MINIREVIEWS

Contributions of transgenic mouse studies on the research of hepatitis B virus and hepatitis C virus-induced hepatocarcinogenesis

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Abstract

Transgenic mouse technology has enabled the investigation of the pathogenic effects, including those on development, immunological reactions and carcinogenesis, of viral genes directly in living organism in a real-time manner. Although viral hepatocarcinogenesis comprises multiple sequences of pathological events, that is, chronic necroinflammation and the subsequent regeneration of hepatocytes that induces the accumulation of genetic alterations and hepatocellular carcinoma (HCC), the direct action of viral proteins also play significant roles. The pathogenesis of hepatitis B virus X and hepatitis C virus (HCV) core genes has been extensively studied by virtue of their functions as a transactivator and a steatosis inducer, respectively. In particular, the mechanism of steatosis in HCV infection and its possible association with HCC has been well studied using HCV core gene transgenic mouse models. Although transgenic mouse models have remarkable advantages, they are intrinsically accompanied by some drawbacks when used to study human diseases. Therefore, the results obtained from transgenic mouse studies should be carefully interpreted in the context of whether or not they are well associated with human pathogenesis.

Core tip: Transgenic technology offers researchers several advantages over *in vitro* experiments, including the ability to trace the pathogenic effects of viral genes in living organisms. Transgenic mouse studies have provided evidence that the direct action of viral genes, especially the genes encoding hepatitis B virus

X and hepatitis C virus core proteins, is involved in hepatocarcinogenesis. However, such results should be considered carefully as transgenic mouse experiments have intrinsic advantages and drawbacks. As such, the results including phenotypes and molecular mechanisms from transgenic mouse studies must always be verified by comparing them to those of human studies for evidence of an association.

INTRODUCTION

Primary liver cancer has a high mortality rate worldwide and ranks 5th as the most common cancer among men and 7th among women; as such, therapeutic options to cure this disease are urgently needed[1,2]. Hepatocellular carcinoma (HCC) accounts for the majority of primary liver cancers. The most prevalent etiological agents of HCC are hepatitis B virus (HBV) and hepatitis C virus (HCV) infections. Once HBV- or HCV-related cirrhosis is established, HCC develops with an annual rate of approximately 4.3% or 7.1%, respectively in Japan, and 2.2% or 3.7%, respectively, in Western countries[3,4].

Individual viral proteins may play significant roles in and confer characteristics that are peculiar to the viral pathogenesis of HBV or HCV. Transgenic mouse strategies are applied to explore the functions of viral genes *in vivo* and can provide significant information on the mechanisms of viral pathogenesis by allowing the elucidation of the effects of individual viral proteins[5-7]. In this review, we summarize the past contributions of transgenic mouse studies to HBV- and HCV-induced viral pathogenesis with a particular focus on the evaluation of how well the results of these transgenic mouse studies correlate with human pathogenesis and how useful they are in the development of therapeutic strategies for viral disease, especially hepatocarcinogenesis.

MECHANISMS OF HEPATOCARCINOGENESIS DUE TO HBV AND HCV

When considering the contribution of transgenic mouse studies to the research on hepatocarcinogenesis caused by HBV or HCV, it is important to outline the mechanisms of the disease and consider for which mechanisms transgenic mouse studies can be applied to provide pathogenic and therapeutic contributions.

First, the common mechanism between HBV and HCV is as follows: Once these viruses infect liver, they skillfully evade host immune surveillance and induce chronic necroinflammation. These injuries cause fibrosis and result in liver cirrhosis. Hepatocytes, using their intrinsic regenerative capability, continue to proliferate in order to compensate for the necrotic tissues. Genetic alterations continuously accumulate during these processes, resulting in the formation of a pathogenic state such as cirrhosis from which HCC frequently arises.

Second, viral genes may be involved in hepatocarcinogenesis by directly affecting cellular machineries. The most representative genes of this type that have drawn clinical attention are the genes for HBV X (HBx) and HCV core protein. HBx is multifunctional and may induce the transactivation of many cellular genes[8]. On the other hand, the HCV core protein causes steatosis in the liver and subsequent HCC[9]. Transgenic mouse studies can shed light on the mechanisms of HBx and HCV core protein by enabling assays on the direct actions of these viral genes *in vivo*.

Third, a mechanism specific to HBV is its integration into the cellular DNA of the host; this may increase the genomic instability and cis-activation of the adjacent cellular genome that may possibly be involved in the regulation of the cell cycle[10]. Importantly, most integrated viral DNA retain the sequences encoding HBxAg, and the HBxAg expressed from the integrated HBV DNA further promotes genetic instability of the host by a variety of mechanisms[10].

ADVANTAGES AND LIMITATIONS OF RESEARCH USING TRANSGENIC MICE

Advantages

The mechanism of viral hepatocarcinogenesis comprises a number of complex factors[11]. Although the majority of research in this field has been performed using human samples, human resources have limitations in both ethical and quantitative terms, and it is sometimes difficult to extract significant conclusions from the final results that are dependent of a combination of vastly complex molecular events. The cell lines used for *in vitro* experiments are mainly derived from HCC cells in which carcinogenic events have already finished and little information on the real-time carcinogenic process can be obtained. Transgenic mouse research can compensate for these drawbacks.

Limitations

Transgenic technology offers the researchers several advantages over *in vitro* experiments[12,13]: It enables the investigation of the pathogenic effects, including those on development, immunological reactions, and carcinogenesis, of viral genes directly in living organisms in a real-time manner. Viral genes can be designed to be placed under their own or another appropriate promoter for specific expression in permissive cell types or tissues[14,15]. Thus, it is possible to identify particular cell types or organs that allow the expression of viral

proteins. In addition, it allows a sufficient number or amount of experimental material to be obtained at any specific condition, enabling the analysis of comprehensive and objective data.

Unfortunately, there are also several drawbacks to transgenic mouse research. First, because viral proteins of HBV or HCV, which normally only infect human or chimpanzee, are forced to be expressed in mouse tissue, the resulting protein-protein interactions may not be the same as those that would occur in the natural hosts and unexpected molecular reactions may result. Second, expressed viral proteins may become immune-tolerant in mice and cannot induce immunological response[16], and as eliciting an immune response is the main mechanism of viral liver pathogenesis, it becomes difficult to completely simulate this human disease; however, this may actually be an advantage as it may allow the evaluation of the direct role of viral proteins on liver pathogenesis without local inflammation[6]. Third, because transgenes are randomly integrated into the genome, the expression of transgene can be affected by the adjacent genomic structures. Phenotypes should be confirmed by the results obtained in mouse lines established using several founder mice independently. It is extremely difficult to establish viral replication in mice and cell culture systems are more suitable for analyzing viral replication, including the identification of viral receptors. Thus, because transgenic mouse models for viral diseases are not multipotent and can produce results that are easily hampered by experimental artifact, the obtained results should always be strictly scrutinized and evaluated in both scientific and clinical aspects. Namely, confirmation as to whether the phenotypes really correlate with the pathogenesis of human liver diseases is needed.

HBV AND TRANSGENIC MOUSE

HBV is a DNA virus with a length of 3.2 kb that replicates *via* an RNA intermediate for viral replication. Similar to retroviruses, which undergo reverse transcription for replication, it is integrated into the host genome.

Hepatitis B surface antigen transgenic mouse: Immunopathology of HBV

Chisari's group has made vast contributions to the clinical field by performing transgenic mouse studies to investigate immune-mediated hepatitis and hepato-carcinogenesis[17,18]. In the 1980s, they produced a transgenic mouse model that overexpressed the large protein of the hepatitis B surface antigen (HBsAg). It induced severe, prolonged hepatocellular injury that was characterized by inflammation and regenerative hyperplasia, resulting in the development of HCC[19]. This was the first transgenic mouse model in which the development of HCC was observed from the function of a single viral protein. However, these results were considered to be the outcome of the storage effect of the large HBsAg, since overexpression of the HBV

core, precore, X, small or middle envelope protein was not associated with any evidence of liver disease in the transgenic mouse model due to immunological tolerance for the inherently expressed viral proteins[16]. In order to better mimic the HBV pathogenesis seen in human where immunological reactions to viral proteins are essential, HBsAg-specific cytotoxic T cells were transferred to the mice to induce viral antigen-mediated acute hepatitis; this provided direct evidence that hepato-cellular injury in HBV infection may be immunologically mediated[20,21]. Moreover, further induction of cytokines such as interferon (IFN)-γ after acute liver injury may magnify the degree of inflammation, resulting in ful-minant hepatitis[22]. These transgenic mouse studies clarified the immunological aspects of HBV infection, showing that the balance between viral load and the strength of the immunological reactivity towards HBV antigen determines the fate of the disease[18]. As for the immune pathogenesis of HCC, the adoptive transfer of CD8 lymphocytes to HBsAg transgenic mice generated a pathogenesis that closely resembled that of human chronic viral hepatitis and finally resulted in the development of HCC[23]. This model strengthened the notion that only immune-mediated hepatocellular injury, and not insertional mutagenesis from the integration of the HBV genome or the expression of the *HBx* gene, could cause hepatocarcinogenesis.

In the early era of transgenic mouse studies, several reports showed a high level of HBV replication *in vivo*, injecting a duplicated HBV plasmid[24-26]. These results demonstrated that the HBV genome integrated into the mouse chromosome acted as a template for viral gene expression, allowing viral replication. Although these mice did not show the HCC phenotype, they contributed to the detailed studies of the replication and expression of HBV and to pathological studies of hepatitis. In addition, HBs large envelope protein expression in transgenic mouse showed the inhibition of HBsAg secretion, suggesting an inhibitory effect of the pre-S-containing domain of the large envelope peptide[27].

HBxAg transgenic mouse: Direct hepatocarcinogenic role of HBV

The *HBx* gene is a known oncogene with pleiotropic functions that transactivate multiple cellular genes; it has attracted extensive clinical attention and has been regarded as a suitable target for transgenic mouse research[8,28,29]. Kim *et al*[30] first observed the occurrence of HCC in HBx transgenic mouse. Koike *et al*[31,32] further showed that HBx induced hepatocyte proliferation and contributed to hepatocarcinogenesis. Inspired by these studies, many researchers have attempted to produce the HCC phenotype in HBx transgenic mice, but most of them failed[33-36]. These inconsistent observations in terms of HCC development among transgenic mice might be partly due to differences in the sequences[37] or subtypes[38] of HBV or the genetic background of the mice used. However, strong and continuous expression of HBx might be a requirement for observing the HCC

phenotype[38,39]. Nonetheless, it remains unclear whether this high level of HBx expression is physiologically relevant to human pathogenesis[8]. Moreover, there is still a lack of clinical and molecular evaluation methods that can be used to measure how much the direct carcinogenetic function of HBx contributes to human HBV hepatocarcinogenesis where necrosis-regeneration sequence of hepatocytes by immunological reactions to HBV is generally considered to be an essential mechanism. How a single HBx gene is involved in this huge complex pathogenesis process remains to be clarified.

It is has been reported that fibrosis levels of liver complicated with HBV-HCC is milder than that of HCV-HCC[40-42]. In our cohort of 57 patients with HBV-HCC who were treated surgically, 25 (44%) did not have cirrhosis, and 22 (39%) had a mild level of fibrosis (F1 or 2) (Unpublished results). Therefore, the direct carcinogenic role of HBx or the dysregulation of the cell cycle due to insertional mutagenesis by the HBV genome might play a large role, especially in those who have a mild level of fibrosis, than cirrhosis, which is the final state of necrosis-regeneration sequence. In addition, our past studies have shown that the HBx transgenic mouse is a good model for testing the anti-hepatocarcinogenic function of IFN-β[29,43].

HCV AND TRANSGENIC MOUSE

HCV and steatosis: Close association with human pathogenesis

While HBx transgenic mouse research has been confronted by difficulties in correlating the research results with specific clinical or molecular landmarks, HCV transgenic mice have provided fruitful experimental observations in terms of steatosis[6,44] which is commonly observed in both HCV-infected humans and HCV-transgenic mice.

Steatosis has been reported to be a characteristic finding of chronic HCV infection[45-47]. Moriya *et al*[9] observed steatosis in HCV core gene transgenic mice at as early as 3 mo of age and found that about a quarter of the mice developed HCC in their late life, demonstrating that the HCV core protein itself has a direct role in hepatocarcinogenesis by virtue of steatosis[9]. Lerat *et al*[48] also observed hepatic steatosis and HCC in transgenic mouse models that express complete viral and structural proteins without immunological reactions.

It is well known that HCV genotype 3 directly induce steatosis in liver[44,49], supporting the observations obtained in transgenic mouse. Importantly, an association between steatosis and fibrosis has also been demonstrated in a meta-analysis of chronically infected patients with HCV[50], and hepatic steatosis is a risk factor for HCC in chronic hepatitis C patients[51,52]. These results indicate that the findings obtained from transgenic research are well associated with findings from human research.

HCV core protein and PRARα

In addition, HCV core gene transgenic mice became the base for further studies which explored the mechanisms of steatosis and its relationship with HCC development. Tanaka *et al*[53] generated peroxisome proliferator-activated receptor alpha (PRARα-homozygous, -heterozygous, and -null mice with HCV core protein expression and showed that severe steatosis developed in mice that had both PRARα alleles, revealing that the expression of PRARα, which is important in maintaining triglyceride homeostasis, was essential for the development of HCV core protein-induced steatosis and HCC[53]. Moriishi *et al*[54] showed that a knockout of the proteasome activator 28 gamma (*PA28γ*) gene induces the accumulation of HCV core protein in the nucleus of hepatocytes of HCV core gene transgenic mice and disrupts the development of both hepatic steatosis and HCC, thus revealing that PA28γ plays a crucial role in the development of HCV-induced liver pathogenesis[54].

HCV core protein and reactive oxygen species

Transgenic mouse studies have further provided significant findings on the mechanisms of the progression from steatosis to hepatocarcinogenesis. Okuda *et al*[55], reported that HCV core protein increases the production of reactive oxygen species (ROS) *via* a direct effect on the mitochondrial electron transport system. Korenaga *et al*[56] reported that reduced activity of electron transport complex 1 enhances the production of ROS in HCV core gene transgenic mouse. Consistent with these observations, both mitochondrial dysfunction[57] and high levels of oxidative stress have been demonstrated in HCV-infected patients[58,59]. 8-Hydroxy-2'-deoxy-guanosine which is generated by ROS and leads to an increased frequency of mutations, accumulates in HCV core gene transgenic mouse[60] and causes mutations in cellular genes[61]. In addition to its direct effect on mitochondria, HCV core protein has been shown to cause endoplasmic reticulum stress that results in an oxidized redox state in hepatocytes, interfering with immune responses and potentiating fibrosis and carcinogenesis[62]. Moreover, Klopstock *et al*[63] used HCV transgenic mice that were crossed with Mdr2-knockout mice to demonstrate that the HCV transgene accelerates inflammation-associated hepatocarcinogenesis, which has a pathogenesis similar to that of human HCV-induced carcinogenesis[63].

It was also reported in a transgenic mouse study that the production of ROS induces high levels of iron deposition in liver, resulting in an increased risk of HCC[64]. A strong correlation between hepatic DNA damage and iron overload has been confirmed in a human study[65]. Mitochondrial ROS may be linked to metabolic disorders such as insulin resistance, hepatic steatosis, and hepatic iron accumulation, all of which are characteristic features of chronic HCV infection[66].

Direct role of HCV core protein in hepatocarcinogenesis

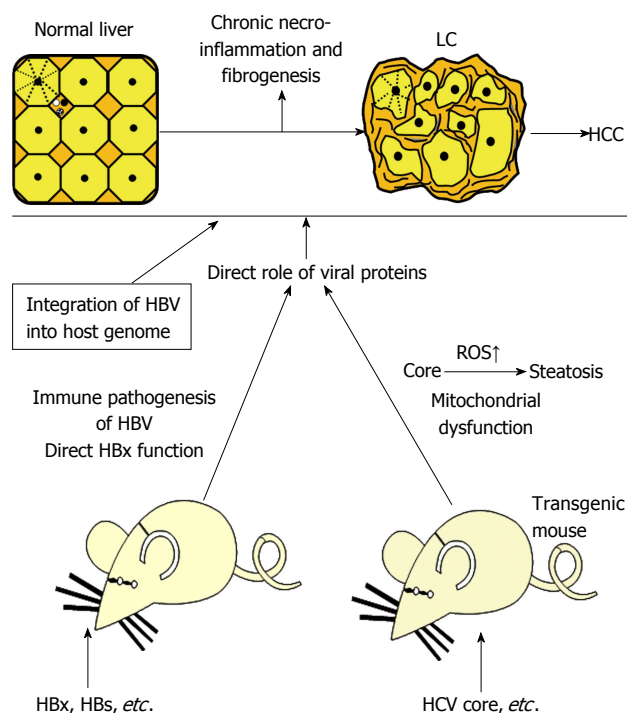The mainstream mechanism of HCV-induced hepato-

Figure 1 Outline of the overall aspects of this review. The mainstream mechanism of HBV- or HCV-induced hepatocarcinogenesis is necroinflammation and hepatocyte-regeneration sequences that eventually result in cirrhosis where HCC frequently develops. However, transgenic mouse studies have clarified the significant contribution of viral proteins to hepatocarcinogenesis by directly affecting cellular machinery. A close association of HCV core protein and hepatic steatosis has been established by transgenic mouse studies. In addition to the inherent insertional mutagenesis mechanism of HBV, results from transgenic mouse studies have also suggested the direct involvement of HBV proteins in hepatocarcinogenesis. HBV: Hepatitis B virus; HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma; ROS: Reactive oxygen species; HBs: Hepatitis B surface; HBx: Hepatitis B virus X.

carcinogenesis is persistent necroinflammation that induces the irregular regeneration of hepatocytes, allowing the accumulation of genetic or epigenetic alterations. In addition to this, transgenic mouse studies have shown that the HCV core protein plays a significant role in hepatocarcinogenesis by inducing steatosis *via* the production of ROS from by the dysregulation of lipid metabolism or functional abnormalities of the mitochondria[5]. Thus, transgenic mouse studies have shown that viral proteins, especially the HCV core protein, directly interact with lipid-metabolizing pathways and contribute to HCC development; these are the major achievements of transgenic mouse studies on HCV-induced carcinogenesis.

CONCLUSION

The mechanisms of hepatocarcinogenesis common between HBV and HCV include persistent necroin-flammation and the regeneration of hepatocytes that allows the accumulation of genetic changes. However, there are yet no reports on common genetic changes that can fully explain these complex pathways; rather, multiple dysfunctions resulting from abnormalities in a number

of signal transduction pathways appear to converge to produce the common HCC phenotype. However, the use of transgenic mouse technology has clarified that even a single viral gene, such as the gene for HBx or HCV core protein can directly affect the cellular machinery and impact the mainstream mechanism of persistent necroinflammation-induced hepatocarcinogenesis (Figure 1). Especially for HCV, a good correlation has been found between the experimental findings from transgenic mouse studies and clinical observations. Thus, transgenic mouse models may provide an efficient method for evaluating the effectiveness of anti-hepatocarcinogenesis agents in the future.

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