.

The study of HCV in chimpanzees has provided a wealth of knowledge regarding the mechanism of infection, replication, and both innate and humoral antiviral immune responses. Chimpanzees infected with HCV display elevations of aminotransferases and liver biopsies show necroinflammatory changes after acute infection. However, chimpanzees differ from humans in that their course of infection is milder; chronic carriers do not develop cirrhosis or fibrosis and only one chimpanzee has been reported to have developed HCV-related hepatocellular carcinoma^[3]. Other differences include lack of efficacy of interferon (IFN) treatment as evidenced by constant viral loads despite administration of this agent. Alternative studies of direct antiviral agents are currently being studied in chimpanzees. For example, Olsen *et al.*^[4] showed that administration of a nucleoside analogue and protease inhibitor resulted in viral load decline in HCV-infected chimpanzees. Together with recent clinical trials and use of novel HCV protease inhibitors, success in the treatment of HCV-infected chimpanzees has potential to spark new human clinical trials using antiviral agents without concurrent use of pegylated-IFN and ribavirin.

Chimpanzees offer a valuable animal model for active immunization studies as well as for investigating mechanisms of innate and cell-mediated antiviral activity. Through studies on chimpanzees that have naturally

cleared infection, Nascimbeni *et al.*^[5] have described the role of memory T-cell (both CD4 and CD8) responses that may help prevent infection upon re-challenge with virus. The varying quantity and quality of this cell mediated response helps explain differing responses to re-infection among individual chimpanzees. Barth *et al.*^[6] recently highlighted the importance of neutralizing antibodies to prevent early viral replication. They also showed that heightened CD8+ and natural killer (NK) cell activity increased production of IFN stimulating genes and IFN I / II, thus further supporting the role of adaptive immunity in limiting viral re-infection. Results of vaccination studies in HCV-infected chimpanzees have proven difficult to interpret for a variety of reasons including heterogeneity of genotypes, the error-prone RNA polymerase that creates mutations resistant to neutralizing antibodies, and downregulation of NK and T-cell responses *via* gpE2 interaction with CD81. Important information can nonetheless be gathered from both therapeutic and prophylactic vaccination studies^[7]. Meta-analyses of HCV therapeutic vaccination studies in chimpanzees by Dahari *et al.*^[8] concluded that vaccinations that included non-structural HCV proteins were less effective in achieving HCV clearance in comparison to inclusion of structural proteins in vaccines, which were hypothesized to heighten T-cell responses. However, successful vaccination data should be interpreted carefully, because most studies use endpoints as reduction in clinical disease rather than sustained virological response. The search for a prophylactic vaccination for HCV has been challenging. The mechanism of protective vaccination is usually the generation of neutralizing antibodies. In HCV, neutralizing antibodies have been observed to coexist with high HCV titers, thus suggesting their presence does not limit HCV entry into cells *in vivo*. Neutralizing antibody levels also tend to decrease after infection resolves, indicating a lack of a memory response or capability to prevent re-infection. More recent work has subsequently focused on generating a reliable T-cell (CD4+ and CD8+) response in attempts to protect against the development of infection with exposure to the virus. Folgori *et al.*^[9] developed a prophylactic vaccination strategy in chimpanzees using adenoviral vectors and electroporated plasmid DNA encoding the HCV non-structural region. Through stimulation of a cross-reactive T-cell response, chimpanzees were capable of resolving infection when challenged with virus differing from the vaccine by more than 13% at the amino acid level. Vaccination studies in chimpanzees are ongoing. There are many disadvantages to the use chimpanzees as models, including cost, ethics, and most recently, an NIH ban on the use of chimpanzees for biomedical research^[10].

Tupaia infection with HCV

Tupaia belangeri is a tree shrew native to Southeast Asia. Tupaia has been shown to be susceptible to a variety of human viruses including herpes simplex virus, rotavirus,

and HBV. In 2002, Zhao *et al.*¹¹¹ demonstrated effective hepatitis C replication and virion synthesis in primary tupaia hepatocytes. This group plated and infected primary tupaia hepatocytes with serum or plasma derived HCV from infected humans. Infection and effective replication was confirmed by reverse transcription polymerase chain reaction detection of negative strand RNA in hepatocytes as well as secretion of viral particles in culture medium. HCV RNA could be detected up to 14 d after plating. The enveloped virions produced were shown to be resistant to degradation by ribonuclease and could infect previously uninfected tupaia hepatocytes.

In 2010, Amako *et al.*¹²² reported a longitudinal study which followed HCV-infected tupaia over a three year period after inoculation with hepatitis C from a patient or viral particles from full length cDNA. The animals demonstrated mild inflammation and viremia during the acute infection followed later by development of liver steatosis, cirrhotic nodules and tumorigenesis. Moreover, serum from infected tupaia was harvested and inoculated into naïve tupaia resulting in acute infection, demonstrating effective replication and potential transmission of HCV.

HCV entry in tupaia has been shown to support current knowledge of human essential entry receptors. In 2011, Tong *et al.*¹³³ cloned tupaia CD81, scavenger receptor class B member 1 (SCARB- I or SR-B I), claudin-1 (CLDN1) and occludin and demonstrated that entry of HCV pseudoparticles or cell culture-derived HCV was permitted by these molecules. Inhibition of CD81 or SR-B I blocked HCV entry. However, there may be structural variations between human and tupaia receptors that may allow for differences in efficiency of viral entry. For example, the subtle structural difference between human and tupaia CD81 was shown to alter the ability of the extracellular loop to bind to HCV glycoprotein E2 in a study by Tian *et al.*¹⁴⁴. This information may be helpful in elucidating potential drug targets to block viral entry in humans. Tupaia is, therefore, a promising and effective model for the ongoing study of HCV entry and replication. A disadvantage of the tupaia model is that unlike humans with HCV, these animals rarely maintain sustained viremia^{12,15}.

Mouse models for HCV replication

Much research has been devoted to understanding why murine hepatic cells are naturally resistant to hepatitis C viral cell entry, and permit only inefficient replication in cell culture and in animals. Ultimately, overcoming this resistance could provide a valuable mouse model for HCV replication as well as development of potential immune strategies to block HCV entry and replication in humans.

Mouse hepatocytes have been shown to allow HCV entry in cell culture in cells genetically engineered to express human HCV-specific entry molecules including CD81 and occludin. However, in addition to the barrier to entry, murine cells also have a resistance towards rep-

lication, assembly and release of HCV. This was demonstrated by Long *et al.*¹⁶⁶ who used a transcomplementation system of mouse hepatoma cell lines that contained a subgenomic HCV replicon for ectopic expression of HCV structural proteins, p7, NS2 and apolipoprotein E (ApoE). They were able to demonstrate that assembly and release occurred successfully in murine cells with expression of the aforementioned proteins including ApoE.

In order to overcome the barrier to murine infection by HCV, Washburn *et al.*¹⁷⁷ developed a model consisting of a humanized mouse engineered by engraftment of human hepatocyte progenitors and human CD34+ human hematopoietic stem cells. This model was generated by the use of a fusion of the FK506 binding protein and caspase 8 under the control of the albumin promoter. This construct induced apoptotic elimination of host hepatocytes in Balb/C Rag2 (-/-) C-null mice. Ultimately these humanized mice were shown to harbor both human hepatocytes and T cells. Upon infection with HCV, the mice developed liver inflammation and fibrosis. A human T-cell immune response to HCV was observed. This study was limited by low levels of HCV infection demonstrated by absence of HCV RNA in the serum, and a lack of a B cell immune response due to absence of an engraftment of a complete immune system.

Similarly, Mercer *et al.*¹⁸¹ and Kneteman *et al.*¹⁹¹ developed a chimeric severe combined immune deficient (SCID)/urokinase-type plasminogen activator mouse model. This immune deficient mouse model has been shown to support proliferation of transplanted human hepatocytes, and more importantly, sustained HCV infection as demonstrated by detection of viral RNA within hepatocytes after intravenous inoculation. Production and release of viral particles were demonstrated by successful passage of infection through three generations of mice. The studies showed that when the human hepatocytes comprise the majority of liver cells (at least 80% of total hepatocytes) within chimeric SCID mice, infection with HCV can occur, and can result in liver failure. Further research revealed that human apolipoprotein (ApoB) and cholesterol ester transfer protein may play a role in allowing HCV infection in the chimeric SCID mice, and thus may offer a target to prevent viral entry within humans²⁰¹. HCV-infected SCID mice have also been used to study direct antiviral agents including IFN alpha-2 anti-NS3 and anti-NS5B proteases. Responses were shown to parallel that of humans. More recently, Kamiya *et al.*²¹¹ tested anti-NS3-4A (telaprevir) in HCV-infected SCID mice to evaluate the pharmacokinetics and dynamics of the drug as it related to a dose-dependent reduction in HCV serum RNA. Studies on SCID mice thus offer the potential to serve as a bridge between *in vitro* and clinical trials for HCV antiviral agents. Limiting factors of the SCID mouse model include a lack of a complex immune system, and inability to achieve a fully humanized liver. Recent studies have shown promise with use of a herpes simplex virus type-1 thymidine kinase/ganciclovir system

for cell specific ablation as means of obtaining exclusive growth of human hepatocytes in the SCID mouse^[22].

In order to create a mouse model permissive to HCV infection while maintaining complex immunity, Dorner *et al.*^[23] developed a humanized mouse model using genetic engineering to study viral entry and immunity. Mice were genetically engineered to express HCV-specific entry factors including CD81, occludin, SCARB-I, and CLDN1. Using this model, it was demonstrated that human-specific CD81 and occludin were essential to all HCV entry into murine hepatocytes. Expression of SCARB-I heightened HCV entry when expressed in combination with CD81 and occludin. Because this model used an immunocompetent mouse, viral replication and persistence of infection was limited. However, this model was also used to study passive immunization by administration of anti-CD81 antibodies and anti-E2 antibodies both of which decreased HCV infection. This model may offer future study of passive immunization or vaccination strategies to prevent acute infection of HCV before or after exposure.

Rat model for HCV infection

As discussed above, the search for an immunocompetent rodent host for HCV infection has been difficult. Although transplantation of human hepatocytes has been possible in mice, long-term HCV infection has only been shown in the setting of immunodeficiency, which limits study of immune responses. Ouyang *et al.*^[24] sought to overcome these barriers by creating an immunocompetent rat model that was tolerant to human hepatocytes. In order to achieve this, Huh7 human hepatoma cells were injected into the peritoneal cavities of fetal rats between gestation ages of 15-17 d. By injecting human hepatocytes during the development of the fetal immune system of the rat, specific tolerance to that specific cell type was achieved. Subsequent transplantation of human Huh7 cells into newborn tolerant rats resulted in survival and limited growth of the human cells without evidence of rejection as demonstrated by mixed lymphocyte assays. Colonies of cells bearing human liver cell markers increased in size, and were visualized within rat livers by immunohistochemical staining. Human albumin was detected in liver cells and in serum, and human hepatic mRNA was detected in hepatocytes, thus demonstrating active synthetic function of transplanted human Huh7 cells.

To study HCV infection, HCV isolated from human serum was inoculated into immunocompetent, tolerant, Huh7 cell-transplanted rats^[25]. HCV RNA levels of 7.0×10^3 copies/mL were detectable in serum by week 4, and peaked at 20×10^3 copies/mL by week 12 after infection. Levels decreased thereafter. Moreover, biochemical evidence of hepatic inflammation was demonstrated by elevations of serum alanine aminotransferase beginning at 4 wk, and peaked at $3 \times$ the baseline level by the 13th week, after which levels declined. Light microscopy of liver sections showed mononuclear infiltrates in portal

and central regions at times coinciding with detectable viremia. Controls without transplanted cells, tolerization, or HCV inoculation lacked any markers of HCV infection or hepatitis. The limitations of this rat model include low numbers of transplanted human hepatocytes as well as relatively low levels of viremia (22 500 copies/mL), in comparison to that in a typical human infection. However, this model offers a rodent model large enough to tolerate repeated blood and tissue sampling to study viral entry, replication, and immune-mediated hepatic injury as well as a screening tool to evaluate novel antiviral agents.

PROSPECTIVE

Hepatitis C viral infection is a growing health concern that leads to liver failure and hepatocellular carcinoma. The study of HCV has been limited due to a lack of appropriate and reliable animal models. However, much of our understanding of viral infection, replication and host immune responses has been gathered from animal data. Animal studies have historically utilized chimpanzees, but alternative models such as tupaia, mice and rats are now viable models for research.

Chimpanzees are advantageous models given their close genetic resemblance to humans, intact immune system which offers potential for study of innate and adaptive immune responses, and potential to study both treatments and prophylactic vaccinations. Additionally, important research with chimpanzees has resulted in the development of molecular clones of the virus for use in molecular and cellular research. Disadvantages include high cost, ethical constraints, and low susceptibility to chronic infection, thus limiting the study of HCV-related cirrhosis and hepatocellular carcinoma. Currently, chimpanzees are not available for biomedical research^[10].

Advantages of the tupaia model include low cost, ease of propagation, immunocompetence, and capability to study cirrhosis and tumorigenesis. On the other hand, tupaia and humans are genetically very different, potentially limiting the applicability of tupaia data on viral infectivity and disease to humans. Reliability and reproducibility of infection with tupaia have been also been debated.

Xenograft mice constructed with human hepatocytes are useful models given the demonstrated persistent viremia, and development of cirrhosis. These models provide a unique opportunity to study viral activity within human hepatocytes in a living host. Limitations include difficulty with mouse reproduction for repeated sampling, and immunodeficiency that limits the study of the natural immunological response.

The immunocompetent mouse and rat are promising models given their relatively low cost, ease of propagation, and intact immune system. There is great potential for the study of immunologic mechanisms as well as treatment and vaccination agents using these models. Disadvantages include relatively low levels of viremia,

and possible limitation of persistent infection by a competent immune response.

Our knowledge of HCV and related disease has grown significantly from its discovery nearly three decades ago. Together with molecular and cellular approaches, continued animal research will undoubtedly play a critical role in development of new antiviral therapies, and our understanding of mechanisms involved in the pathogenesis of both acute and chronic HCV infections. Further research is needed to enhance the potential of the currently available models in order to optimize the cost of maintenance and propagation, viremia, and maximize the similarity of infection models as they relate to human infection.

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