Repeated hepatocyte injury promotes hepatic tumorigenesis in hepatitis C virus transgenic mice

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Although hepatitis C virus (HCV) is a well-known causative agent of hepatocellular carcinoma (HCC), the mechanism by which HCV induces HCC remains obscure. To elucidate the role of HCV in hepatocarcinogenesis, a model of hepatocyte injury was established using HCV core transgenic mice, which were developed using C57BL/6 mice transfected with the HCV core gene under control of the serum amyloid P component promoter. After 18–24 months, neither steatosis nor hepatic tumors were found in transgenic mice. The extent of hepatocyte injury and tumorigenesis were then examined in transgenic mice following repeated administration of carbon tetrachloride (CCl_4) using various protocols (20%, 1/week; 10%, 2/week and 20%, 2/week). Serum alanine aminotransferase (ALT) levels did not differ among HCV core transgenic mice and non-transgenic littermates; however, after 40 weeks, hepatic adenomas preferentially developed in transgenic mice receiving 20% CCl_4 once weekly. Moreover, HCC was observed in transgenic mice receiving 2 weekly injections of a 20% solution of CCl_4, and was not observed in the non-transgenic control mice. In conclusion, the HCV core protein did not promote hepatic steatosis or tumor development in the absence of hepatotoxicity. However, the HCV core protein promoted adenoma and HCC development in transgenic mice following repeated CCl_4 administration. These results suggest that hepatotoxicity resulting in an increased rate of hepatocyte regeneration enhances hepatocarcinogenesis in HCV-infected livers. Furthermore, this experimental mouse model provides a valuable method with which to investigate hepatocarcinogenesis. (Cancer Sci 2003; 94: 679–685)

Hepatitis C virus (HCV) infection is known to cause persistent infection and chronic hepatitis, cirrhosis and hepatocellular carcinoma (HCC).[1, 2] Although a relationship between HCV infection and hepatocarcinogenesis has been clearly established on the basis of clinical experience, the mechanism by which HCV induces HCC is not well understood. HCC is mainly observed in patients with chronic hepatitis or cirrhosis. Thus, chronic liver inflammation creating a cycle of repeated hepatocyte destruction and regeneration may contribute to the development of HCC. The relationship between chronic inflammation and carcinogenesis, such as that between chronic hepatitis and HCC, is also observed in other inflammatory conditions, including ulcerative colitis (colon cancer), and *Helicobacter pylori*-induced gastritis (gastric cancer).[3, 4] Chronic inflammation in these organs leads to carcinogenesis through genomic damage. However, chronic hepatitis in the absence of hepatitis virus infection, such as occurs with autoimmune hepatitis, carries a low risk for the development of HCC.[5, 6] Thus, it is thought that, in addition to the effects of chronic inflammation, viral proteins must play a substantial role in HCV-associated hepatocarcinogenesis.

HCV, a member of the Flaviviridae family, has a positive sense single-stranded RNA genome consisting of about 9600 nucleotides.[7, 8] It encodes a single polyprotein of about 3000 amino acid residues.[7, 8] This polyprotein is processed into three structural proteins and six non-structural proteins. Of these HCV proteins, the core protein has the most highly conserved sequence among all the structural proteins, and has been shown to affect multiple host cell functions, including apoptosis, transcription, signal transduction, immune presentation, and cell transformation.[9–13] Moreover, a direct relationship between the HCV core protein and hepatocarcinogenesis has recently been reported.[14] This report describes spontaneous development of HCC in transgenic mice harboring HCV core protein gene without evidence of chronic inflammation.

In this study, we developed a model of liver cell injury in HCV core transgenic mice, and assessed the relationship between hepatocarcinogenesis and HCV core protein expression under conditions mimicking chronic hepatitis, i.e., repeated administration of carbon tetrachloride (CCl_4). In this model, preferential hepatic tumorigenesis was observed in HCV core transgenic mice, leading to the development of adenomas and HCC.

Materials and Methods

Production of transgenic mice. HCV cDNA (clone TH, nt 342–914, genotype 1b) was isolated from the serum of a patient with chronic hepatitis by PCR with the appropriate primers. The HCV cDNA was inserted into the expression vector pSG-1, containing a human serum amyloid P component promoter and a SV40 intron/polyadenylation sequence (kindly provided by Dr. Ken-ichi Yamamura). This promoter is liver-specific and its expression is known to depend on stage of development.[15] With this promoter, the target protein was undetectable during the fetal period and only became detectable after birth. The constructed expression unit was excised and purified by gel electrophoresis and ultra-centrifugation. Purified DNA was micro-injected into fertilized C57BL/6 mouse eggs (Japan SLC, Inc., Hamamatsu). Potential founders were analyzed for the presence of the transgene by PCR of mouse genomic DNA isolated from ear punch specimens using the HCV-specific primers, S371-20, 5′-ACCAAACGTAACACCAACC-3′, and R536-20, 5′-GATAGGTTGTCGCCTTCCAC-3′. Animal care and experimentation were performed according to study guidelines established by the Subcommittee on Laboratory Animal Care, Tokyo Metropolitan Institute for Neuroscience.

Southern blot analysis. Southern blot analysis of genomic DNA isolated from the livers of transgenic mice was performed as described previously.[16, 17] Briefly, genomic DNA was ex-

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tracted from transgenic mice using a QIAamp Tissue kit (Qiagen, Chatsworth, CA), and copy control DNA containing HCV core region cDNA was digested and separated by agarose gel electrophoresis. After electrophoresis, the DNA was transferred to a Nylon membrane (positively charged, Roche Diagnostics, Basel, Switzerland). The blots were probed with digoxigenin (DIG)-labeled HCV core region cDNA prepared using a DIG-High Prime kit (Roche Diagnostics), according to the manufacturer's instructions. The intensities of the resulting bands were quantified by optical density measurement using a model GS-700 imaging densitometer and molecular analyst software (Bio-Rad, Hercules, CA), and the integrated copy number of the transgene was determined for each lineage.

Western blot analysis. Liver samples were obtained from 8-week-old transgenic mice and non-transgenic littermates. To compare HCV core protein expression levels with those of HCV-infected patients, liver samples from patients with HCV-associated HCC were used. HCC lesions, as well as non-tumor regions, were resected from these patients after having obtained informed consent. *In vitro* transcription and translation of protein from a HCV core expression vector were performed to provide a positive control. The liver samples (approximately 100 mg) were homogenized in 500 µl of radioimmune precipitation assay (RIPA) buffer containing 1% sodium dodecyl sulfate (SDS), 0.5% NP40, 10 m*M* Tris-HCl (pH 7.4), 1 m*M* EDTA, and 150 m*M* NaCl with a Dounce homogenizer (Wheaton, Milliville, NJ), and centrifuged. The supernatant was mixed with an equal volume of 2× sample buffer containing 100 m*M* Tris-HCl (pH 6.8), 4% SDS, 0.15% bromophenol blue, 20% glycerol, and 10% 2-mercaptoethanol. The mixture was heated at 95°C for 5 min and separated on a 12% polyacrylamide gel. After electrophoresis, the proteins were transferred to a polyvinylidene difluoride membrane (Immobilon, Millipore Corp., Bedford, MA) with a semi-dry blotting apparatus (Biocraft, Tokyo). The transferred proteins were incubated with a blocking buffer containing 5% non-fat dry milk (Snow Brand Milk Products Co., Ltd., Sapporo) in phosphate-buffered saline. Monoclonal anti-HCV core antibody C7-50A (1 µg/ml) and horseradish peroxidase-labeled sheep anti-mouse IgG (1:2000 dilution) (Amersham Pharmacia Biotech, Buckinghamshire, UK) were used to detect HCV core protein.[18] The signals were visualized with a chemiluminescence system (ECL Plus, Amersham Biosciences Corp., Piscataway, NJ).

Quantification of HCV core protein. To estimate the distribution of HCV core protein in transgenic mice, HCV core protein concentrations were determined in various organs. Samples from various organs (brain, heart, lung, liver, kidney, intestine, spleen, pancreas and testis) were homogenized in RIPA buffer. Total protein concentrations in these homogenates were determined using the Bradford method (Bio-Rad) and adjusted to 1 mg/ml with normal serum. Aliquots (100 µl) of these homogenates were assayed by the new EIA method described previously.[19] HCV core protein concentrations in the tissue samples were divided by total protein concentrations and expressed as fmol/g of total protein.

Histological analysis. Liver samples were cut into at least 3 segments from different lobes, making sure to include any visible nodules, and these segments were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at a thickness of 5 µm, and stained with hematoxylin and eosin.

Administration of CCl_4. CCl_4 (ultra pure, Wako, Osaka) was dissolved in olive oil to a final concentration of 10% or 20% (v/v). Transgenic mice and non-transgenic littermates were intraperitoneally injected with 1 ml/kg CCl_4 solution once (day 0), or twice (day 0 and day 3), a week (1/W and 2/W, respectively). Among mice administered CCl_4, blood samples were serially collected by puncture of the retrobulbar venous plexus and serum alanine aminotransferase (ALT) levels were measured to evaluate the extent of liver injury. Serum ALT levels were determined using the Transnase Nissui kit (Nissui Pharmaceutical Co., Ltd., Tokyo), according to the manufacturer's protocol.

Statistical analysis. Statistical analyses were performed using the Mann-Whitney *U* test. The criterion of statistical significance was $P<0.05$.

Results

Establishment of the transgenic mice lineages. To analyze the function of the HCV core protein *in vivo*, we established HCV core transgenic mice. Seven animals were identified as harboring the transgene by PCR screening. To establish lineages of transgenic mice, these animals were mated with normal C57BL/6 mice. After confirming transmission of the transgene to the offspring, HCV core protein expression was estimated by western blotting. Three founder lineages, SC 6, SC 11 and SC 14, were identified as expressing HCV core protein in the liver. Of these lineages, those with integrated transgenes were identified by southern blot analysis (Fig. 1). Following densitometric analysis, the copy numbers of the transgene were estimated to be 25, 16 and 8 for lineages SC 6, SC 11 and SC 14, respectively. These mice developed normally and were capable of reproduction. Thus, these 3 lineages were used for further study.

Core protein expression in transgenic mice. To confirm expression of HCV core protein in the livers of transgenic mice, western blot analysis was performed on liver protein samples from transgenic mice from all three lineages and non-transgenic littermates. Following this, expression levels were compared with those of HCV-infected patients. As we have previously reported, two forms of HCV core protein exist, p21 and p23, as identified by western blot analysis.[20] All samples, except for those from non-transgenic mice, showed bands of 21 kDa, similar to the 21-kDa band of the control HCV core protein obtained by *in vitro* transcription and translation of DNA from the vector (Fig. 2a). All samples yielded core protein of the same size as p21 and the expression levels of HCV core protein were almost identical among all 3 lineages of transgenic mice (lanes 2–4). Importantly, these expression levels did not differ significantly from those observed in the liver tissue of HCV-infected patients (lanes 6–8).

Then, the tissue distribution of HCV core protein expression was estimated. Total protein preparations of various tissues (brain, heart, lung, liver, kidney, intestine, spleen, pancreas and testis) from the transgenic mice were subjected to HCV core protein quantification assay using the sensitive EIA method.[19] Expression of the core protein was identified only in the liver and substantially lower levels were noted in other tissues. The levels of core protein expression in other tissues were similar to those observed in non-transgenic littermates (Fig. 2b).

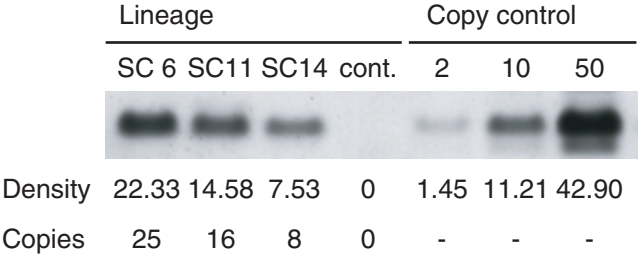

Fig. 1. Southern blot analysis of liver DNA samples from transgenic mice. DNA samples were extracted from the livers of transgenic mice from three lineages (SC 6, SC 11 and SC 14), as well as non-transgenic littermates (cont.). Copy controls (2, 10 and 50 copies) were made from an expression vector containing HCV core region cDNA.

Core protein expression levels were compared among two of the lineages, SC 11 and SC 14, because they appeared different by western blot analysis and HCV core protein quantification assay. Total protein preparations from the livers of eight 8-week-old male transgenic mice from each lineage, SC 11 and SC 14, were subjected to EIA HCV core protein quantification assay. The mean core protein expression levels were 298.22±119.68 fmol/g in the SC 11 lineage, and 65.05±63.92 fmol/g in the SC 14 lineage.

Histopathological findings after 18 months. To analyze the incidence of hepatic steatosis and tumorigenesis in transgenic and control mice, histological examination of liver specimens from 49 transgenic and 39 control mice was performed at the age of 18 to 24 months. Among the 3 lineages of transgenic mice, liver steatosis was observed in 11.1% to 18.2% of males and 0% to 14.3% of females (Table 1). The incidence of steatosis among the 3 lineages did not differ significantly from that of non-transgenic littermates, occurring in 12.5% of males and 4.3% of females, respectively. Among the samples obtained from transgenic mice and non-transgenic littermates, no hepatic tumors, adenomas or hepatocellular carcinomas were observed, although a single pre-neoplastic focus was found in a transgenic mouse at 18 months of age in the SC 14 lineage (data not shown). Thus, we did not observe steatosis or hepatic tumors in our HCV core transgenic mouse model, despite achieving a similar level of HCV core protein expression in the liver to that observed in the livers of infected humans.

Short-term observations following CCl_4 administration. Although the reason why we did not observe steatosis or hepatic tumors is unclear, one possible explanation is that the effect of transgene expression in mice might be weaker than previously reported.[14] We then analyzed the additive or synergistic effect of HCV core protein in the setting of liver inflammation. To deter-

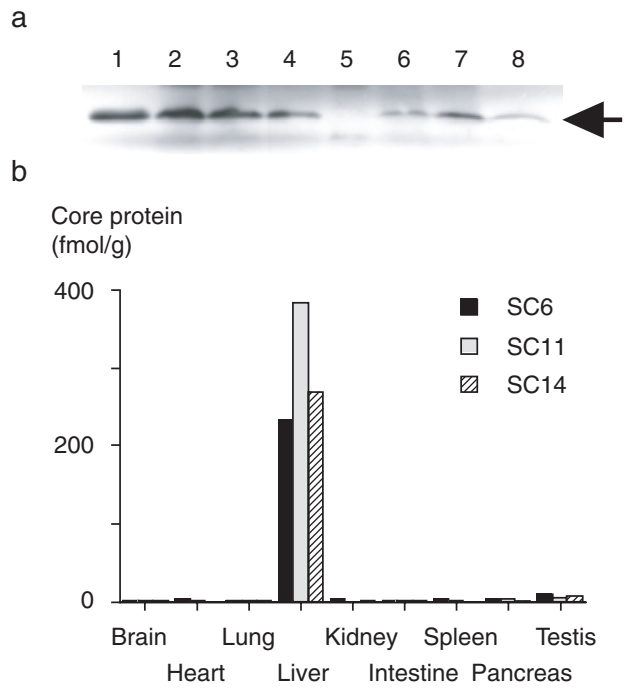

Fig. 2. (a) Western blot analysis of liver protein samples from transgenic mice and HCV-infected patients. Lane 1: positive control made by *in vitro* translation and transcription of the HCV core expression vector. Lanes 2–4: liver protein samples from 8-week-old transgenic mice (lane 2, SC 6; lane 3, SC 11; lane 4, SC 14). Lane 5: sample from a non-transgenic littermate. Lanes 6–8: samples from HCV-infected patients. The arrows indicate the position of the HCV core protein. (b) Tissue distribution of the HCV core protein. HCV core protein expression was measured in various tissue samples from 8-week-old transgenic mice from each lineage by the EIA method.

Table 1. Incidence of steatosis and hepatic tumors in transgenic mice

Lineage	Sex	No.	Steatosis	Hepatic tumor
SC 6	M	9	1 (11.1%)	0
	F	7	1 (14.3%)	0
SC 11	M	11	2 (18.2%)	0
	F	13	0	0
SC 14	M	6	1 (16.7%)	0
	F	3	0	0
Cont.	M	16	2 (12.5%)	0
	F	23	1 (4.3%)	0

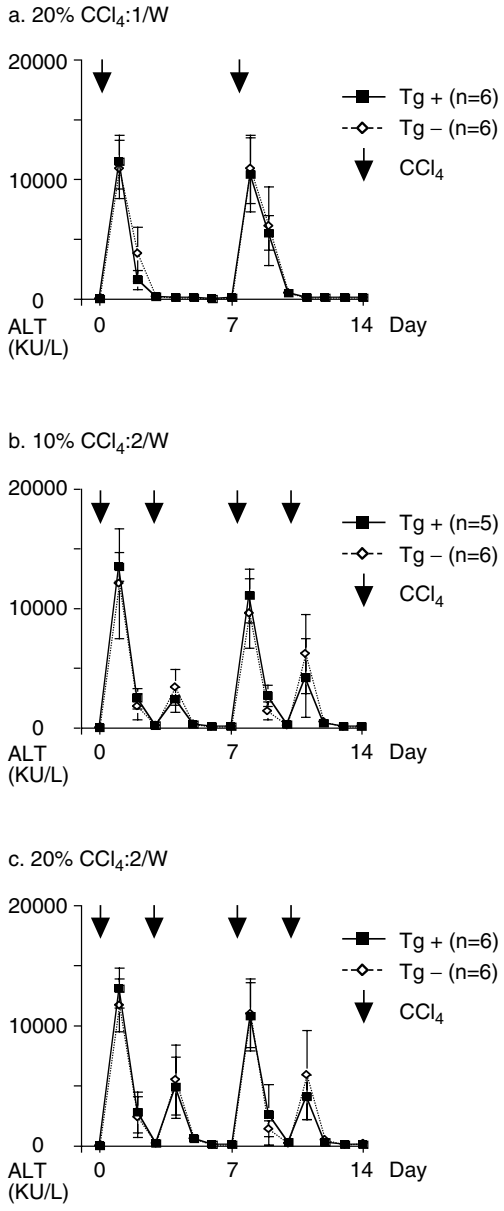

Fig. 3. Serum ALT elevations following CCl_4 administration in transgenic mice and non-transgenic littermates. The closed squares represent ALT levels in transgenic mice and the open diamonds represent those in non-transgenic littermates. Values are indicated as the mean±SD. The downward arrows indicate CCl_4 administration (a) 20%, 1/W; (b) 10%, 2/W; (c) 20%, 2/W.

mine the contribution of the HCV core protein to liver cell injury, CCl_4 was administered to transgenic mice and non-transgenic littermates and serum ALT levels were measured every day for 2 weeks. Five or 6 transgenic mice (lineage SC 11) and 6 non-transgenic littermates were assigned to receive one of three protocols of CCl_4 administration: 10% CCl_4 twice weekly (10%: 2/W), 20% CCl_4 once weekly (20%: 1/W), or 20% CCl_4 twice weekly (20%: 2/W) (Fig. 3). In the study group that received an injection of 20% CCl_4 once weekly, single peaks in ALT levels were observed during the week after administration, and maximum values of 11,456.5±2278.9 KU/liter and 10,856.9±2479.2 KU/liter were obtained in transgenic mice and non-transgenic littermates, respectively (Fig. 3a). Four days after administration of CCl_4, ALT levels returned to the normal range. During the second week of administration following this protocol, ALT levels rose to the same level as observed in the first week. In the study group that received an injection of 10% CCl_4 twice weekly, serum ALT levels peaked twice during the week after administration (Fig. 3b). The first peak did not differ from that observed upon administration of 10% CCl_4 once weekly, which reached 13,480.1±1225.9 KU/liter in transgenic mice and 12,080.4±4619.3 in non-transgenic littermates. However, the second peak was less than the first, reaching 2367.3±1076.9 KU/liter in transgenic mice and 3394.2±1483.8 in non-transgenic littermates. In the study group that received 20% CCl_4 twice weekly, two peaks in serum ALT were observed during the week after administration (Fig. 3c). The first peak reached 13,137.1±1621.8 KU/liter in transgenic mice and 11,685.3±2207.4 KU/liter in non-transgenic littermates, and the second peak reached 4352.9±2536.3 KU/liter in transgenic mice and 5502.8±2862.6 in non-transgenic littermates (Fig. 3). Among the 3 protocols examined, administration of 20% CCl_4 twice weekly caused the most damage, followed by administration of 10% CCl_4 twice weekly, while administration of 20% CCl_4 once weekly had only a mild effect. No significant differences in peak ALT levels were observed among transgenic and non-transgenic mice in any of the 3 protocols examined. Thus, CCl_4 administration induced the same degree of liver injury in HCV core transgenic and control mice.

Long-term effects of CCl_4 administration. To elucidate the involvement of HCV core protein and accelerated liver regeneration in hepatocarcinogenesis, long-term follow-up of mice administered CCl_4 according to 3 different protocols was performed. Both transgenic mice (n=41 and n=18, for the SC 11 and SC 14 lineages, respectively) and non-transgenic littermates (n=36) were followed. Three different protocols of CCl_4 administration were continued for 40 weeks and serum ALT levels were measured every 4 weeks as a parameter of hepatotoxicity. Although peaks in ALT levels were observed over the

Table 2. Incidence of hepatic tumors by administration with CCl_4

CCl_4	Lineage	Period	No.	Adenoma		HCC	
				Incidence	Tumors[1)]	Incidence	Tumors[1)]
20%, 1/W	SC 11	40 W	14	8 (57.1%)	11[2)]	0	—
	SC 14	40 W	8	1 (12.5%)	2	0	—
	Cont.	40 W	13	3 (23.1%)	3[2)]	0	—
10%, 2/W	SC 11	40 W	11	10 (90.1%)	15	0	—
	Cont.	40 W	11	8 (72.7%)	17	0	—
20%, 2/W	SC 11	40 W	11	10 (90.9%)	17	1 (9.1%)	1
	SC 14	40 W	10	10 (100%)	17	1 (10.0%)	1
	Cont.	40 W	12	10 (83.3%)	17	0	—

1) Total number of tumors (multiple tumors developed in some mice).
2) P<0.05.

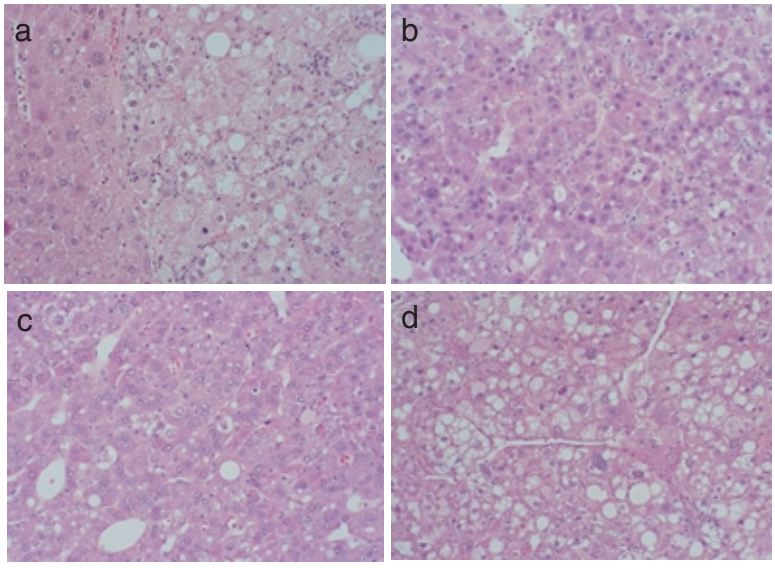

Fig. 4. Histopathological findings in the livers of HCV transgenic mice following CCl_4 administration. (a) Hepatic adenoma that developed in a transgenic mouse administered 20% CCl_4 once weekly. (b and c) HCC that developed in a transgenic mouse from lineage SC 11 administered 20% CCl_4 twice weekly. (d) HCC that developed in a transgenic mouse from lineage SC 14 administered 20% CCl_4 twice weekly.

course of 40 weeks, they did not differ among transgenic mice and non-transgenic littermates (data not shown). Following administration of a 20% solution of CCl_4 once weekly, hepatic adenomas were found in 8 of 14 transgenic mice of the SC 11 lineage (57.1%), 1 of 8 transgenic mice of the SC 14 lineage (12.5%), and 3 of 13 non-transgenic littermates (23.1%) (Table 2). Hepatic adenomas were observed as distinct nodules compressing the adjacent parenchyma, occasionally exhibiting thin fibrovascular connective tissue, and their diameters ranged from 5–10 mm. They consisted of cells containing clear or vacuolated cytoplasm or cells resembling normal hepatocytes (Fig. 4a). Although the incidence of adenoma formation did not differ significantly among transgenic mice and non-transgenic littermates, a significantly higher number of hepatic tumors was observed among transgenic mice of the SC 11 lineage (Table 2). Following administration of 10% or 20% CCl_4 twice weekly, most animals, regardless of whether or not they were transgenic, developed hepatic adenomas. However, after 40 weeks of administration of 20% CCl_4 twice weekly, HCC was observed only among transgenic mice, including 1 of 11 transgenic mice (9.1%) from the SC 11 lineage (Fig. 4, b and c), and 1 of 10 transgenic mice (10.0%) from the SC 14 lineage (Table 2). The HCC lesion from the liver of the SC 11 transgenic mouse was 20 mm in diameter. It was composed of well-differentiated cells with a trabecular pattern. The liver cell plates were more than one cell layer thick and irregular (Fig. 4, b and c). The HCC lesion from the liver of the SC 14 transgenic mouse was 10 mm in diameter. It was composed of moderately differentiated cells with vacuolated cytoplasm and karyomegaly. In this nodule, fat droplets were observed (Fig. 4d).

Discussion

The transforming capacity of a gene is often investigated as an indicator of its potential for oncogenesis. A high degree of transformation has not been observed with any of the HCV viral genes or proteins. However, substantial transforming capacity has been observed when the HCV core protein is present along with other genes, including *ras*, *myc*, or *STAT 3*.[10, 21, 22] Thus, we investigated the possibility that the HCV core protein might be oncogenic in co-operation with up-regulated liver regeneration *in vivo*. We established a model of hepatic tumorigenesis using HCV core transgenic mice with chemically induced liver damage. Spontaneous HCC development was not observed in HCV core transgenic mice in the absence of chemically induced liver damage, although these mice did express similar levels of core protein to those seen in HCV-infected patients. Therefore, repeated administration of CCl_4 was used to induce hepatic injury mimicking chronic hepatitis in the transgenic mice. In this model, CCl_4 administration induced adenoma and HCC development in HCV core transgenic mice. Thus, although a weak independent effect of HCV core protein on hepatocarcinogenesis was found in the present experiment, this effect was markedly enhanced when HCV core protein was paired with a high rate of cell turnover in the liver.

Recent studies have indicated a direct role of HCV viral proteins in oncogenesis.[23–25] Transgenic mice harboring HCV core, structural, or full-length genes exhibit marked liver steatosis and spontaneous HCC development after a long latency period in the absence of inflammation.[14, 26] However, the development of HCC has not been observed in other studies in which transgenic mice harboring HCV core, structural or full-length genes were investigated.[27–31] Likewise, a higher incidence of steatosis was not observed in the present experiment when transgenic mice were compared with non-transgenic mice. Moreover, HCC was not observed in any of the transgenic mice by 24 months of age. The reasons underlying these discrepancies are not clear. There is a possibility that the HCV clone used in this experiment differed from that used to create transgenic mice in which spontaneous development of HCC was observed. Although the HCV clone used in this experiment was of the same genotype (genotype 1b) as the clones used in previous reports, three amino acid substitutions were identified in the core region of the clone (data not shown). These amino acids might play a crucial role in the development of HCC. The core protein is known to alter lipid metabolism and to bind to lipid droplets and apolipoprotein A2.[32, 33] Steatosis due to lipid accumulation within hepatocytes was observed to precede HCC in all reported cases. Thus, the ability of the core protein used in the present experiment to induce lipid accumulation might differ from that of core proteins examined in previous reports in which spontaneous HCC development was observed. However, binding was observed between translated core protein from the HCV clone used in this experiment and lipid droplets in the cultured cells.[34]

Although the sensitivity of HCV core transgenic mice to Fas-mediated hepatocyte injury has been described, the sensitivity of HCV core-transfected mice to CCl_4 administration has not been established.[31] Significant differences in the ALT levels of transgenic and non-transgenic mice were not observed 2 weeks after CCl_4 administration, regardless of the protocol used to administer CCl_4 in the present experiment (Fig. 3). Likewise, no differences in the ALT levels were observed after 40 weeks of CCl_4 administration (data not shown). Thus, transfection of the HCV core protein did not affect hepatocyte susceptibility to CCl_4 toxicity. However, 40 weeks of CCl_4 administration resulted in a higher incidence of hepatic adenoma among transgenic mice that received the low-dose protocol (20%: 1/W). A significantly greater number of adenomas was observed in transgenic mice of the SC 11 lineage than in non-transgenic littermates. This indicates that HCV core protein expression might enhance the development of adenomas following CCl_4 administration, although the promotion of adenoma development by CCl_4 was induced in both transgenic mice and non-transgenic littermates. A significant difference was also observed in the number of adenomas that developed among transgenic mice of the SC 11 and SC 14 lineages. A difference in the core protein expression levels of these lineages was observed, with a substantially lower level of expression noted within livers of mice from the SC14 lineage. Thus, the level of expression of HCV core protein might influence the ability to induce the development of hepatic adenomas. In addition, two mice developed HCC following injection of the 20% solution of CCl_4 twice weekly. One of these mice belonged to the SC 11 lineage and the other to the SC 14 lineage. None of the non-transgenic mice developed HCC, although most of both transgenic and non-transgenic mice developed adenoma following administration of the 20% solution of CCl_4 twice weekly. This result implies that both HCV core protein expression and severe hepatocyte damage induced by high-dose CCl_4 administration might be necessary for HCC development in this mouse model. CCl_4 is not directly genotoxic, but cytochrome P-450 metabolism in the liver produces a metabolite which causes perivenular necrosis and regeneration.[35–38] HCC development with CCl_4 treatment has been reported in human c-H-*ras* transgenic mice, but not in control C57BL/6 mice.[38] Thus, the HCV core protein might enhance the development of HCC in a similar manner to the human c-H-*ras* gene when combined with CCl_4 administration. It is unlikely that the ability of HCV core protein to enhance the development of HCC in CCl_4-exposed mice is due to positional integration of the transgene into the host genome because HCC was observed to develop in mice from two different lineages in this experiment. Interestingly, differences in core protein expression among the livers of mice from different lineages were not observed to affect the development of HCC, though this can not be considered conclusive, be-

cause only two cases of HCC were observed. Importantly, expression of the HCV core protein alone did not promote the development of HCC within the livers of transgenic mice, but rather, it enhanced HCC development under conditions of up-regulated cell proliferation. This indicates that HCV core protein facilitates the development of HCC where chronic hepatitis is present. This observation might explain discrepancies noted among previous experiments with regard to the ability of HCV core protein to promote the development of HCC in transgenic mice. It is possible that some HCV core clones are unable to induce HCC in the absence of chronic inflammation, while others can. This suggests that attempts to reduce inflammation in the liver might prevent the development of HCC in HCV-infected patients. This is consistent with clinical observations suggesting that patients with HCV-associated cirrhosis and high ALT levels develop HCC more quickly than HCV-infected patients with low ALT levels. Moreover, patients receiving IFN therapy with persistently normal ALT levels have a lower risk of developing HCC, even if HCV is not eradicated.[39, 40]

In summary, CCl_4 administration was observed to promote the development of HCC in HCV core protein transgenic mice, regardless of the presence of steatosis. This model was used to mimic a state of up-regulated cell proliferation in the setting of HCV core protein expression and might be useful for further investigation of HCV-associated hepatocarcinogenesis, as well as research into the prevention of HCC in patients with HCV.

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