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Original Article

Hepatic Steatosis Becomes Severe with Age in the Livers of Full-Length HCV Genome Transgenic Mice

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ABSTRACT: Hepatitis C virus (HCV) is a growing public health concern worldwide. Vaccine preventing HCV has not yet been developed due to the hindrance of research on suitable small animal models. Previously, we made full-length HCV genome transgenic mice under the control of human Pol I promoter and these mice could replicate and produce infectious HCV particles in their livers. In the previous study, we have also found that these transgenic mice deposited lipids abnormally in their livers, but in the current study, we extensively examined the livers of these full-length HCV genome transgenic mice between 5 and 16 months of age and found that the excessive lipid deposition started as early as 5 months and persisted almost throughout the life. This excessive lipid deposition in liver is called hepatic steatosis, which is one of the important phenotypes of chronic HCV infection in human patients. In the current study, we have also found that the severity of hepatic steatosis is increased with the increase of age in our HCV genome transgenic mice. Thus, this *in vivo* study in HCV genome transgenic mice can provide us an insight in understanding the HCV related hepatic steatosis and also helps to develop drugs against HCV causing excessive lipid deposition in livers.

KEYWORDS: HCV, transgenic mice, steatosis, H&E, ORO staining.

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INTRODUCTION

Hepatitis C virus causes chronic liver diseases including steatosis, cirrhosis and hepatocellular carcinoma[1]and affects about 3% of the total population worldwide[2]. The usual progression of liver disease in patients with HCV is a process of inflammation accompanied by periportal necrosis and fibrosis. The inflammation that results from the virus causes stimulation of stellate cells which ultimately leads to the deposition of collagen which leads to fibrosis progression within the liver. If this process is rapid and unhindered then the usual outcome is the development of cirrhosis which is the final irreversible stage characterized by parenchymal nodules with encircling fibrous septa. The hepatitis C virus is not considered to directly injure the liver but it rather triggers an HCV-specific lympho proliferation. Through

profuse cytokine production and also a direct cytopathic effect, these T cells result in hepatocyte apoptosis[3]. Many patients with chronic HCV are also noted to have a degree of steatosis present on their liver biopsies. Hepatic steatosis is defined as excessive lipid accumulation within the hepatocyte cytoplasm and has been more recently recognized as a significant cause for cirrhosis in the United States[4]. The etiology for hepatic steatosis can be determined by the distribution and size of the lipid accumulation within the hepatocytes. Micro-vesicular steatosis that is seen in Reye's syndrome and Acute Fatty Liver Disease of Pregnancy occurs due to dysfunctions in β-oxidation of free fatty acids and this can result in acute liver failure[5, 6].

Non Alcoholic Fatty Liver Disease (NAFLD) is mainly associated with obesity[7, 8], diabetes[7, 9, 10], hyperdyslipemia[7, 8, 11, 12] and insulin resistance[12-15] which are the main features of the recently characterized metabolic syndrome. Unfortunately, vaccines to prevent HCV infection are not yet available. Furthermore, the study of HCV is being hampered by the lack of suitable small animal models which will mimic the pathological features of chronic hepatitis C virus infection like humans[16]. More recently, much progress has been made on HCV biology in making transgenic mice for HCV infection study[17]. However, understanding the HCV causing pathogenesis is still hampered by its narrow host range, mostly restricted to humans and chimpanzees. Even though chimpanzee is the widely recognized model for HCV infection but its use is limited by high cost and ethical concerns[18].

In our earlier study, we generated transgenic mouse lines carrying the full-length HCV genome under the control of human Pol I promoter and these mice can replicate, produce infectious viruses[17]. In our previous study, we have also shown hepatic steatosis very briefly. In the current study, we have extensively analyzed the livers of full-length HCV genome transgenic mice and found that these HCV genome transgenic mice develop hepatic steatosis as early as 5 month-old and persisted throughout the life. We have also found that the severity of hepatic steatosis is increased with the increase of age in our full-length HCV genome transgenic mice. This hepatic steatosis is one of the important characteristics of chronic HCV infection in human patients[19]. We thus expect that this current study will be helpful to some extent in understanding the severity stages of hepatic steatosis in chronic HCV infected patients as well as these full-length HCV genome transgenic mice can also be used as good models for discovering drugs against HCV causing excessive lipid deposition in the livers.

MATERIALS AND METHODS

Animal procedures

The full-length HCV genome transgenic mice under the control of human Pol I promoter were generated as described previously[17]. The transgenic mice carrying the full-length HCV genome and the age matched wild type (C57BL/6 background, used as controls) mice were used in the experiments. All mice were bred in a pathogen-free facility and tested routinely for mouse hepatitis virus. In this study, all the mice used in the experiments were approved by the Hamamatsu University School of Medicine Committee of Laboratory Animal Experimentation, Japan.

Tissue preparation for haematoxylin and eosin (H & E) staining

Mice were killed by breaking the necks or spinal cords and immediately, liver tissues were excised from 5, 8, 12 and 16-month-old wild type and HCV genome transgenic mice. The liver tissues were then fixed immediately in 10% neutral-buffered formalin overnight for H & E staining. Place one lobe of fixed livers in a small block containing paraffin wax to impregnate the tissue prior to sectioning. 4 µm thicknesses of liver tissues were cut from paraffin-embedded livers by using a thin tissue sectioning instrument (LEICA RM 2135, Leica Biosystems, Nussloch GmbH).

Tissue preparation for Oil Red O (ORO) staining

Liver tissues were excised from different ages of wild type and HCV genome transgenic mice as described previously, and immediately kept in OCT compound (Cat. No.4583, Tissue-Tek, Torrance, CA, USA) and frozen for Oil Red O staining. The 4 µm thicknesses of frozen liver sections were prepared by using a fine tissue cutter (Microm HM 550, Thermo Fisher Scientific, MA, USA).

Haematoxylin and Eosin Staining

The liver tissue sections (4 µm thicknesses) were then stained with haematoxylin and eosin in accordance with the conventional procedure. We described here the H & E staining in details. First, place tissue sections in haematoxylin (Cat. No.H9627, Sigma-Aldrich Co., Missouri, USA) solution for 4 min at room temperature, rinse in running tab water, differentiate with 0.3% acid alcohol, again rinse in running tab water, stain with eosin (Cat. No.HT110180, Sigma-Aldrich Co., Missouri, USA) for 2 min at room temperature,

dehydrate and add few drops of aqueous mountant (Cat. No. TA-030-FM, Thermo Scientific, CA, USA) before covering the slides with the cover slips. The stained tissue sections were then examined under a microscope (BX51, Olympus, Tokyo, Japan).

Oil Red O Staining

The frozen liver sections (4 µm thicknesses) were then stained with Oil Red O in accordance with the conventional procedure. We described here ORO staining in details. First, air dry the frozen liver sections on the slides, rinse with 60% isopropanol (Cat. No. 166-04836, Wako, Nagoya, Japan), stain with freshly prepared 0.5% ORO (Cat. No.O0625, Sigma-Aldrich Co., Missouri, USA) in isopropanol solution for 15 min at room temperature, again rinse with 60% isopropanol, lightly stain with haematoxylin solution for 1 min at room temperature for nuclear staining, rinse with distilled water, finally mount with an aqueous mountant and cover the slides with coverslips. The stained tissue sections were then examined under a microscope as described before.

Ethical Statement

The experiments conducted in this manuscript were approved by the Recombinant DNA Experiment Safety Committee of Hamamatsu University School of Medicine, Japan.

RESULTS

Vacuolating lesions observed in the livers of full-length HCV genome transgenic mice

Haematoxylin & eosin staining showed that many vacuolating lesions are observed in the cytoplasm of hepatocytes of the full-length HCV genome transgenic mice (HCV-Tg mice) of different ages (Figure 1B, 1D, 1F and 1H, white color observed through H&E staining) and the developments of these vacuolating lesions were started as early as 5 months old in HCV-Tg mice, whereas no vacuolating lesions were observed in the livers of different aged-match wild type control mice (Figure 1A, 1C, 1E and 1G, no white color observed through H&E staining). It was an interesting result and gave us insight that whether these vacuolating lesions were filled with lipids or not, and that is why we did ORO staining for the confirmation of the presence of excessive lipid deposition in those vacuolating lesions of the hepatocytes of liver sections obtained from the different ages of wild type control mice and HCV-Tg mice as described below.

Deposition of excessive lipid (Steatosis) observed in the livers of full-length HCV genome transgenic mice

Following the observance of vacuolating lesions in the cytoplasm of hepatocytes of HCV-Tg mice through haematoxylin & eosin staining, in order to confirm whether these vacuolating lesions were filled with excessive lipids, we did Oil Red O staining of the frozen liver sections obtained from the different ages of wild type control mice and

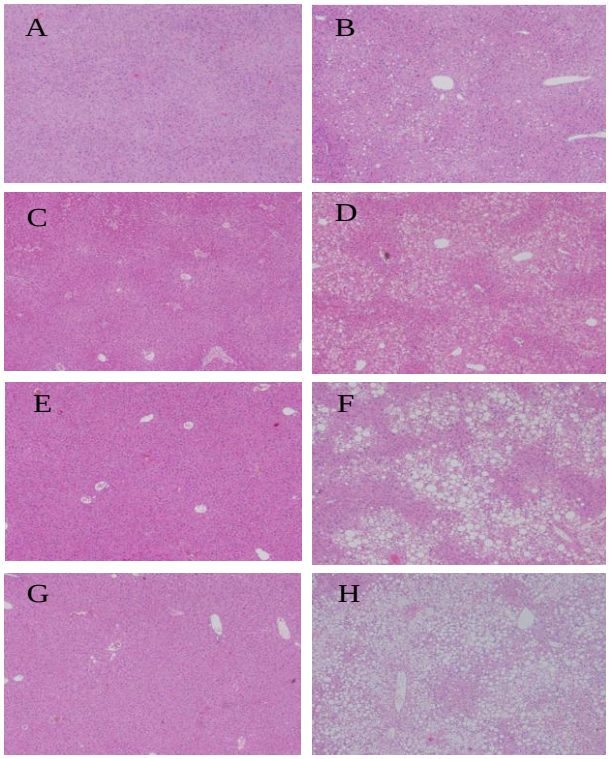

Figure 1. Microscopic examination of the liver sections obtained from wild type and full-length HCV genome transgenic (HCV-Tg) mice through haematoxylin and eosin staining. **(A & B)** 5-month-old male wild type and HCV-Tg mice; **(C& D)** 8-month-old male wild type and HCV-Tg mice; **(E & F)** 12-month-old male wild type and HCV-Tg mice; **(G & H)** 16-month-old male wild type and HCV-Tg mice respectively. Paraffin-embedded liver sections were stained by haematoxylin and eosin. Photographs were obtained at 100x magnifications.

full-length HCV genome transgenic mice. From the microscopic examination of ORO staining, we have found that these vacuolating lesions are filled with excessive lipids which are found as large lipid droplets in the cytoplasm of hepatocytes of liver sections obtained from the different ages of full-length HCV genome transgenic mice (Figure 2B, 2D, 2F and 2H, red color observed through ORO staining). On the other hand, we did not find any lipid droplets in the cytoplasm of hepatocytes of liver sections obtained from the different ages of wild type control mice (Figure 2A, 2C, 2E and 2G, no red color observed through ORO staining). Since these vacuolating lesions are excessively filled with lipids, and this phenotype is called

hepatic steatosis, which is one of the important characteristics of chronic HCV infection. This result also supported one of the previous studies[20].

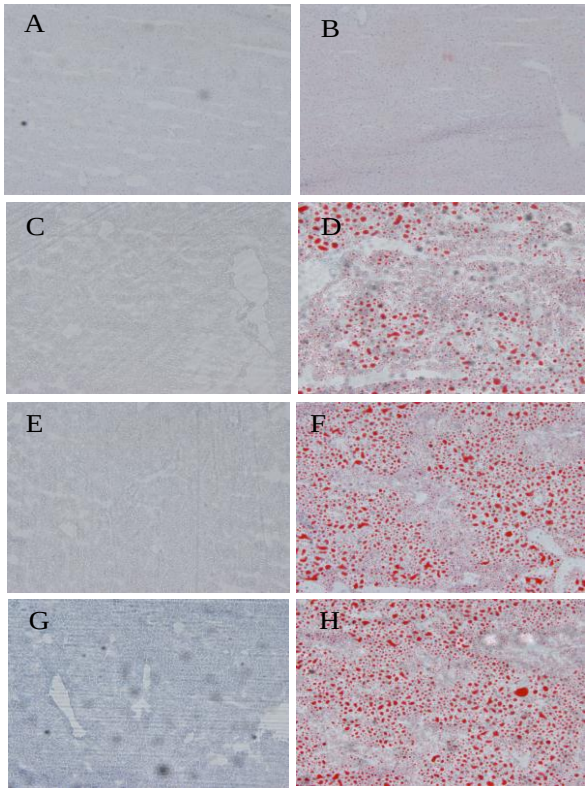

Figure 2. Microscopic examination of the liver sections obtained from wild type and full-length HCV genome transgenic (HCV-Tg) mice through Oil Red O staining. **(A & B)** 5-month-old male wild type and HCV-Tg mice; **(C& D)** 8-month-old male wild type and HCV-Tg mice; **(E& F)** 12-month-old male wild type and HCV-Tg mice; **(G & H)** 16-month-old male wild type and HCV-Tg mice respectively. OCT-embedded frozen liver sections were stained by Oil Red O. Photographs were obtained at 100x magnifications

Severity and frequency of hepatic steatosis increase with the increase of ages of full-length HCV genome transgenic mice

Furthermore, we have found that the lipid deposition is mild in the livers of younger transgenic mice and becomes moderate to severe with the increase of age of transgenic mice, whereas lipid deposition is not obvious in the livers of all the age matched wild type control mice (Figure 2A-H). Sixty percent of the male transgenic mice developed hepatic steatosis in the livers by the age of 5 months, and this percentage increased with the increase of age in transgenic mice (Table 1). In contrast, no hepatic steatosis was observed in the livers of 5, 8, 12 and 16-month-old wild type male control mice (n= 5 for each age category). Since many previous reports showed that hepatic steatosis is usually more frequent in male and in our study no significant hepatic steatosis was observed in the different ages of wild type male mice, so we disregard to analyze the hepatic steatosis in the livers of the different ages of wild type female control mice. In addition, we could not be able to analyze the hepatic steatosis in the livers of 5-month-old of HCV-Tg female mice due to lack of the number of that aged female mice.

Strain, Sex & Age	Mice number	Mice with hepatic steatosis	Incidence
HCV-Tg, ♂, 5 months	5	3	60.0%
HCV-Tg, ♂, 8 months	5	4	80.0%
HCV-Tg, ♀, 8 months	6	3	50.0%
HCV-Tg, ♂, 12 months	10	10	100.0%
HCV-Tg, ♀, 12 months	5	5	100.0%
HCV-Tg, ♂, 16 months	4	4	100.0%
HCV-Tg, ♀, 16 months	4	4	100.0%

Table 1: Incidence of hepatic steatosis in full lenght HCV genome transgenic (HCV-Tg) mice

DISCUSSION

Hepatitis C viral Core and non-structural NS5A proteins were mainly involved for developing hepatic steatosis in transgenic mice[20], but the hepatic steatosis is more evident in the livers of our full-length HCV genome transgenic mice compared to previously reported transgenic mice. Furthermore, the previously reported HCV genome transgenic mice didn't fully mimic the humans in HCV causing pathogenesis because only one gene of whole hepatitis C virus genome was used in making those transgenicmice[20]. On the other hand, we made full-length HCV genome transgenic mice under the control of human Pol I promoter and these mice could replicate and produce infectious viral particles in their livers[17]. With the current study, we have analyzed the livers extensively and found that the abnormal lipid deposition started as early as 5 months old and persisted throughout the life in our full-length HCV genome transgenic mice. Furthermore, we have also found that the severity of lipid deposition is increased with the increase of transgenic mice age. Since the severity may expect that other gene(s) along with Core and NS5A may also be involved for excessive lipid retention in the livers of these transgenic mice. We need further study to find out this involvement. We also expect that our transgenic mice can be used as

a good model for screening to find out the effective remedies against HCV causing hepatic steatosis. Even though, the overall experiment performed in very few number of mice and it is just due to the lack of mice number as well as the maintenance of HCV transgenic mice is very expensive. In order to reduce the overall experiment cost, we performed the experiment as low as number in mice.

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