

Hepatitis C virus core protein induces hepatic steatosis in transgenic mice

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Hepatitis C virus (HCV) is a major cause of chronic hepatitis worldwide, which finally leads to development of hepatocellular carcinoma. Chronic hepatitis C is characterized by several histological features in the liver which discriminate it from other forms of hepatitis: bile duct damage, lymphoid follicles and steatosis (fatty change). Little is known, however, about the role of HCV or its viral proteins in the pathogenesis of hepatitis. Recently, the core protein of HCV has been suggested to have a transcriptional regulatory function, and thereby to

be involved in inducing phenotypic changes in hepatocytes. To clarify whether or not the HCV core protein has an effect on pathological phenotypes in the liver, two independent transgenic mouse lines carrying the HCV core gene were established. These mice developed progressive hepatic steatosis, indicating that the HCV core protein plays a direct role in the development of hepatic steatosis, which characterizes hepatitis C. This transgenic mouse system would be a good animal model for the study of pathogenesis in human HCV infection.

Introduction

Hepatitis C virus (HCV) is the causative agent in most cases of acute and chronic non-A, non-B hepatitis (Choo *et al.*, 1989; Houghton *et al.*, 1991; Saito *et al.*, 1990; Simonetti *et al.*, 1992). Little is known, however, about the role of HCV or its proteins in the pathogenesis of hepatitis. One of the major issues regarding the pathogenesis of HCV-associated liver lesion is whether the HCV proteins have direct effects on pathological phenotypes. Although several strategies have been used to characterize the hepatitis C viral proteins (Grakoui *et al.*, 1993; Harada *et al.*, 1991; Lanford *et al.*, 1993; Matsuura *et al.*, 1992, 1994; Ralston *et al.*, 1993; Santolini *et al.*, 1994), the relationship between protein expression and disease phenotype has not been clarified. We previously established transgenic mouse lines in which the HCV envelope proteins are efficiently expressed in the liver (Koike *et al.*, 1995). Those mice develop no pathological changes in the liver up to the age of 18 months.

The HCV core protein is an unglycosylated protein of about 22 kDa (p22) which is encoded by the 5'-proximal portion of the large open reading frame (Harada *et al.*, 1991).

Typical of flaviviral core proteins, it is rich in arginine and lysine, particularly at its N terminus, and it can bind to the RNA genome to form the nucleocapsid of an HCV virion (Santolini *et al.*, 1994). Besides its genome-packaging function, the core protein has been suggested to have a regulatory effect on the expression of hepatitis B viral genes (Shih *et al.*, 1993) and different cellular genes such as the interferon- β gene (Kim *et al.*, 1994) or *c-myc* oncogene (Ray *et al.*, 1995). Its intracellular localization is cytoplasmic, but it shows nuclear translocation when its C-terminal portion is deleted (Suzuki *et al.*, 1995). These pieces of information imply that the core protein may have a direct effect on the pathogenesis of diseases caused by HCV.

In the present study, we describe transgenic mouse lines carrying the HCV core gene under the control of a regulatory region from the hepatitis B virus. These mice showed stable expression of the core protein and developed progressive steatosis in the liver, one of the characteristic features of chronic hepatitis C (Bach *et al.*, 1992; Lefkowitz *et al.*, 1993; Scheuer *et al.*, 1992).

Methods

■ **Construction of plasmid.** To ensure high-level expression in the liver, expression vector pBEPBgIII (Koike *et al.*, 1995), which contains the hepatitis B virus regulatory elements, was utilized. A 0.6 kb fragment

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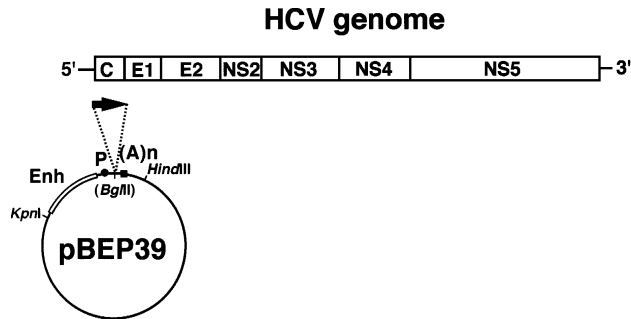


Fig. 1. Schematic representation of the transgene construct. The hepatitis C virus core gene was placed downstream of a transcriptional regulatory region from hepatitis B virus (Koike *et al.*, 1995). A 1.2 kb *KpnI*–*HindIII* fragment was introduced into mouse embryos. Enh, enhancer; P, promoter; (A)_n, polyadenylation signal.

containing the coding region of the core protein was cut from plasmid pSR39 (Harada *et al.*, 1991) by double digestion with *PstI* and *EcoRI*, and ligated into the *BglII* site of plasmid pBEP*BglII* after treatment with T4 DNA polymerase and attachment of *BclI* linkers. In the resulting plasmid pBEP39, the coding region of the core protein was expected to be expressed under the control of hepatitis B virus regulatory element (Fig. 1).

■ **Production of transgenic mice.** A 1.2 kb *KpnI*–*HindIII* fragment from pBEP39 (Fig. 1) was purified by PAGE and microinjected into mouse embryos from the C57BL/6N strain (Clea Japan Inc.) as described previously (Koike *et al.*, 1989). Transgenic mice were identified by subjecting 1 µg of tail DNA to amplification by PCR using a set of oligonucleotides as primers. Oligonucleotides T1 (GCCACAGGAC-GTTAAGTTC) and T2 (TAGTTCACGCCGTCCTCCAG) were used to amplify a 438 bp fragment from the core gene. To further characterize the founders, Southern blotting was performed as described previously (Koike *et al.*, 1989). Mice were cared for according to institutional guidelines, fed with ordinary feed (Funabashi Farms, Funabashi, Japan) and maintained in specific pathogen-free conditions.

■ **Antibodies.** Anti-core rabbit serum and anti-core mouse monoclonal antibody were used in this study. Monoclonal antibody was raised against partially purified recombinant core protein expressed by baculovirus. This monoclonal antibody recognizes aa 105–112 of the core protein. Polyclonal rabbit antibody was prepared using recombinant vaccinia virus.

■ **Western blotting.** Whole tissue homogenates were subjected to 12.5% SDS–PAGE and electro-transferred to nitrocellulose membrane (Schleicher & Schuell) as described previously (Koike *et al.*, 1995). The filter was then reacted with anti-core monoclonal antibody, followed by anti-mouse IgG conjugated with horseradish peroxidase (Vector Labs) and visualized by an ECL kit (Amersham).

■ **Histological and immunohistochemical methods.** Tissue sections (5 µm thick) either fixed in 10% neutral-buffered formalin or frozen were used for haematoxylin and eosin staining, Sudan III staining or immunostaining. The HCV core protein was stained with anti-core or normal rabbit serum. For detection, biotinylated anti-rabbit IgG followed by avidin–biotin peroxidase (Vector Labs) was used. Specificity control of immunostaining was carried out by testing liver tissues and other organs from a normal littermate mouse with immune serum. Transgenic mouse liver was tested with normal rabbit serum.

■ **Serum lipid levels.** Serum total cholesterol and triglycerides levels

were determined using Fuji dry-chem 5500 auto-analyser (Fuji film, Tokyo, Japan).

■ **TUNEL assay.** We applied the terminal deoxynucleotidyl transferase-mediated dUTP nick and labelling (TUNEL) assay (Gavrieli *et al.*, 1992) using a TACS2 TdT *in vitro* apoptosis detection kit (Trevigen, Gaithersburg, MD). Paraffin-embedded sections of liver were subjected to TUNEL assay according to the manufacturer's protocol.

Results

Transgenic mouse lineages

Three transgenic mouse lineages which contained the HCV core gene were derived (C18, C21 and C49). These transgenic mouse lines had the HCV core gene of genotype 1b (Takeuchi *et al.*, 1990) under the control of hepatitis B virus regulatory elements (Fig. 1), which have been shown to allow high-level expression of genes in transgenic mice without interfering with mouse development (Koike *et al.*, 1994, 1995). Southern blotting of *StuI*-digested genomic DNA revealed a distinct integration pattern for each lineage (data not shown). The observed pattern is compatible with the presence of at least one full-length transgene. Founder mice were back-crossed to C57BL/6N. The integrated transgenes were successfully transmitted to offspring, and F1 and F2 heterozygous mice from lineages C21 and C49, both of which had three to four copies of the transgene, were investigated in this study.

Expression of the core protein

Western blot analysis with anti-core mouse monoclonal antibody on lysates from the transgenic mouse liver showed the core protein of 22 kDa (Fig. 2*a*), which is compatible with the size of the core protein previously reported (Harada *et al.*, 1991; Matsuura *et al.*, 1994; Santolini *et al.*, 1994). There was no significant difference in the levels of core protein in the liver between the C21 and C49 lines. Total protein preparations from tissues including the brain, salivary glands, lung, liver, intestine, spleen and kidney from a mouse of the C21 line were then immunoblotted to study the tissue distribution of the core protein. The core protein was detected only in the liver (Fig. 2*b*). Such tissue distribution is distinct from that in transgenic mice carrying HCV envelope genes under the control of the same promoter, in which E1 and E2 proteins were detected in several tissues in addition to the liver (Koike *et al.*, 1995). This difference in tissue distribution may simply be the result of a difference in sensitivities between the anti-envelope and the anti-core antibodies. Alternatively, the core protein may affect the function of transcriptional regulatory regions (Kim *et al.*, 1994; Ray *et al.*, 1995; Shih *et al.*, 1993), thus influencing the tissue of expression preference.

Histological changes in the liver of the transgenic mice

Following expression of the core protein from birth, vacuolating lesions appeared in the cytoplasm of hepatocytes

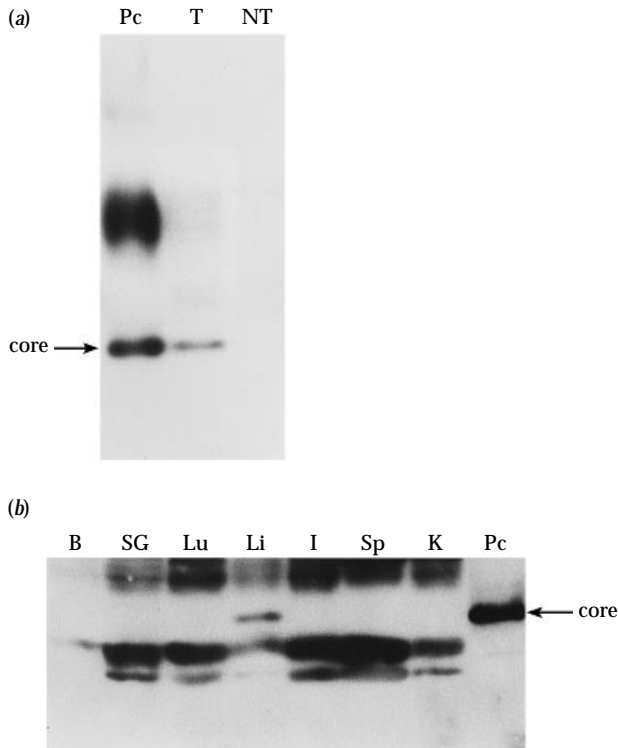


Fig. 2. (a) Detection of the core protein by Western blot analysis. Pc, positive control core protein expressed in insect cells using a baculovirus vector; T, a liver sample from a hepatitis C virus core transgenic mouse, C21 line male; NT, a liver sample from a non-transgenic littermate. (b) Tissue distribution of the core protein in a 6-month-old C21 male mouse. B, brain; SG, salivary glands; Lu, lung; Li, liver; I, intestine; Sp, spleen; K, kidney; Pc, positive control core protein expressed in insect cells using a baculovirus vector. Arrows indicate the positions of the core protein.

(Fig. 3A–D) from the age of 2 months. These vacuolations were stained positive by Sudan III, indicating that these lesions are steatosis (Fig. 3E). Staining with periodic acid–Schiff was negative (data not shown). Steatosis was mild in younger mice (Fig. 3B, 3 months) and became moderate to severe with the increasing age of the mice (Fig. 3C–D, 6 and 9 months, respectively). The lipid droplets were small in young mice, while large droplets became predominant in older mice. All the male and approximately 50% of the female transgenic mice developed steatosis in the liver by the age of 6 months. In contrast, no steatosis was observed in the livers of control littermates. Because obesity is a major cause of steatosis in humans, we determined the body weights of the mice. However, there was no significant difference between the body weights of the HCV core transgenic mice and those of non-transgenic littermates [males: 40.1 ± 5.0 g ($n = 22$) vs 43.0 ± 4.7 g ($n = 20$); females: 30.1 ± 4.3 g ($n = 18$) vs 28.9 ± 3.2 g ($n = 15$); statistically not significant]. In addition, there was no significant difference in weights of the livers between the transgenic mice and non-transgenic littermates. There was no significant difference in the levels of total cholesterol and triglycerides in the HCV core transgenic mice

and those in normal control mice. No hepatocyte necrosis, bile duct damage, lymphoid follicle formation, preneoplastic focus or neoplasia was observed in the transgenic mice up to the age of 12 months.

We then examined the tissue localization of the core protein by immunostaining. Frozen sections of transgenic mouse liver stained with anti-core rabbit serum revealed that the core protein was present in the cytoplasm of hepatocytes principally located around the central veins, but was also present within lobules (Fig. 3F). The core protein was invariably localized in the cytoplasm of hepatocytes (Lo *et al.*, 1995; Santolini *et al.*, 1994; Suzuki *et al.*, 1995). Control mouse liver did not show staining with anti-core rabbit serum (Fig. 3G), and transgenic mouse liver did not show staining with normal rabbit serum.

Absence of increased apoptosis in the liver

Although we did not observe necroinflammatory reactions in the livers of the transgenic mice, we applied the TUNEL method (Gavrieli *et al.*, 1992) in order to detect the presence of apoptosis, which may not be detected by conventional microscopic examination. However, the incidence of TUNEL-positive hepatocytes in transgenic mouse liver was as low as that in the livers of non-transgenic littermates. There was no elevation of serum alanine aminotransferase levels by the age of 12 months in the transgenic mice.

Discussion

The steatosis which occurred in the liver of the HCV core transgenic mice is characteristic of chronic hepatitis C. When compared with chronic hepatitis B or autoimmune chronic active hepatitis, chronic hepatitis C has several characteristic histological features in the liver. These include bile duct damage, lymphoid follicles and steatosis (Bach *et al.*, 1992; Lefkowitz *et al.*, 1993; Scheuer *et al.*, 1992). Bach *et al.* (1992) reported that steatosis was observed in 72% of 50 patients with chronic hepatitis C compared to 19% with autoimmune chronic active hepatitis. Lefkowitz *et al.* (1993) reported that large-droplet fatty change was observed at a significantly higher rate in patients with chronic hepatitis C than in those with chronic hepatitis B. Our results confirm the existence of a relationship between chronic hepatitis C and steatosis, and suggest a direct role for the HCV core protein in inducing steatosis in the liver.

The hepatic steatosis observed in the transgenic mice is not the result of uptake of lipids from the blood, because there was no elevation in the levels of serum triglycerides or cholesterol. The core protein may have a specific effect on hepatocytes, causing the formation of fat droplets. It is noteworthy that there is no histological phenotype in the liver of mice transgenic for HCV envelope genes, E1 and E2, which are expressed in the cytoplasm of hepatocytes under the control of

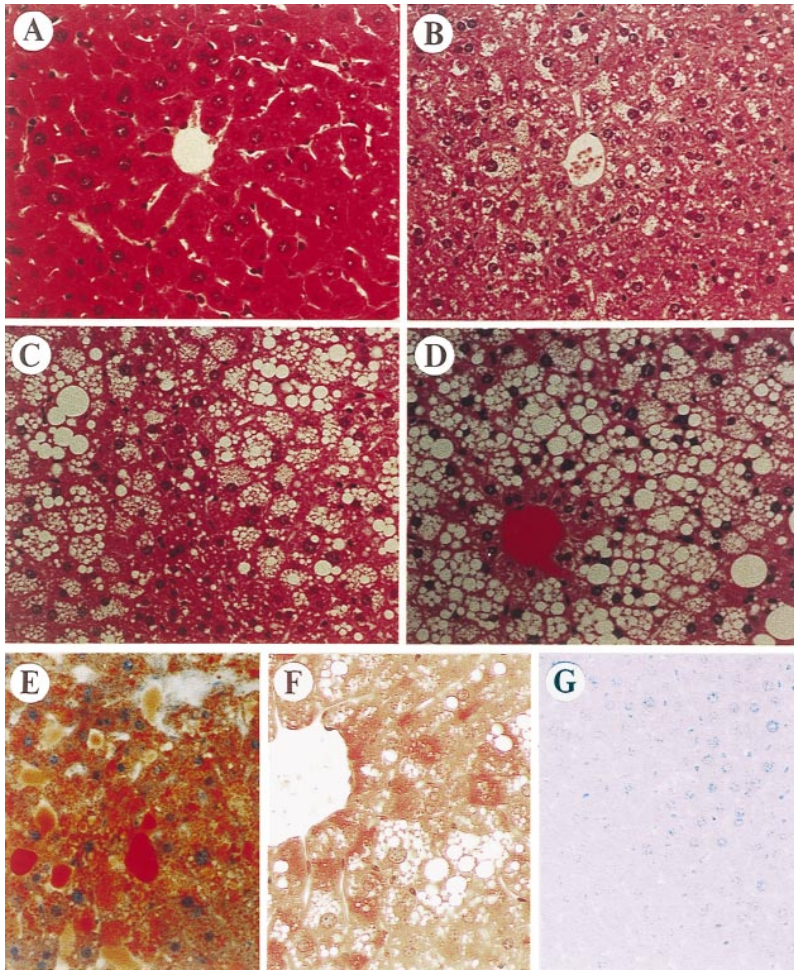


Fig. 3. Microscopic examination of the liver from HCV core transgenic mice. (A) A 6-month-old male non-transgenic mouse; (B) a 3-month-old male transgenic mouse from the C21 line; (C) a 6-month-old male transgenic mouse from the C49 line; (D) a 9-month-old male transgenic mouse from the C21 line; (E) a 9-month-old male transgenic mouse from the C21 line; (F) a 6-month-old male transgenic mouse from the C21 line; (G) a 6-month-old male non-transgenic mouse. Paraffin-embedded sections of liver were stained by haematoxylin–eosin (A–D). Frozen liver sections were stained by Sudan III (E) or immunostained with anti-core rabbit serum by the ABC method (F, G), and counterstained by methyl green (E–G). Magnifications: $\times 150$ (A, D), $\times 200$ (B, C, E), $\times 300$ (F, G).

the same promoter used in this study (Koike *et al.*, 1995). The core protein may bind to enzymatic molecules or apolipoproteins which are involved in the metabolism of lipids, and lead to the formation of fat droplets in hepatocytes. It is interesting that the HCV core gene is associated with the formation of cytoplasmic vacuoles containing triglycerides when it is transfected into cultured cells (Barba *et al.*, 1997; Moradpour *et al.*, 1996). It should be noted that the HCV core protein is also detectable in the liver of some chronic hepatitis C patients by our Western blot analysis, and that the expression level in the transgenic mouse liver is only a few times higher than those in the livers of patients (H. Fujie and K. Koike, unpublished results).

Other features characterizing histology of hepatitis C, bile duct damage and lymphoid follicle formation, were not observed in the HCV core gene transgenic mice. Moreover, no increased incidence of cell death compared to that of normal littermate mice and no apparent lymphocytic infiltrations were observed in the livers of the transgenic mice. The HCV core protein per se is not likely to cause hepatitis, although there remains a possibility that the expression level of the core protein may be too low to induce cell death or hepatitis in

transgenic mice. Other HCV proteins may be responsible or the immunological response of the host may be essential for the induction of hepatitis in HCV infection.

Our current results support the existence of a relationship between chronic HCV infection and hepatic steatosis, and suggest a direct role for the viral core protein in the steatotic changes that are frequently observed in chronic HCV infection. This mouse system would be a good animal model for studying the pathogenesis of HCV infection.

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