Characterization of liver histopathology in a transgenic mouse model expressing genotype 1a hepatitis C virus core and envelope proteins 1 and 2

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Hepatitis C virus (HCV) is a major cause of chronic hepatitis and hepatocellular carcinoma worldwide. The purpose of this study was to determine how the HCV structural proteins affect the dynamic structural and functional properties of hepatocytes and measure the extra-hepatic manifestations induced by these viral proteins. A transgenic mouse model was established by expressing core, E1 and E2 proteins downstream of a CMV promoter. HCV RNA was detected using RT-PCR in transgenic mouse model tissues, such as liver, kidney, spleen and heart. Expression of the transgene was analysed by real-time PCR to quantify viral RNA in different tissues at different ages. Immunofluorescence analysis revealed the expression of core, E1 and E2 proteins predominantly in hepatocytes. Lower levels of protein expression were detected in spleen and kidneys. HCV RNA and viral protein expression increased in the liver with age. Histological analysis of liver cells demonstrated steatosis in transgenic mice older than 3 months, which was more progressed with age. Electron microscopy analysis revealed alterations in nuclei, mitochondria and endoplasmic reticulum. HCV structural proteins induce a severe hepatopathy in the transgenic mouse model. These mice became more prone to liver and lymphoid tumour development and hepatocellular carcinoma. In this model, the extra-hepatic effects of HCV, which included swelling of renal tubular cells, were mild. It is likely that the HCV structural proteins mediate some of the histological alterations in hepatocytes by interfering with lipid transport and liver metabolism.

INTRODUCTION

Hepatitis C virus (HCV) is a major cause of chronic human liver disease. Approximately 170 million people worldwide are infected with HCV (Cohen, 1999). HCV infection often progresses to chronic hepatitis, liver cirrhosis and hepatocellular carcinoma (HCC). Although death from fulminant hepatitis C is rare, it accounts for the deaths of at least 8000–10 000 Americans each year (Alter, 1997). HCV belongs to the family Flaviviridae and has a positive single-stranded RNA genome that encodes a 3000 aa polyprotein precursor. The structural proteins core, E1 and E2, which form viral particles, are located at the

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amino-terminal end of the precursor protein. Downstream are non-structural proteins P7, NS2, NS3, NS4A, NS4B, NS5A and NS5B, which are involved in virus replication (Choo et al., 1991; Grakoui et al., 1993; Bartenschlager et al., 1994; Lin et al., 1994; Lo et al., 1996).

The exact pathological processes of HCV infection are not completely understood. Following an incubation period of 3–12 weeks, HCV clinical manifestations are often mild. However, as many as 85% of the infected individuals develop a chronic infection, frequently with severe long-term liver pathology (Hoofnagle, 1997). HCV infection is also associated with the development of extra-hepatic manifestations, such as type II mixed cryoglobulinemia, glomerulonephritis and B-cell non-Hodgkin’s lymphoma (Chan et al., 2001; Gasparotto et al., 2002; Ishikawa et al., 2003).

Chronic HCV infection has been treated with interferon-α but less than 20% of patients achieve a sustained response with this drug. Combination therapy of interferon-α and a nucleoside analog, ribavirin, has increased the rate of sustained response to more than 30% (Gretch et al., 1996; Davis et al., 1998; Poynard et al., 1998). The broad genetic variability of the virus genome has resulted in six identified genotypes with more than 50 subtypes (Simmonds et al., 1993). Also, in individuals infected with HCV, the virus becomes a quasispecies, a population of closely related variants (Bukh et al., 1995; Kato, 2000). Thus, the understanding of the biology of the infection, the diagnosis and the development of a vaccine against HCV is complex.

Little is known about the mechanism of HCV pathogenesis. However, both immune-mediated mechanisms and direct viral cytotoxicity have been suggested as factors in the pathology associated with hepatitis C (Chisari, 1997). The lack of an appropriate viral culture system and a small animal model that can support virus replication has hampered detailed analysis of HCV pathogenesis and vaccine development (Chisari, 1997). Combined research efforts with different small animal models will facilitate detailed analysis of HCV in vivo and clarify HCV pathogenesis.

A previous study has shown that HCV proteins are not directly cytopathic on liver cells and that the host immune response plays an important role in hepatitis C infection (Wakita et al., 1998). In contrast, other studies have provided evidence that HCV structural proteins play a direct role in the development of liver steatosis, and increase the risk of liver cancer in transgenic mice (Lerat et al., 2002). These transgenic mouse models utilize organ-specific promoters, which preferentially express viral proteins in the liver. In the present study, a transgenic mouse model that expressed the HCV structural proteins core, E1 and E2 under the control of a universal CMV promoter was developed. The model shared several features of human HCV infection, such as severe hepatopathy characterized by the development of steatosis as well as liver and lymphoid tumours. This animal model could be used to study pathogenesis and novel treatment modalities for HCV infections and liver cancer.

**METHODS**

**Construction of expression vector.** Total RNA was extracted from the plasma of a patient infected with HCV genotype 1a. The RNA was used as a template to amplify core, E1 and E2 genes. The HCV fragment containing core, E1 and truncated E2 genes, encoding for amino acid residues 1–683 (2049 nt), was constructed by RT-PCR using the forward primer 5′-ACCATGAGCAGGAATCCTAAAACCTC-3′ and the reverse primer 5′-TGGTAGGGTTGTGGAAGGAACAGC-3′. The amplified fragment was cloned into the EcoRI sites of pCR 2.1 vector using the TOPO-TA cloning kit (Invitrogen). The nucleotide sequence was verified by DNA sequencing using the University of Ottawa DNA sequencing facility. The core, E1 and E2 fragments were subsequently subcloned into pVAX downstream of a cytomegalovirus (CMV) promoter (Fig. 1a). The pVAX-HCV (core, E1, E2) was linearized by MfeI restriction enzyme digestion.

**Production of transgenic mice.** The linearized plasmid containing HCV genes encoding core, E1 and E2 proteins was micro-injected into the pro-nuclei of fertilized eggs from a B6CF1 mouse strain (Charles River Laboratories). The injected eggs were surgically implanted into the oviducts of pseudopregnant mothers. Founder mice harbouring HCV fragments were identified as transgenic via PCR using DNA isolated from tail biopsies according to the DNeasy Tissue kit protocol (Qiagen). Five founder mice that demonstrated a high copy number of the transgene were used to establish transgenic lines. Transgenic mice were maintained by breeding with non-transgenic mice to develop a heterozygous colony. All mice were fed ordinary chows and were maintained in a specific, pathogen-free state and received standard care following guidelines established in the animal care facility at the Faculty of Medicine, University of Ottawa.

**RT-PCR.** Total RNA was extracted from different mouse tissues (liver, spleen, kidney, brain, lung and heart) using the RNeasy mini kit (Qiagen). Reverse transcription of total RNA was performed with M-Mul reverse transcriptase (Applied Biosystems) and random hexamers (Applied Biosystems) to generate cDNA, according to the manufacturer’s instructions. The cDNA was amplified by PCR using the primers described above. RNA extract from the liver of a non-transgenic littermate was used as a negative control.

**Immunofluorescence analysis.** Transgenic mice and non-transgenic littermates were sacrificed between the ages of 3 and 18 months. Mice were anaesthetized and then perfused with normal saline followed by 4% paraformaldehyde. Liver, kidney, heart, spleen, lung, salivary glands and brain tissues were removed surgically and placed in 4% paraformaldehyde overnight at 4°C. A piece of tissue was placed onto a plastic mould, covered with tissue embedding medium and then frozen in isopentane on dry ice. Frozen tissue sections of 5 μm thickness were cut using a cryostat and placed onto lysine-coated slides. Frozen tissue sections were incubated with blocking buffer (5% normal goat serum and 0.1% Triton X-100 in PBS) for 1 h at room temperature. Rabbit anti-core E1, E2 polyclonal antibody (prepared in our laboratory according to the University of Ottawa animal care facility protocols for antibody production) or anti-core monoclonal antibody (Biogenesis) were applied at dilutions of 1:50 and 1:500, respectively, for 1 h at room temperature. After washing with PBS, FITC-conjugated anti-rabbit IgG (Sigma) or FITC-conjugated anti-mouse IgG (Sigma) were incubated at a dilution of 1:200 for 1 h at room temperature. After washing with PBS, the tissue sections were mounted with Vectashield mounting medium (Vector). In addition to using tissues from non-transgenic littermates as negative controls, we also performed parallel immunostaining experiments using pre-immunized rabbit serum, as well as secondary anti-rabbit FITC-labelled antibody alone.
Real-time RT-PCR assay. Total RNA was extracted from mouse liver using the RNeasy mini kit (Qiagen). There were four groups of three mice (n=3) of different ages. Each mouse liver was tested in triplicate by real-time RT-PCR. For the reverse transcription reaction, the RNA was reverse transcribed using random hexamers (Applied Biosystems) and MuLV reverse transcriptase (Applied Biosystems), following the manufacturer’s instructions. Reverse transcription was performed for 1 h at 37°C using 1 μg RNA per reaction in a 20 μl reaction volume. In order to stop this reaction, the cDNA samples were incubated for 15 min at 72°C. The real-time PCR was performed in special optical tubes in 36-well microtitre plates (Perkin-Elmer/Applied Biosystems) with an iCycler (Bio-Rad). Fluorescent signals were generated using the Quantitect SYBR Green PCR kit (Qiagen). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control gene with the following sense and antisense primer sequences, 5’-ATGTTCCGGTCGGTCTCTGA-3’ and 5’-TTGAAGTCGGAGAGAC- AACCT-3’, respectively. The HCV core gene was analysed using the following oligonucleotide primers (300 nM): forward 5’-ACCATGAGCAATCCTAAACCTC-3’ and reverse 5’-GCAACAAGTAAACTCCACCAACGA-3’. The core and GAPDH genes were amplified using the primers mentioned above and a cDNA template from both transgenic and non-transgenic animals. Target samples were added in individual reactions to a total volume of 50 μl and no cDNA was added to the negative control. For each amplification using real-time PCR, the protocol included 15 min at 95°C and 40 cycles of 15 s at 94°C, 30 s at 66°C and 30 s at 72°C. All PCR experiments were performed with hot start and were run in 2187
triplicate. The iCycler software (Bio-Rad) detected the threshold cycle \( (C_T) \) for each amplicon. Normalization was performed using the \( 2^{-\Delta\Delta C_T} \) method (Livak & Schmittgen, 2001). Experimental controls including nonreverse-transcribed RNA samples were performed.

**Histological staining.** Mouse tissues were fixed in 4% paraformaldehyde and embedded in paraffin. Sections of 5 μm thickness were stained with haematoxylin and eosin, Sudan black B or Masson’s trichrome staining according to standard methods used in the Department of Pathology and Laboratory Medicine at the Faculty of Medicine, University of Ottawa. Tissue examination was performed by one of us (S.G.) and interpreted according to standard histopathological morphology. To determine the extent of steatosis a semi-quantitative method was used, in which 1+ represented lipid droplets in sporadic hepatocytes; 2+ represented moderate steatosis, in which hepatocytes with lipid droplets were seen clearly in more than two of five fields examined; 3+ represented moderate to severe steatosis, in which lipid droplets were extensively distributed in most areas of the liver; 4+ very severe steatosis, in which steatosis was observed everywhere in the liver. Characterization of the tumours was performed according to standard morphological histology. For example, the diagnosis of lymphosarcoma in the liver and nodes was made based on the undifferentiated nature of the cell morphology, which resembled early embryonic cells; the presence of many mitotic figures and the invasiveness represented by a lack of tumour capsule, tumour size and invasion of nodes and liver. In addition, a large portion of normal hepatic tissue was replaced by tumour cells. In contrast, the diagnosis of adenoma was based on cell morphology, including the well differentiated nature of cells with few mitotic figures and the lack of invasion of extra-hepatic tissues.

**Electron microscopy.** The electron microscopy study was done according to the standard procedures at the electron microscopy facility at the Children’s Hospital of Eastern Ontario in Ottawa. Approximately 0.25 mm thick liver tissue slices were post-fixed briefly in 2% osmium tetroxide in 0.1 M cacodylate buffer, dehydrated in graded ethanol and embedded in Spur epoxy resin. Ultrathin 60–70 nm sections were cut using a Leica Ultracut R ultra-microtome, mounted on 200 mesh copper grids and stained with 10% uranyl acetate in 50% methanol, followed by Reynold’s lead citrate. Grids were examined and micrographed using a JEOL 1010 transmission electron microscope at 60 KV.

**RESULTS**

**In vitro expression of the transgene**

An HCV expression vector containing the sequences encoding the core, E1 and E2 proteins was constructed from a PCR product obtained from the serum of a patient infected with genotype 1a HCV. The amplified fragment was inserted into the pVAX vector containing a CMV promoter (Fig. 1a). To measure the HCV protein expression in vitro, Chinese hamster ovary (CHO) cells were transiently transfected with pVAX core, E1 and E2, and then cells were analysed by immunolabelling with anti-core monoclonal antibody or with anti-core, E1 and E2 rabbit polyclonal antibody using immunofluorescence analysis. Recombinant viral proteins were present mainly in the cytoplasm, and no protein expression was detected in the nuclei (Fig. 1c, d). Cell culture of the transfected CHO cells expressing the HCV recombinant proteins demonstrated no apparent morphological cellular changes.

**Generation of transgenic mice**

Five transgenic founder (F0) mice were used to establish five transgenic lines. Transgenic mice were analysed for HCV transgene expression by PCR using genomic DNA extracted from the tails. Founder mice were crossed with B6C3F1 mice to expand the mouse colony, and ensure the integrated transgene was successfully transmitted to the offspring. F1 (first generation) and F2 (second generation) heterozygous mice from the five lines were used as models in this study.

**Expression of the core, E1 and E2 transgenes**

Transgenic mice expressed the HCV core, E1 and E2 transcript in all tissues including, liver, kidney, spleen, heart, lung and brain (Fig. 1b), but the viral proteins were mainly expressed in the liver (Fig. 2a, b) demonstrating selective expression in certain tissues. Viral protein detection was not observed in either the heart or the brain. Spleen cells, as well as epithelial cells in the renal microtubules of the kidney, demonstrated relatively low levels of viral protein expression. The liver demonstrated the highest level of viral protein expression with a centrilobular distribution of groups of cells expressing the viral proteins. Tissues from non-transgenic littermates did not show HCV protein expression. When fetal tissue was analysed by immunofluorescence to detect the viral proteins, the fluorescent signal was restricted to the salivary glands (Fig. 2e, f). However, salivary glands of adult mice did not express HCV proteins (data not shown). In addition, there was no detectable viral protein expression in fetal livers (data not shown). There was no difference in the levels of protein expression between the five lines. Core, E1 and E2 protein expression was tested in all tissues using both rabbit anti-core, E1, E2 polyclonal and anti-core monoclonal antibodies.

**Viral proteins accumulate in hepatocytes with age**

The number of hepatocytes expressing HCV proteins was directly associated with the age of transgenic animals. Hepatocytes expressing the HCV proteins were detected only in mice 2–3 months of age or older (Fig. 2a, b). These hepatocytes were scattered around the central veins in a centrilobular pattern. Fluorescent cells appeared confluently in the renal microtubules of the kidney, demonstrated relatively low levels of viral protein expression. The liver demonstrated the highest level of viral protein expression with a centrilobular distribution of groups of cells expressing the viral proteins. Tissues from non-transgenic littermates did not show HCV protein expression. When fetal tissue was analysed by immunofluorescence to detect the viral proteins, the fluorescent signal was restricted to the salivary glands (Fig. 2e, f). However, salivary glands of adult mice did not express HCV proteins (data not shown). In addition, there was no detectable viral protein expression in fetal livers (data not shown). There was no difference in the levels of protein expression between the five lines. Core, E1 and E2 protein expression was tested in all tissues using both rabbit anti-core, E1, E2 polyclonal and anti-core monoclonal antibodies.
Fig. 2. Immunofluorescence staining of HCV structural proteins in frozen formalin-fixed transgenic mouse tissues. (a) Liver section of a 2-month-old transgenic mouse showing the expression of HCV structural proteins in hepatocyte cytoplasm (arrows). (b) Liver section from a 2-month-old non-transgenic mouse showing no evidence of HCV structural proteins. Propidium iodide used as a counter stain. (c) Liver of 1-year-old transgenic mouse showing an increase in the number of hepatocytes expressing HCV structural proteins in the cytoplasm (arrows). Cells without green fluorescence in the middle of the section are not expressing the protein. (d) Liver section of a 1-year-old non-transgenic mouse showing no expression of HCV structural proteins. (e–f) Immunofluorescence detection of HCV structural proteins in frozen formalin-fixed sections of salivary glands of a transgenic mouse fetus stained with a rabbit polyclonal antibody to HCV core, E1 and E2 proteins; (e) is low magnification (× 20), (f) high magnification (× 40). Proteins are detected in both the epithelial cell layer – arranged as wedges forming an acinus around a central lumen – and in the myoepithelial cell layer surrounding them. Rabbit polyclonal antibody against core, E1 and E2 was used as primary antibody. FITC-conjugated antibody was used as a secondary antibody. For panels (a–d), magnification × 40. Bars, 50 μm.
mouse (Table 1). There was a 1.9-, 274- and 776-fold increase in HCV transcript concentration at 6, 14 and 18 months, respectively, when compared with 4-month-old mice.

**Differential viral RNA expression in transgenic mouse tissues**

The viral RNA expression varied not only according to age, but also according to individual tissues (Table 2). The HCV core, E1 and E2 expression was higher in the liver than in any other tissues examined by real-time PCR. Hepatic viral RNA expression was increased more than 68-fold over the brain. In contrast, spleen and kidneys showed approximately the same level of expression of viral RNA.

**Histopathological observations in transgenic mice**

Post-mortem examination of transgenic mice demonstrated that the gross morphology of organs was normal, with the exception of the livers, which showed hepatomegaly and yellow discolouration. Transgenic mice (n = 4) had a ratio of liver to body mass of 6.1 ± 1.6 % while the non-transgenic counterpart (n = 5) demonstrated a ratio of 4.1 ± 0.6 % (P < 0.01). To determine if the expression and accumulation of HCV proteins induced morphological alteration in hepatocytes, paraffin-embedded liver sections were stained with haematoxylin and eosin. Transgenic hepatocytes showed several morphological alterations including increased cell volume and chromatin disorganization. Normal hepatocytes showed a polygonal shape that was well delineated and had arranged hepatic plate. In contrast, transgenic hepatocytes were round and swollen to about three times the size of normal cells. There was a loss of hepatic cell borders and a loss of hepatic plate arrangement in transgenic hepatocytes as well as non-uniform staining in the nuclei (Fig. 3a, b). Cytoplasmic vacuolation characteristic of steatosis was prominent in hepatocytes of transgenic mice at 3 months of age or older (Fig. 3b). A percentage of 45.8% transgenic mice that developed steatosis were male, while 54.2% were female (Table 3). Both microvesicular and macrovesicular steatosis were observed. There was a centrilobular distribution of macrovesicular steatosis, while the microvesicular pattern was more generally distributed in the liver. Sudan black staining confirmed that the observed cytoplasmic vacuolation in hepatocytes were in fact lipid deposits in transgenic mice (Fig. 3c, d). The extent

**Table 1. Relative expression of RNA in the livers of transgenic mice of different ages detected by real-time RT-PCR**

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<th>Age (months)</th>
<th>Mouse no.</th>
<th>HCV core CT</th>
<th>GAPDH CT</th>
<th>ΔC_T (average core CT− average GAPDH CT)</th>
<th>ΔΔC_T (average ΔCT− average ΔC_Tcontrol)</th>
<th>Normalized core relative to 4 months (2^−ΔΔCT)</th>
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<tr>
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**Table 2. Relative expression of RNA in various tissues detected by real-time RT-PCR**

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<tr>
<th>Transgenic mouse tissue</th>
<th>HCV core CT</th>
<th>GAPDH CT</th>
<th>ΔC_T (average core CT− average GAPDH CT)</th>
<th>ΔΔC_T (average ΔCT− average ΔC_Tcontrol)</th>
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<td>Lung</td>
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<td>11.45</td>
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of the steatosis became more severe in older mice, which resulted in involvement of more than 90% of hepatocytes at the age of 12 months. There was minimal or no inflammatory cell infiltration in liver sections of transgenic mice. Trichrome staining demonstrated deposits of collagen in portal areas and the collapse of lobular architecture of the liver in older mice (12–18 month-old) (Fig. 4f). Histological abnormalities were also found in the kidneys of transgenic mice. Microtubular and glomerular epithelial cells were swollen and demonstrated some cytoplasmic vacuolation (data not shown).

Evidence of lymphoproliferative disorders and tumours in transgenic mice

Several mice developed nodular hyperplasia in liver, spleen and lymph nodes, as well as development of lymphosarcoma in the liver. Out of 109 transgenic mice investigated, 17 (15.59%) transgenic mice developed various tumours. Of these mice, 12 (70.5%) were male suggesting that tumour formation occurred more readily in males compared with females (Table 3). Mice developing lymphoproliferative disorders were 18 months of age or older. Transgenic mice developed different hepatic tumours (Fig. 4), including adenomas (29%) (data not shown), hemangiomas (17.6%) (Fig. 4a–c), lymphoid tumours (29%) (Fig. 4d) and HCC (23%) (Fig. 4e). All corresponding tissues from non-transgenic littermates did not demonstrate tumours or any evidence of lymphoproliferative disorders.

Subcellular abnormalities in transgenic hepatocytes

Electron microscopy demonstrated several abnormalities in the intracellular organelles of hepatocytes in transgenic mice (Fig. 5a, b). A loss of the rough endoplasmic reticulum pattern was observed as well as swelling of mitochondria and loss of mitochondrial cristae. Additionally, the nucleus had lost the normal organization of chromatin, which

Fig. 3. Hepatic steatosis in transgenic mice. (a) Haematoxylin and eosin-stained paraffin-embedded liver section showing normal liver histology in a 1-year-old non-transgenic mouse. (b) One-year-old transgenic liver section showing both macrovesicular (green arrows) and microvesicular (yellow arrows) steatosis. (c–d) Hepatic steatosis in transgenic mice as detected by Sudan black B staining. (c) Four-month-old non-transgenic mouse liver cryosections (5 μm) showing no black stained lipid droplets. (d) Four-month-old transgenic mouse liver section showing black stained lipid droplets indicated by arrows. The nucleus of the cell is stained red. Magnification × 20. Bars, 50 μm.
Fig. 4. Liver tumours in transgenic mice. (a–b) Haematoxylin and eosin-stained paraffin-embedded liver section showing hemangioma in a murine transgenic liver. Lesion appears consistent with early endothelial cell proliferation and peliosis – proliferating endothelial cells (arrows) aggressively invading hepatic sinusoids in the focal area – but there is more focal angiopathy rather than neoplasm, characteristic of benign hemangioma. (a) Shows the edge of the hemangioma. (b) Shows the centre of the hemangioma (magnification ×40). (c) Photomicrograph of a liver tumour in HCV-transgenic mouse stained with Masson’s trichrome stain showing blue stained collagen trapped between hepatocytes. Red blood cells are shown in light brown colour (magnification ×60). (d) Haematoxylin and eosin staining of a liver section demonstrating infiltration by a lymphoid tumour. This mouse also developed tumours in the spleen and in cervical lymph nodes as well (magnification ×20). (e) Trichrome staining of a transgenic liver section demonstrating development of HCC. Polymorphic nuclei and invasion of steatotic liver by anaplastic cells. Cells are arranged in macrotrabecular patterns – where trabeculae are eight or more cells thick – differentiating it from adenoma. Note the ground glass appearance of tumour cells indicating protein synthesis (magnification ×20). (f) Trichrome staining of a liver section in an 18-month-old transgenic mouse. Capsular fibrosis due to disappearance of hepatocytes and approximation of the remaining reticulin fibres demonstrating collapse of the lobular structure, collagen deposition and inflammatory infiltrates. It involves large lobular areas and multiple lobules, producing approximation of portal fields together, which can still be recognized with a stain for elastic fibres (magnification ×20). Bars, 50 μm.
appears uncondensed and uneven in contrast to a normal nucleus.

**DISCUSSION**

The structural proteins core, E1 and E2 of HCV were expressed in a transgenic mouse model using a universal promoter to elucidate the effects of HCV on hepatic and extra-hepatic tissues. Initially, the vector was transfected into CHO cells, in order to confirm authentic expression of the viral proteins and a high level of expression from this vector. The HCV transgenic mice expressed RNA encoding the structural proteins in all tissues examined. However, in adult mice viral proteins were expressed selectively in the

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*Fig. 5.* Electron micrograph of a normal (a) and transgenic mouse liver (b). In the transgenic, extensive abnormalities are observed in the hepatocyte organelles, with abnormal mitochondrial shape, swelling and loss of cristae as well as chromatin disorganization and lipid droplets in the cytoplasm (magnification $\times 7500$). L, Lipid; M, mitochondria; N, nucleus; GC, Golgi complex.
liver, but detected at low concentrations in both the kidney and the spleen. It has been shown that chronic infection with HCV can lead to the immune complex syndromes of cryoglobulinemia and membranoproliferative glomerulonephritis. Also hepatitis C is a complicating factor among patients with end-stage renal disease and renal transplants (Morales, 2004). The differential expression of viral proteins in murine tissues suggests that viral protein expression may be controlled via translational mechanisms. Immunohistochemistry suggested that the level of viral proteins in the liver increased with age. This was confirmed by real-time RT-PCR, which was performed in transgenic mice livers of different age groups and in various tissues.

Our study showed that fetal transgenic mice expressed viral protein only in salivary glands. This expression was not observed in adult transgenic mice. Several studies have shown that HCV can replicate in several tissues other than the liver, such as lymphocytes and salivary glands (Koike et al., 1997; Toussirot et al., 2002; Ishikawa et al., 2003). This may have important implications for the pathogenesis of HCV infections. There appears to be a strong association of salivary gland lesions, lymphocytic capillaritis and lymphocytic adenitis with chronic hepatitis C. HCV infection of lymphocytes may be related to HCV-associated lymphoma and/or autoimmune disorders. Similarly, salivary gland infection may be associated with Sjögren’s syndrome (Haddad et al., 1992; Toussirot et al., 2002).

The HCV structural proteins caused profound structural abnormalities in the liver. Progressive steatosis, mitochondrial swelling and nuclear abnormalities became more severe with age. This hepatopathy was more widespread in the livers of older mice. Eventually, there was increasing fibrosis and liver cancer in older mice. Thus, this transgenic model of HCV represents many of the liver abnormalities observed in human infections. Moriya et al. (1997) described an HCV transgenic mouse model that expressed only the core protein. Their results showed that mice, 16 months of age, developed hepatic tumours, first appearing as adenomas with fat droplets in the cytoplasm. Additionally, they demonstrated neoplasia development in an adenoma, a ‘nodule-in-nodule’ formation. The authors concluded that HCV core protein may have a primary role in HCC development in transgenic mice and suggested that steatosis may develop as early as 3 months of age. Similarly, our transgenic mouse model developed steatosis as early as 3 months and developed adenoma and carcinoma after 1 year of age. In contrast to the HCV core transgenic, our transgenic model expressed the three HCV structural core, E1 and E2 proteins.

The three HCV structural proteins are important for the viral life cycle, specifically in the entry and formation of the viral capsid (Santolini et al., 1994; Penin, 2003). The significance of including core, E1 and E2 as a polyprotein in this mouse model allows for the analysis of the impact of these three proteins as a complex in the liver over the life span of the transgenic mice. While younger transgenic mice have no detectable HCV proteins, at 3 months of age an increasing number of hepatocytes expressing these proteins were observed around central veins. Viral protein expression and steatosis appeared in a similar time frame. Transgenic mice did not develop steatosis until they were at least 3 months of age with both severe microvesicular and macrovesicular steatosis eventually developing. The accumulation of lipids in hepatocytes is indicative of a disturbance in the lipid metabolism that may be caused by the accumulation of viral proteins and it is likely related to defects in mitochondrial and peroxisomal fatty acid oxidation and biosynthesis (Sabine et al., 1999; Hope & McLauchlan, 2000; Moriya et al., 2001; Perlemutter et al., 2002; Lerat et al., 2002). Our results demonstrate aggravation of liver abnormalities with increasing age (Fig. 3a and b). Previous studies have demonstrated steatosis is associated with a decreased antioxidant effect in the ageing liver. Similarly, a study performed by Lerat et al. (2002) also indicated that steatosis increased with age. However, their mouse model rarely developed steatosis before 10 months of age.

A cross-sectional study by Kumar et al. (2002) suggested that the HCV genotype 3 but not genotype 1 was cytopathic and induced hepatic steatosis in HCV-infected patients. However, our mouse model expressing HCV genotype 1a caused severe steatosis, which demonstrates that this genotype is also involved in the development of this abnormality. Both Rubbia-Brandt et al. (2000) and Adinolfi et al. (2001) suggested that steatosis in patients infected with HCV genotype 1 is not of viral origin. However, the model described in this study suggested that HCV genotype 1a may induce steatosis, and it is of viral origin. Steatosis caused by obesity was ruled out in this study, as the transgenic mice were not obese. More compelling evidence included that the non-transgenic littermates did not show steatosis.

In contrast to earlier studies by Lerat et al. (2002) using an albumin liver-specific promoter and Moriya et al. (1997) using an exogenous promoter, the results in the present study reflect the activity of a CMV promoter that has a universal transcriptional regulator. Thus, protein expression was illustrated in extra-hepatic tissues. After establishing the presence of HCV in older mice, fetal transgenic mice were analysed. In fetal mice, HCV structural proteins were detected only in the salivary glands (Fig. 2). Koike et al. (1995) confirmed this in another study. In contrast to Koike et al. (1995), there was no saladenitis present in our transgenic mouse model. However, Koike et al. (1995) developed mice that expressed only E1 and E2. In addition, the expression of HCV proteins in the salivary glands of older mice seemed to be lost. The unstable expression of HCV proteins in extra-hepatic tissues may explain the lack of saladenitis in our model. Therefore, our transgenic mouse model may not be as useful for characterizing extra-hepatic sites of pathogenesis associated with persistent HCV infection.

Several mice older than 18 months of age demonstrated lymphoproliferative disorders with nodular hyperplasia and lymphoma developing in the liver. A recently described
transgenic mouse model, expressing HCV core, developed lymphomas and liver adenomas at 20 months of age or older (Ishikawa et al., 2003). Similarly, HCV patients may develop two types of lymphoproliferative disorders, cryoglobulinemia and non-Hodgkin’s B-cell lymphomas in the liver (Chan et al., 2001; Gasparotto et al., 2002). Clonal expansion of B-cells has been detected in bone marrow, blood and liver of HCV-infected patients. One study suggests that HCV E2 could play a pathogenic role by stimulating a strong humoral response and the clonal expansion of B-cells, resulting in lymphoproliferative disorders (Gasparotto et al., 2002). However, our model is tolerant to the transgenes and chronic lymphocyte stimulation may therefore originate by other mechanisms. It is likely that the core protein may be involved in oncogenicity and lymphoproliferation. However, the study of this model may be useful to define the pathogenesis of HCV-associated lymphoproliferative disorders.

Recent publications have illustrated that HCV confers oncogenic potential to hepatocytes (Ikeda et al., 1993; Takano et al., 1995; Chiba et al., 1996; Silini et al., 1996; Bruno et al., 1997; Shibata et al., 1998; Ueno et al., 2001). In addition, HCC tumorigenesis in patients infected with HCV is correlated with steatosis (Lemon et al., 2000; Ohata et al., 2003). Transgenic mice with steatosis displayed dysplastic growth of cells evolving into tumour formation. Furthermore, transgenic mice showed deposition of collagen and progressive fibrosis (Kato et al., 2003). These transgenic mice expressing core with a liver-specific serum amyloid P regulator did not demonstrate HCC. Therefore, the other two structural proteins of HCV (E1 and E2) used in this study may be a requisite for HCC development. Although the underlying mechanism of HCC progression in HCV transgenic mice remains to be understood, our transgenic mice are acceptable models to elucidate this role.

Murine models are critical for understanding HCV pathogenesis and to explore the molecular interface between HCV, the liver and other tissues. Recently, transplantation of healthy human hepatocytes that carry a plasmogen activator transgene (Alb-uPA) into SCID mice was carried out (Mercer et al., 2001). This model may be useful in acute studies of antiviral activity against HCV. In summary, expression of core, E1 and E2 in the liver of transgenic mice may contribute to the development of liver disease including steatosis, fibrosis and HCC. Further studies are required in order to explain the role of the structural proteins in mitochondrial injury and more generally in lipid metabolism. The transgenic mice presented in this study are suitable models to study the pathogenesis of HCV.

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