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Liver target delivery of small interfering RNA to the HCV gene by lactosylated cationic liposome☆

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Background/Aims: **RNA interference has considerable therapeutic potential, particularly for anti-viral therapy. We previously reported that hepatitis C virus (HCV)-directed small interfering RNA (siRNA; siE) efficiently inhibits HCV replication, using HCV replicon cells. To employ the siRNA as a therapeutic strategy, we attempted *in vivo* silencing of intrahepatic HCV gene expression by siE using a novel cationic liposome.**

Methods: **The liposomes consisted of conjugated lactose residues, based on the speculation that lactose residues would effectively deliver siRNA to the liver *via* a liver specific receptor. The lactosylated cationic liposome 5 (CL-LA5) that contained the most lactose residues introduced the most siRNA into a human hepatoma cell line, which then inhibited replication of HCV replicons.**

Results: **In mice, the siRNA/CL-LA5 complexes accumulated primarily in the liver and were widespread throughout the hepatic parenchymal cells. Moreover, siE/CL-LA5 specifically and dose-dependently suppressed intrahepatic HCV expression in transgenic mice without an interferon response.**

Conclusions: **The present results indicate that the CL-LA5 we developed is a good vehicle to lead siRNA to the liver. Hence, CL-LA5 will be helpful for siRNA therapy targeting liver diseases, especially hepatitis C.**

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Keywords: **siRNA; HCV; Cationic liposome; Lactosylation; Target delivery; Hepatocyte; Liver; Transgenic mouse; Interferon**

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Abbreviations: HCV, hepatitis C virus; RNAi, RNA interference; siRNA, small interfering RNA; IFN, interferon; UTR, untranslated region; CL-LA, lactosylated cationic liposome; siE, HCV-directed siRNA.

1. Introduction

Hepatitis C virus (HCV) is a major etiological agent that causes chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma [1]. Although combination therapy with pegylated interferon-α and ribavirin has markedly improved the clinical outcome, less than half of the patients with chronic hepatitis C can be expected to respond favourably to currently available agents [2]. Therefore, developing a specific reagent against HCV is a major public health objective.

RNA interference (RNAi) is one type of post-transcriptional gene silencing [3,4]. The effector of RNAi is

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short interfering RNA (siRNA) duplexes (~21–23 nt), which play a key role in the specific degradation of target mRNA. Currently, RNAi technology is widely used as a tool for gene function analysis. In addition, it is expected to be a powerful therapeutic agent to silence pathogenic gene products associated with disease, including cancer, viral infections and autoimmune disorders [5–10]. Previously, we and others reported that synthetic siRNA efficiently and specifically inhibits HCV replication *in vitro* [11–20] and suggested the potential for siRNA as a novel HCV agent.

In fact, the RNAi machinery has been shown to work *in vivo* by injection of siRNA [21]. However, safety and delivery remain the main obstacles to achieving *in vivo* gene silencing by RNAi technology. Currently, viral vectors [22], hydrodynamic injection [23] and cationic liposomes [24] have been the main methods of introducing siRNA *in vivo*. However, the mechanism of action of viral vectors has not been clarified and may result in severe side effects. Furthermore, hydrodynamic injection cannot be used for human therapy. On the other hand, since the physical properties of cationic liposomes are well understood, the use of these liposomes holds the best promise for clinical application. In addition, cationic liposomes do not elicit an immune response, which is a great advantage for drug targeting in that multiple administrations of siRNA are possible, which is crucial for an siRNA therapeutic effect. Moreover, cationic liposomes are easily modified and improved.

In HCV therapy, it is important that reagents are specifically led to the liver. Thus, to specifically and effectively transfer siRNA into hepatocytes, we designed lactosylated cationic liposomes, as Ohishi et al. reported that lactosylated polyion complex micelles enhanced the delivery of oligonucleotides into hepatoma cells [25]. Based on their observations we expected that siRNA complexed with lactosylated cationic liposomes would be superficially trapped in the liver by lactose-specific receptors and therefore effectively introduced into hepatic parenchymal cells *in vivo*.

Here, we report that siRNAs with cationic liposomes containing lactose residues were largely transfected into hepatocytes *in vitro* and *in vivo*, where they efficiently suppressed intrahepatic HCV expression in transgenic mice. Furthermore, this system did not activate the interferon (IFN) system. Our results strongly suggest that lactosylated cationic liposomes have an appropriate mechanism by which to deliver siRNA as a therapy for liver disease.

2. Materials and methods

2.1. siRNAs

The design of HCV-directed siRNA has been described previously [11]. Briefly, we designed nine siRNAs that target the 5′-UTR and 3′-UTR of the HCV genome and examined the efficiency of their inhibition of HCV replication *in vitro*. Among the nine siRNAs, the most effective siE was used in the present study and was directed toward nucleotides 325–344 of the HCV genome. The target sequence was 5′-GUCUC GUAGACCGUGCAUCAUU-3′. The p53m siRNA (sip53m) [11] and GL3-M1 siRNA (siGL3-M1) were used as the negative controls. The sense sequence of siGL3-M1, which is sequence-specific for firefly luciferase mRNA, was 5′-GCUAUGAAACGAUAUGGGCUU-3′.

2.2. Preparation of cationic liposomes and siRNA/cationic liposome complexes

The cationic liposomes were composed of three lipids: a cationic lipid, phosphatidylcholine (PC), and lactosylated phosphatidylethanolamine (LA-PE). The preparation of the cationic liposomes [24] and the synthesis of the LA-PE [26] have been described previously. The ratio of the two neutral lipids, PC and LA-PE in the liposomes was as follows: CL-LA0, 5:0; CL-LA1, 4:1; CL-LA2, 3:2; CL-LA3, 2:3; CL-LA4, 1:4; and CL-LA5, 0:5. Each siRNA was mixed with 16 times the amount of cationic liposome, resulting in siRNA/CL-LA. The size of every siRNA/CL-LA was controlled as an average 150 nm.

2.3. Inhibition assay of HCV replication in replicon cells

We used two kinds of HCV replicon cells [27]; FLR3-1 (genotype 1b, Con-1; Fig. 1a) [28] and R6FLR-N (genotype 1b, strain N) [11]. siRNA/CL-LA was added to the medium of the HCV replicon cells, FLR3-1 or R6FLR-N, at a final concentration of 30 nM. For positive control [11], HCV replicon cells were transfected with siRNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). After 72 h incubation, we performed luciferase assays using the Bright-Glo luciferase assay system (Promega, Madison, WI).

2.4. Immunoblotting

Cells were harvested using lysis buffer [11]. Then 5 μg of protein was separated by 10% SDS–PAGE, and electro-blotted onto a PVDF membrane (Immobilon-P; Millipore, Billerica, MA). Rabbit polyclonal anti-HCV nonstructural protein 3 (NS3) antibody (R212) prepared in our laboratory and mouse monoclonal anti-β-actin antibody (Sigma, St. Louis, MO) were used as the primary antibodies.

2.5. Transfection efficiency of siRNA by CL-LA in vitro

The transfection efficiencies of Cy3-labeled siRNA (Cy3-siRNA) by CL-LA were determined using confocal laser microscopy (Zeiss, Jena, Germany). HCV replicon cells were seeded in the Lab-Tek II Chamber Slide-System (Nalge Nunc International, Rochester, NY) at 2.0×10^4 cells per well. The siRNA was labeled with Cy3 using a Silencer siRNA Labeled Kit (Ambion, Austin, TX). After incubation for 24 h, the cells were fixed in 4% buffered formalin and the nuclei stained using DAPI.

2.6. Animals

Male BALB/c mice at 8 weeks of age were purchased from Japan SLC (Hamamatsu, Japan). BALB/c mice and CN2-29 transgenic mice received human care according to guidelines of the National Institutes of Health. Animal experiment protocols performed in accordance with The Tokyo Metropolitan Institute of Medical Science Animal Experiment Committee.

2.7. siRNA delivery by CL-LA in vivo

Alexa-546 or Alexa-568 labeled siE/CL-LA was intravenously injected into BALB/c mice. After 5 and 30 min, the liver, lung, spleen, and kidney were extirpated from the mouse. Sections of these tissues were then stained with DAPI and slides examined using confocal laser microscopy (Zeiss).

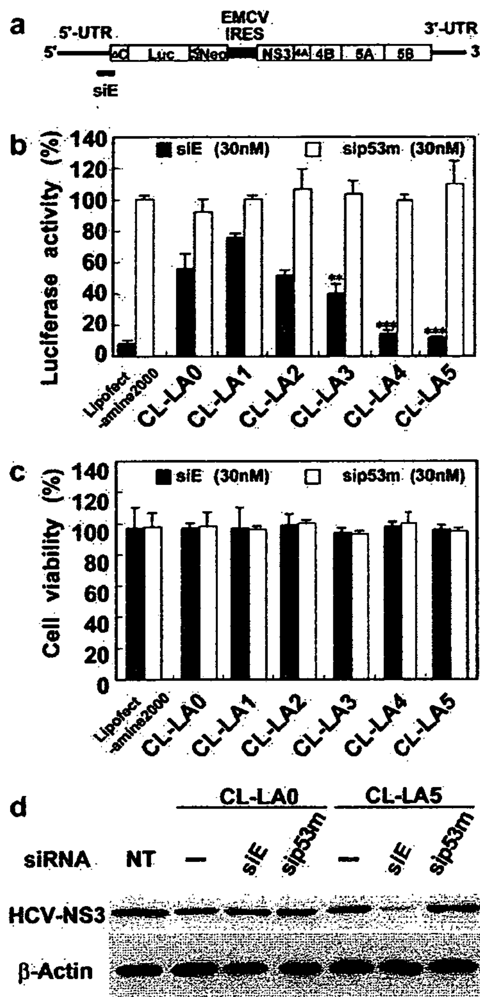


Fig. 1. Effect of siRNA/CL-LA in HCV replicon cells. (a) Schematic representation of HCV replicon RNA and siE position. UTR, untranslated region; ΔC, truncated HCV core region (nucleotides 342–377); Luc, firefly luciferase gene; 2A, 2A genes of foot-and-mouth disease virus; Neo, neomycin resistant gene; EMCV, encephalomyocarditis virus; IRES, internal ribosomal entry site; NS, HCV nonstructural protein; siE, HCV-directed siRNA. (b) FLR3-1 replicon cells were treated with 30 nM siRNA/CL-LA. Luciferase activity was measured after 72 h. Data represent means ± SD compared with mock-transfected cells ($n = 5$). The average luciferase activities were analyzed by Dennett's test. **$P < 0.01$ vs. CL-LA0 and ***$P < 0.001$ vs. CL-LA0. sip53m was used as the negative control. Commercial transfection agent Lipofectamine 2000 was used as the relative positive control. (c) Cell viability was determined after 72 h. Data represent means ± SD ($n = 5$) of WST conversion compared with mock-transfected cells. (d) Immunoblot analysis of HCV-NS3 and β-actin. NT, non treatment.

2.8. Gene silencing of HCV genome expression in vivo by siRNA

We used 8- to 10-week-old, 20 g CN2-29 transgenic mice, which contain conditional HCV cDNA, the expression of which is regulated by the Cre/loxP-system (Fig. 4a) [29]. Expression of HCV core protein is regulated by Cre DNA recombinase in the liver, which can be expressed by administration of adenovirus encoded Cre DNA recombinase (AxCANCre). AxCANCre was intravenously administered at 2×10^9 pfu per body 1 h prior to siRNA (2.5, 5 or 10 mg/kg) injection. After 48 h, expression levels of HCV core protein in the liver were detected using the Ortho HCV core protein ELISA kit (Eiken Chemical, Tokyo, Japan).

2.9. Detection of interferon-β induction by administration of siRNA/CL-LA5 complex

Poly(I):poly(C) was purchased from Yamasa-shoyu (Chiba, Japan). siRNA/CL-LA5 or poly(I):poly(C)/CL-LA5 (200 μg) was intravenously injected into the CN2-29 mice. After 6 h, the livers were extirpated and total RNA was extracted by the acid guanidinium–phenol–chloroform method. cDNA was synthesized from 1 μg of the total RNA using TaqMan reverse transcription reagents (ABI, Foster City, CA). Expression levels of IFN-β mRNA were determined using a TaqMan gene expression assay kit (ABI) according to the manufacturer's instructions [30,31].

2.10. Statistical analysis

The data are expressed as means ± SD. Statistical analysis was conducted using the analysis of variance with the Dennett's test for multiple comparisons. Statistical significance was established at $P < 0.05$.

3. Results

3.1. Optimization of amount of lactosylated phosphatidylethanolamine (LA-PE) included in the cationic liposomes

To optimize the amount of LA-PE in lactosylated cationic liposomes (CL-LA), we initially prepared six kinds of cationic liposomes containing various amounts of lactose residues, and investigated the inhibitory effects of siE/CL-LA against HCV replication in FLR3-1 replicon cells. The CL-LA strengthened the inhibitory efficiency of siE by increasing the amount of LA-PE. The siE/CL-LA5, which contained LA-PE but not PC, had the strongest inhibitory effect. On the other hand, none of the sip53m/CL-LA affected luciferase activity reflecting the HCV replication (Fig. 1b). To access cytotoxicity of a complex of siRNA and CL-LA, cell viability was measured by the WST-8 assay [11]. None of siE/CL-LA or sip53m/CL-LA showed any cytotoxicity (Fig. 1c). A luciferase assay in another replicon cell line, R6FLR-N replicon [11], showed similar results for HCV-specific silencing (data not shown). Immunoblot analysis showed that the levels of HCV NS3 protein that were translated from the HCV replicon were decreased by siE/CL-LA5, but not by sip53m/CL-LA5 (Fig. 1d). These results indicated that siE/CL-LA5 inhibited HCV replication the most effectively *in vitro* and that this inhibition was not due to nonspecific reduction caused by the complex of siRNA and CL-LA.

3.2. Transfection efficiency of siRNA by CL-LA5 into HCV replicon cells

To investigate whether lactose residue enhances the transfection of siRNA, we observed fluorescent-labeled siE introduced into RLR3-1 replicon cells. After a 24 h incubation with siE/CL-LA0 or siE/CL-LA5, the cells were observed by fluorescence microscopy (Fig. 2). The lactosylated cationic liposome CL-LA5 transfected siE into replicon cells more effectively than the non-lactosylated cationic liposome CL-LA0. Moreover, fluorescence of siE transfected by CL-LA5 was observed mainly in the cytoplasm, and was more effective than that with the commercial agent Lipofectamine 2000. These results demonstrated that the lactose residue very strongly enhanced the transfection efficiency of siRNA into replicon cells, particularly in the cytoplasm.

3.3. Delivery of siRNA by CL-LA5 in mice

Next, we investigated the delivery of siRNA by CL-LA5 in BALB/c mice, which were intravenously injected with fluorescent-labeled siE/CL-LA0 or siE/CL-LA5 (Fig. 3). At 5 and 30 min after injection *via* the orbital vein, the livers of the mice were extirpated and observed by fluorescence microscopy. The fluorescence intensity of siE/CL-LA5 at 5 min was clearly stronger than that of siE/CL-LA0. At 30 min after injection, fluorescence of siE/CL-LA5 was equally spread throughout the hepatic parenchymal cells, although that of siE/CL-LA0 could be patchily detected in parts (Fig. 3a). These results demonstrated that CL-LA5 could more easily take siRNA into the cytoplasm of parenchymal liver cells. Furthermore, we also examined the tissue distribution of siRNA delivered by CL-LA5 (Fig. 3b). At 30 min after injection, the liver, spleen, kidney and lung of another mouse were excised and the intensity of fluorescence of labeled-siE in these tissues calculated. Although the relative fluorescence of siE/CL-LA0 accumulated in the liver and spleen, that of siE/CL-LA5 accumulated primarily in the liver alone, and the residual fluorescence of siE was equally diffused in other tissues. Taken together, these results indicated that CL-LA5 was able to trap siRNA primarily in the mouse liver, where it could be efficiently taken up into the hepatocytes.

3.4. Down-regulation of HCV protein expression by siE/CL-LA5 in transgenic mouse liver

To extend our findings of the *in vitro* silencing effect by siE/CL-LA5 and *in vivo* siRNA delivery by CL-LA5, we performed an additional study in an HCV transgenic mouse model [29]. We administered siE/CL-LA5 to CN2-29 mice after inducing HCV protein expression by AxCANCre (Fig. 4b). The mice were sacrificed on the second day after injection, and expression of HCV core protein in the liver measured by ELISA. The siE/CL-LA5 decreased the amount of core protein in a dose-dependent manner. The maximal dose of HCV unrelated siGL3-M1/CL-LA5 did not inhibit the expression of HCV core protein. These results demonstrated that siE/CL-LA5 specifically inhibited HCV protein expression in mouse liver.

3.5. IFN response by siRNA/CL-LA5 in vivo

It has been reported that siRNA can activate the cellular interferon (IFN) pathway, especially when delivered by cationic liposome transfection reagents [32,33].

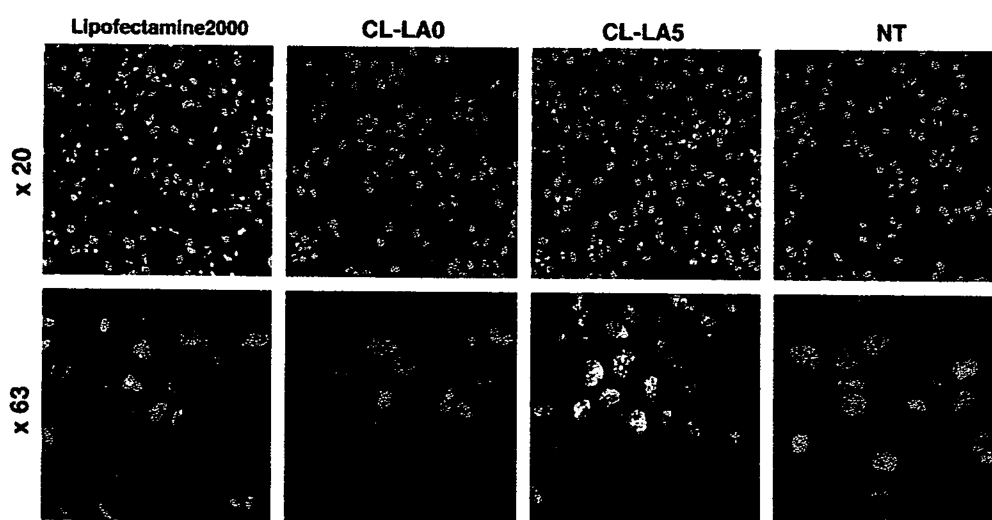
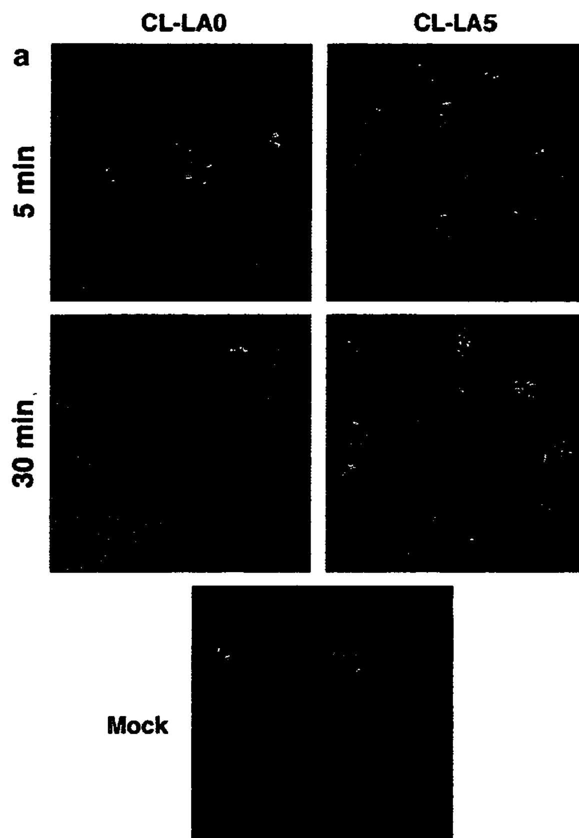


Fig. 2. Transfection efficiency of fluorescent-labeled siRNA into HCV replicon cells. FLR3-1 replicon cells were treated with CL-LA0, CL-LA5, or Lipofectamine 2000 complexed with Cy3-labeled siE (100 nM). After incubation for 48 h, the cells were observed by fluorescence microscopy. The nuclei were stained with DAPI. NT, non treatment.



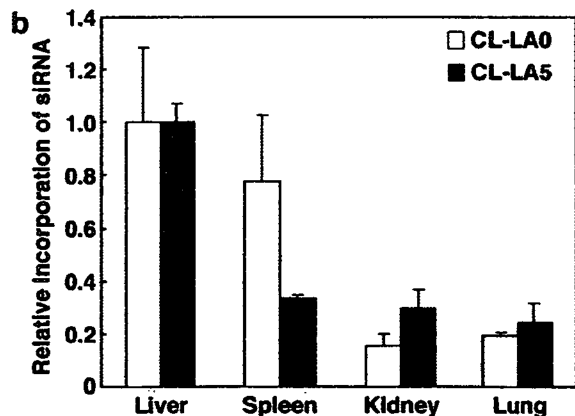


Fig. 3. siRNA delivery in mice. (a) Distribution characteristics of siRNA in liver. Alexa-546 labeled siE/CL-LA0 or siE/CL-LA5 (4.3 mg/kg) was injected intravenously into the orbital veins of BALB/c mice. The liver was observed by fluorescence microscopy at 5 or 30 min after injection. The nuclei were stained with DAPI. Mock; 10% (w/v) maltose solution. (b) Tissue distribution of Alexa-568 labeled siRNA delivered by CL-LA. The liver, spleen, kidney, and lung were examined at 30 min after injection, and the intensity of fluorescence of labeled-siE/CL-LA0 or siE/CL-LA5 was then calculated at 3 locations in each tissue specimen. The relative ratio for incorporation of siE was obtained by setting the liver intensity as control. Data represent means ± SD.

To examine whether a type I IFN response was caused by siRNA/CL-LA5, we measured IFN-β mRNA levels in the liver of CN2-29 mice. Poly(I):poly(C)/CL-LA5

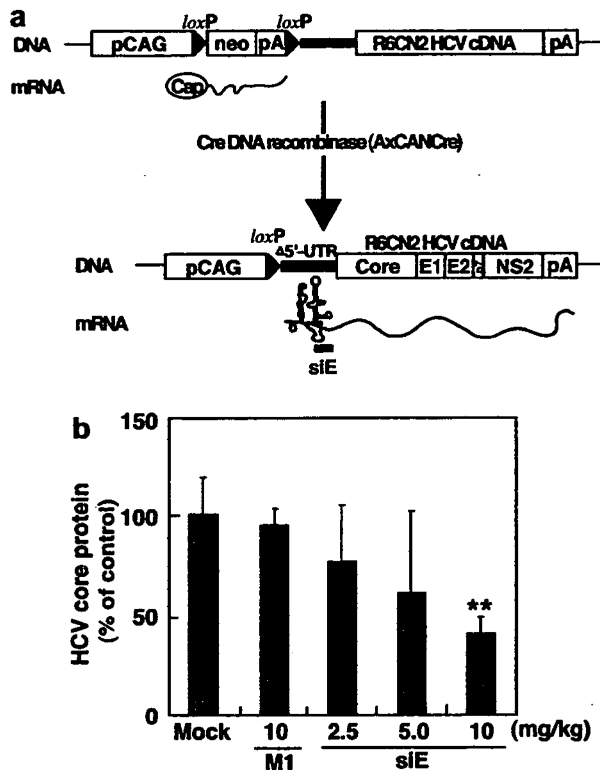


Fig. 4. Effect of siRNA on intrahepatic HCV expression in mice. (a) The CN2-29 transgenic mice contain part of the HCV gene (part of the 5'-UTR to NS2 protein). HCV protein expression can be controlled by infection of adenovirus encoding Cre DNA recombinase (AxCANCre). (b) siRNA/CL-LA5 was intravenously injected into CN2-29 mice at 1 h after AxCANCre infection. HCV core protein expressed in the liver was detected by ELISA after 48 h ($n = 3$). The average HCV core proteins were analyzed by Dennett's test. $^{**}P < 0.01$ vs. Mock control livers. Mock; 10% (w/v) maltose solution, M1; siGL3-M1.

drastically increased the IFN-β mRNA level to 10^6–10^7 copies per 1 μg total RNA, whereas siRNA/CL-LA5 induced only 10^1–10^2 copies per 1 μg total RNA

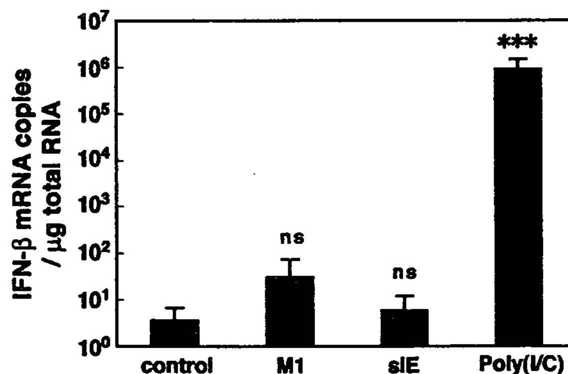


Fig. 5. IFN response *in vivo*. siRNA complexed with CL-LA5 was intravenously injected into CN2-29 mice ($n = 3$). At 6 h after injection, the IFN-β mRNA copy number was measured using real-time detection PCR. The average IFN-β mRNA copies were analyzed by Dennett's test. $^{***}P < 0.001$ and ns vs. non-treated control mice livers. ns, not significant; M1, siGL3-M1; Poly(I/C), poly(I):poly(C).

(Fig. 5). Moreover, siE/CL-LA5 induced only below 10^1 copies per 1 μg total RNA, equal to non-treated mouse liver. These results indicated that CL-LA5 was able to lead siRNA and poly(I):poly(C) to the liver by systemic intravenous injection. Subsequent administration of siE/CL-LA5 was then unable to activate the IFN response in mouse liver.

4. Discussion

Many studies of delivery systems for siRNA based on cationic liposomes have already been reported [24,34–40]. In those studies, the major problem of liposome as an siRNA carrier appears to have been a limitation to specific cell types, which resulted in unwanted tissue distribution *in vivo*. To address this problem, ligand or receptor mediated siRNA delivery systems were developed and these were able to increase uptake into the target cells [41–43]. In this study, to achieve liver specific delivery of siRNA, we designed a lactosylated cationic liposome as a carrier of siRNA and evaluated its delivery ability. The galactose terminus of lactose is a ligand of the asialoglycoprotein receptor, which is specifically expressed on the surface of hepatocytes. Thus, we expected liver specific delivery of siRNA would be enabled *via* this receptor-mediated endocytotic pathway [44]. As expected, CL-LA5, composed of cationic lipid and lactosylated phosphatidylethanolamine, effectively delivered the siE, which then inhibited HCV gene expression *in vitro* and *in vivo*.

siRNA is able to activate the cellular interferon pathway, especially when delivered with cationic liposome transfection reagents [32,33]. In addition, recent reports have revealed that siRNAs containing the 5′-UGUGU-3′ sequence are able to induce a toll-like receptor-mediated IFN response only when they are delivered *in vivo* with cationic lipid through intravenous administration [45,46]. These issues have raised concerns about the future of siRNA therapeutics. In fact, we found that the siE/CL-LA5 barely activated the type I IFN response, but that siGL3-M1/CL-LA5 weakly induced this response in mouse liver, although neither agent contained the 5′-UGUGU-3′sequence. Although the reasons for these phenomena are unclear, siE/CL-LA5 is likely to be tolerated by innate *in vivo* immunity and to have therapeutic potential for HCV.

On the other hand, we used transgenic mice expressing HCV RNA (encoding the IRES to NS2 protein region) to measure the knockdown efficiency of siE/CL-LA5. The target RNA is not replicable. During the course of an HCV infection, the virus exists as quasispecies composed of multiple variants [47]. Due to this physiological condition, mutants resistant to the siRNA may arise rapidly [48,49]. Although we believe that siE/CL-LA5 has the potential to silence natural HCV RNA, further investigations with an actual HCV infection system [50] are required.

Furthermore, siRNA/CL-LA5, a systemic method of delivery of siRNAs to liver tissue, would provide a means to introduce siRNAs into hepatocytes to achieve maximal therapeutic benefit, decrease the amount of drug required, and avoid nonspecific silencing and IFN response. Although further optimization of siRNA stability and safety profile characterizations are required for its practical application in humans, our delivery system of siRNA with CL-LA5 is a promising and feasible therapeutic strategy for liver disease associated with pathogenic gene products such as HCV.

Acknowledgements

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Pathogenesis of HCV-associated HCC: Dual-pass carcinogenesis through activation of oxidative stress and intracellular signaling

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Overwhelming lines of epidemiological evidence have indicated that persistent infection with hepatitis C virus (HCV) is a major risk toward development of hepatocellular carcinoma (HCC). It remains controversial, however, in the pathogenesis of HCC associated with HCV, whether the virus plays a direct role or merely an indirect one. The studies using transgenic mouse models by us and others, in which the core protein of HCV has oncogenic potential, indicate that HCV is directly involved in hepatocarcinogenesis, albeit other factors such as continued cell death and regeneration associated with inflammation would play a role, as well. The downstream events of the core protein are segregated into two components. One is the augmented production of oxidative stress along with the activation of scavenging system including catalase and glutathion (GSH) in the putative preneoplastic stage with steatosis in the liver. Thus, oxidative stress production in the absence of inflammation by the core protein would partly contribute to the development of HCC. The generation of oxidative stress is estimated to originate from mitochondrial dysfunction in hepatocytes by HCV infection. The other is the alteration of intracellular signaling cascade of MAPK (JNK), AP-1, cyclin D1, and CDK4. The combination of these pathways, collective with HCV-associated alterations in lipid and glucose metabolism, would lead to the frequent development of HCC in persistent HCV infection. Our results suggest that there would be a mechanism for hepatocarcinogenesis in persistent HCV infection that is distinct from those for other cancers. Similar to the pathogenesis of other cancers, the accumulation of a set of genetic aberrations may also be necessary for multistage development of HCC. However, HCV core protein, to which an oncogenic potential is ascribed, may allow some of the multiple steps to be bypassed in hepatocarcinogenesis. Therefore, unlike other cancers, HCV infection can elicit HCC in the absence of a complete set of genetic aberrations. Such a scenario, "non-Vogelstein-type" carcinogenesis, would explain the unusually high incidence and multicentric nature of HCC development in HCV infection.

Key words: hepatitis C virus, hepatocarcinogenesis, intracellular signaling transduction, oxidative stress, transgenic mouse

INTRODUCTION

WORLDWIDE, HEPATITIS C virus (HCV) infects hundreds of millions of people persistently, and induces a spectrum of chronic liver diseases.[1] Hence, it affects society in a number of domains including medical, sociological, and economic. Hepatocellular carcinoma (HCC) has become the most frequent cause of death in individuals persistently infected with HCV. In particular, HCV has been given increasing attention because of its wide and deep penetration in the community, coupled with a very high incidence of HCC in persistent HCV infection. Once liver cirrhosis is established in hosts infected with HCV, HCC develops at a yearly rate of 5–7%.[2] Knowledge of the mechanism of HCC development in chronic HCV infection, therefore, is imminently required for the prevention of HCC.

UNIQUENESS OF HCC DEVELOPMENT IN HCV INFECTION

HOW HCV INDUCES HCC is not yet clear, despite the finding that more than 70% of patients with HCC in Japan are infected with HCV.[1,3,4] HCV infection

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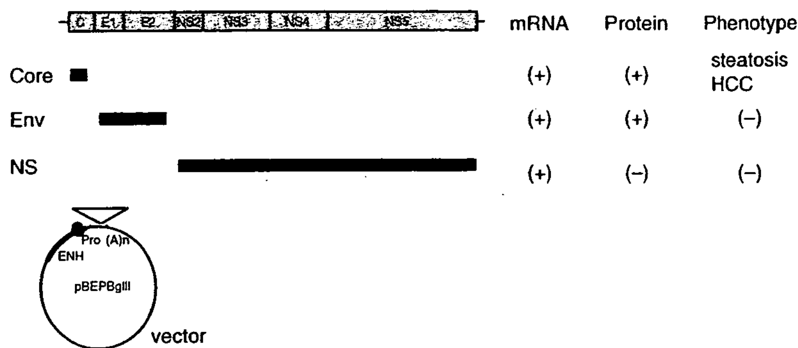

Figure 1 Hepatitis C virus (HCV) transgenic mouse lines. Among the three different transgenic mouse lines established, only the transgenic mice carrying the HCV core gene develop hepatocellular carcinoma (HCC) after an early phase with hepatic steatosis in two independent lineages. The mice transgenic for the envelope genes or non-structural genes do not develop HCC. core, core genes, env, envelope genes; NS, non-structural genes.

is also common in patients with HCC in other countries, albeit to a lesser extent. These lines of evidence obligate hepatologists to a considerable task of determining the role of HCV in hepatocarcinogenesis. Inflammation induced by HCV, manifesting in various forms of hepatitis, should be considered in a study on the carcinogenic capacity of hepatitis viruses. It has been proposed repeatedly that the necrosis of hepatocytes caused by chronic inflammation and ensuing regeneration enhances mutagenesis in host cells, the accumulation of which culminates in HCC. This theory presupposes an indirect involvement of hepatitis viruses in HCC *via* hepatic inflammation. However, this leaves specialists in hepatology with a serious question: can inflammation *per se* result in the development of HCC in such a high incidence or multicentric nature in HCV infection? The secondary role of HCV would have to be weighed against an extremely rare occurrence of HCC in patients with autoimmune hepatitis in whom severe inflammation in the liver persists indefinitely.

This background and reasoning lead to a possible activity of viral proteins for inducing HCC. This possibility has been evaluated by introducing genes of HCV into hepatocytes in culture with little success. A difficulty in using cultured cells is the carcinogenic capacity of HCV, if any, which would be weak and would take a long time to manifest. It takes 30–40 years for HCC to develop in individuals infected with HCV. Another constraint common to studies on carcinogenesis is the development of HCC by transformed cells that might have gone out of growth control and escaped surveillance of the host. Should this be the case, the analysis of transformed cells would not be sufficient for solving the mystery of carcinogenesis. On the basis of these viewpoints, we started tackling carcinogenesis in chronic viral hepatitis by transgenic mouse technology.

CORE PROTEIN OF HCV HAS ONCOGENIC ACTIVITY *IN VIVO*

AS ILLUSTRATED IN Figure 1, transgenic mouse lines with parts of the HCV genome were engineered by introducing the genes excised from the cDNA of the HCV genome of genotype 1b.[5,6] The background of the mouse lines is a C57BL/6 strain, which is known for a rare spontaneous occurrence of HCC.[7] Established are three different transgenic mouse lines, which carry the core gene, envelope genes, or non-structural genes, under the same transcriptional control element. Among these mouse lines, only the transgenic mice carrying the core gene develop HCC in two independent lineages (Fig. 1).[6] The envelope gene transgenic mice do not develop HCC, despite high expression levels of both E1 and E2 proteins.[8,9] The transgenic mice carrying the entire non-structural genes have not developed HCC.

The transgenic mice carrying the core gene express the core protein of an expected size, approximately 21 kDa, the level of which in the liver is similar to that in the liver of chronic hepatitis C patients. Early in life, these mice develop hepatic steatosis, which is a histologic characteristic of chronic hepatitis C, along with lymphoid follicle formation and bile duct damage.[10] Thus, the core gene transgenic mouse model well reproduces this feature of chronic hepatitis C. Of note, significant inflammation is not observed in the liver of this animal model. Late in life, these transgenic mice develop HCC. Most hepatic nodules disclose a pathology characterized by "nodule-in-nodule", and HCC with a low degree of differentiation develops within adenoma as well as within HCC with a higher degree of differentiation.[6] Although numerous lipid droplets are found in cells forming adenoma, as in non-tumorous cells, they are rarely observed in HCC cells. These histologic features

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closely resemble those observed in HCC developing in chronic hepatitis C patients, in which prominent lipid droplets are found in small differentiated HCC and its precursors; poorly differentiated HCC without lipid droplets develops from within differentiated HCC.[6] Notably, the development of steatosis and HCC has been reproduced by other HCV transgenic mouse lines, which harbor the entire HCV genome or structural genes including the core gene.[11] These outcomes indicate that the core protein of HCV has an oncogenic potential when expressed *in vivo*.

MECHANISM OF HEPATOCARCINOGENESIS IN MOUSE MODEL FOR HCV-ASSOCIATED HCC

IT IS DIFFICULT to sort out the mechanism of carcinogenesis even for our simple model, in which only the core protein is expressed in otherwise normal liver tissue. There is a notable feature in the localization of the core protein in hepatocytes; while the core protein predominantly exists in the cytoplasm associated with lipid droplets, it is also present in the mitochondria and nuclei.[6,12] On the basis of this finding, the pathways related to these two organelles, the mitochondria and nuclei, were meticulously analyzed.

One activity of the core protein is an increased production of oxidative stress in the liver. The production of oxidative stress is increased in our transgenic mouse model in the absence of inflammation in the liver (hepatitis). This reflects a state of an overproduction of reactive oxygen species (ROS) in the liver, or predisposition to it, which is staged by the HCV core protein without any intervening inflammation.[13,14] The overproduction of oxidative stress results in the generation of deletions in the mitochondrial DNA, an indicator of genetic damage. Thus, the core protein induces oxidative stress overproduction in the absence of inflammation, and may, at least in part, contribute to hepatocarcinogenesis in HCV infection. If inflammation is induced in the liver with the HCV core protein, the production of oxidative stress is escalated to an extent that can no longer be scavenged by a physiologically antagonistic system. This indicates that the inflammation in chronic HCV infection would have a characteristic different in quality from those of other types of hepatitis, such as autoimmune hepatitis. The basis for the overproduction of oxidative stress may be ascribed to mitochondrial dysfunction.[13,15] The function of the electron transfer system of the mitochondrion is suggested in association with the presence of the HCV core protein.[16] Hepatic steatosis in hepatitis C may work as fuel for oxidative stress overproduction.[14,17,18]

Table 1 Biomolecular alterations with core protein expression observed in the transgenic mouse model

1.	Induction of cytokines including TNF-α and IL-1β[19]
2.	Activation of MAPK pathway and enhancement of AP-1 activation[19,20]
3.	Overproduction of oxidative stress or ROS in the absence of inflammation[13]
4.	Synergy of HCV core and alcohol in inducing oxidative stress and activating MAPK[13,20]
5.	Interaction of HCV core and RXR-α and PPAR-α[21]
6.	Induction of insulin resistance[17]
7.	Development of steatosis by inhibiting MTP activity[5,14,22]
8.	Interaction of HCV core and proteasome activator PA28γ[23]
9.	Inhibition of SOCS-1[24]

AP-1, activated protein-1; HCV, hepatitis C virus; IL-1β, interleukin-1β; MAPK, mitogen-activated protein kinase; MTP, microsomal triglyceride transfer protein; PPAR-α, peroxisome proliferator agonist receptor-α; ROS, reactive oxygen species; RXR-α, retinoid X receptor; SOCS-1, suppressor of cytokine signaling; TNF-α, tumor necrosis factor.

Other possible pathways are the alteration of the expression of cellular genes, interacting with cellular proteins, and modulation of intracellular signaling pathways (Table 1). For example, tumor necrosis factor (TNF)-α and interleukin-1β (IL-1β) have been found transcriptionally activated.[19] The core protein has also been found to interact with some cellular proteins, such as retinoid X receptor (RXR)-α, that play pivotal roles in cell proliferation and metabolism.[20] The mitogen-activated protein kinase (MAPK) cascade is also activated in the liver of the core gene transgenic mouse model. The MAPK pathway, which consists of three routes, c-Jun N-terminal kinase (JNK), p38, and extracellular signal-regulated kinase (ERK), is involved in numerous cellular events including cell proliferation. In the liver of the core gene transgenic mouse model prior to HCC development, only the JNK route is activated. Downstream in the JNK activation, transcription factor AP-1 activation is markedly enhanced.[19,21] Far downstream, both the mRNA and protein levels of cyclin D1 and CDK4 are increased. Thus, the HCV core protein modulates the intracellular signaling pathways and gives advantage for cell proliferation to hepatocytes.

Such an effect of the core protein on the MAPK pathway, combined with that on oxidative stress, may

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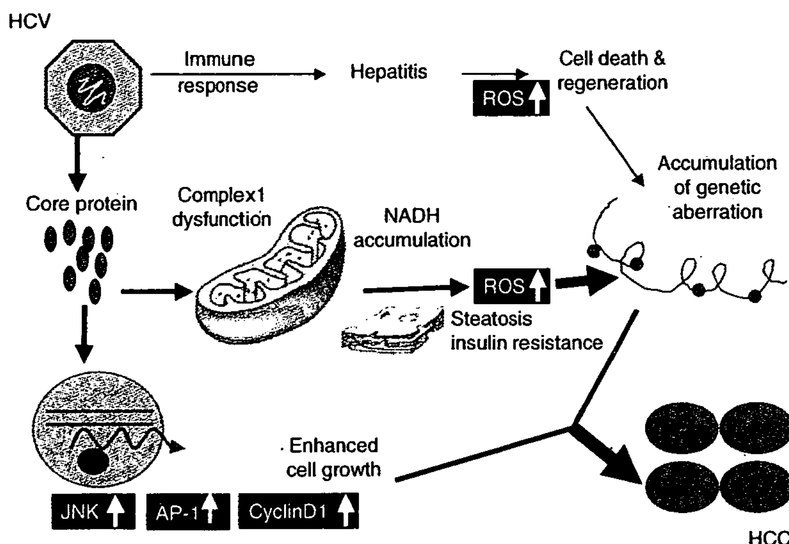

Figure 2 Mechanism of hepatitis C virus (HCV)-associated hepatocarcinogenesis. Inflammation should contribute to hepatocarcinogenesis by producing genetic aberrations via continual cell death and regeneration. In the case of HCV infection, the virus would contribute to hepatocarcinogenesis via two pathways: (i) the core protein acts on the function of mitochondrial electron transfer system, leading to the overproduction of oxidative stress. Inflammation may act synergistically with the core protein in inducing oxidative stress. The presence of steatosis and insulin resistance would enhance the production of oxidative stress; and (ii) modulation of cellular gene expression and signal transduction, which would give a growth advantage to hepatocytes. The combination of these alterations would escalate the development of hepatocellular carcinoma (HCC) in HCV infection. AP-1, activated protein-1; JNK, Jun N-terminal kinase; NADH, nicotinamide adenine dinucleotide; ROS, reactive oxygen species.

explain the extremely high incidence of HCC development in chronic hepatitis C.

HEPATOCARCINOGENESIS INDUCED BY HCV INFECTION: MECHANISM DISTINCT FROM OTHER CANCERS

THE RESULTS OF our studies on transgenic mice indicated a carcinogenic potential of the HCV core protein *in vivo*; thus, HCV may be directly involved in hepatocarcinogenesis.

In research studies of carcinogenesis, the theory by Kinzler and Vogelstein[25] has gained wide popularity. They proposed that the development of colorectal cancer is induced by the accumulation of a complete set of cellular gene mutations. They deduced that mutations in the *APC* gene for inactivation, those in *K-ras* for activation and those in the *p53* gene for inactivation accumulate, which cooperate toward the development of colorectal cancer.[25] The theory has been extended to the carcinogenesis of other cancers as well, called "Vogelstein-type" carcinogenesis (Fig. 2).

On the basis of results we obtained for the induction of HCC by the HCV core protein, we introduce a mechanism different from that of Kinzler and Vogelstein[25] for hepatocarcinogenesis in HCV infection. We allow multistages in the induction of all cancers; it would be mandatory for hepatocarcinogenesis that many mutations accumulate in hepatocytes. Some of these steps, however, may be skipped in the development of HCC in HCV infection to which the core protein would contribute (Fig. 3). The overall effects achieved by the expression of the viral protein would be the induction of HCC, even in the absence of a complete set of genetic aberrations, required for carcinogenesis.

By considering such a "non-Vogelstein-type" process for the induction of HCC, a plausible explanation may be given for many unusual events happening in HCV carriers.[26] Now it does not seem so difficult as before to determine why HCC develops in persistent HCV infection at an outstandingly high incidence. Our theory may also give an account of the non-metastatic and multicentric *de novo* occurrence characteristics of HCC, which would be the result of persistent HCV infection.

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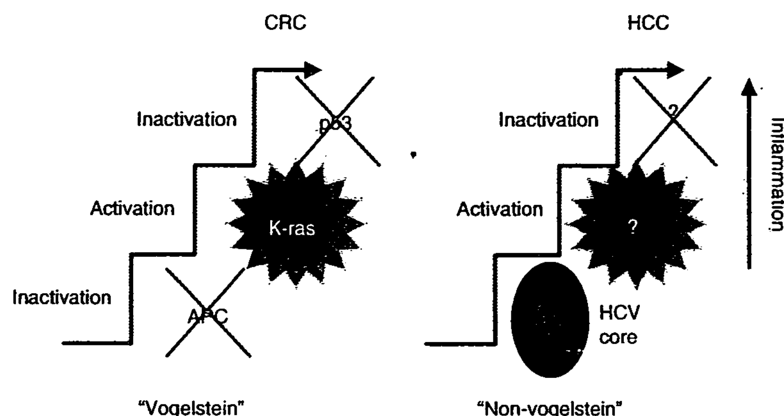

Figure 3 Hepatitis C virus (HCV)-associated hepatocarcinogenesis is a non-Vogelstein-type. Multiple steps are required in the induction of all cancers; it would be mandatory for hepatocarcinogenesis that genetic mutations accumulate in hepatocytes. However, in HCV infection, some of these steps may be skipped in the development of hepatocellular carcinoma (HCC) in the presence of core protein. Overall effects achieved by the expression of core protein would be the induction of HCC, even in the absence of a complete set of genetic aberrations, required for carcinogenesis. By considering such a "non-Vogelstein-type" process for the induction of HCC, a plausible explanation may be given for many unusual events in HCV carriers. CRC, colorectal cancer.

CONFLICT OF INTEREST

NO CONFLICT OF interest has been declared by the author.

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Short Communication

Prevalence of hepatitis B virus infection in Japanese patients with HIV

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Patients with HIV infection are frequently infected with hepatitis viruses, which are presently the major cause of mortality in HIV-infected patients after the widespread use of highly active antiretrovirus therapy. We previously reported that approximately 20% of HIV-positive Japanese patients were also infected with hepatitis C virus (HCV). Hepatitis B virus (HBV) infection may also be an impediment to a good course of treatment for HIV-infected patients, because of recurrent liver injuries and a common effectiveness of some anti-HIV drugs on HBV replication. However, the status of co-infection with HIV and HBV in Japan is unclear. We conducted a nationwide survey to determine the prevalence of HIV–HBV co-infection by distributing a questionnaire to the hospitals belonging to the HIV/AIDS Network of Japan. Among the 5998 patients reported to be HIV positive, 377 (6.4%) were positive for the hepatitis B surface antigen. Homosexual men accounted for two-thirds (70.8%) of the HIV–HBV co-infected patients, distinct from HIV–HCV co-infection in Japan in which most of the HIV–HCV co-infected patients were recipients of blood products. One-third of HIV–HBV co-infected patients had elevated serum alanine aminotransferase levels at least once during the 1-year observation period. In conclusion, some HIV-infected Japanese patients also have HBV infection and liver disease. A detailed analysis of the progression and activity of liver disease in co-infected patients is needed.

Key words: co-infection, hepatitis B, HIV, liver disease.

INTRODUCTION

HEPATITIS B VIRUS (HBV) infection is a major public health problem worldwide, along with hepatitis C virus (HCV) and HIV infections. In the USA, the estimated prevalence of HBV is less than 1%, but approximately 1 million people are persistently infected.[1] The prevalence of HIV in the USA is also <1%, and the virus is estimated to have infected approximately 800 000 people.[2] Because of the common transmission routes, that is, parenteral transmission routes, many people with HIV infection are also infected with HBV. Among the HIV-positive people in the USA, the prevalence of HBV co-infection is 6–14%.[1,2] Before the introduction of highly active antiretroviral therapy (HAART) in 1996, most patients with HIV infection died of HIV-associated opportunistic infections, such as *Pneumocystis jiroveci* pneumonia and cytomegaloviral infection. Since the widespread use of HAART, the mortality associated with HIV infection has declined. However, the reduction in mortality due to opportunistic infection, has left patients co-infected with HIV and hepatitis viruses faced with the menace of progressive liver diseases due to HBV infection,[3,4] in addition to HCV infection.[5]

HBV co-infection or superinfection of HIV-infected patients leads to several problematic situations. First, HBV infection tends to develop into persistent infection in HIV-infected patients,[1,6,7] which is a rare event in healthy adults, although it substantially depends on the genotype of HBV.[8] It results in the acceleration of the development of cirrhosis and eventually hepatocellular carcinoma. Second, some nucleoside reverse transcriptase inhibitors (NRTI) used in HAART also have

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inhibitory effects on the replication of HBV.[9-12] A careless administration or discontinuation of NRTI on HIV-HBV co-infected patients may cause reactivation and/or aggravation of hepatitis B. In addition, the administration of anti-HBV drugs in HIV–HBV co-infection may lead to the development of drug resistance.[11,12] Third, liver injury occurs more frequently in patients on HAART who are co-infected with HIV and HBV than those infected with HIV only.[9,10]

Importantly, co-infection with HIV and HCV increases the morbidity and mortality of HIV-infected patients in Japan,[13] where the prevalence of HIV infection is increasing linearly, and is exceptionally high among developed countries.[14] There are more than 14 000 HIV-positive people in Japan as of 2006, according to the AIDS National Survey in Japan,[14] and approximately 0.8 million chronic HBV carriers.[15] However, the prevalence of co-infection with HIV and HBV in Japan has not been clarified to date. Therefore, we conducted a nationwide study by distributing a postal mail-based questionnaire to the hospitals belonging to the HIV/AIDS Network of Japan.

PATIENTS AND METHODS

IN THE QUESTIONNAIRE, the following information was obtained from the hospitals regarding the number of patients who visited the hospitals at least once between January and December in 2006: (i) the number of HIV-positive patients; (ii) the number of hepatitis B surface antigen (HBsAg)-positive patients among (i); (iii) the number of patients among (ii) who were determined at least once to have a serum alanine aminotransferase (ALT) level higher than 100 IU/L; (iv) the number of HIV-positive patients that contracted HIV from blood products; (v) the number of HBsAg-positive patients among (iv), (vi) the number of patients among (v) who were determined at least once to have a serum ALT level higher than 100 IU/L; (vii) the number of HIV-positive patients among homosexual men, (viii) the number of HBsAg-positive patients among (vii), (ix) the number of patients among (viii) who were determined at least once to have a serum ALT level higher than 100 IU/L; (x) the number of HIV-positive patients that contracted HIV through intravenous drug use (xi) the number of HBsAg-positive patients among (x), (xii) the number of patients among (xi) who had at least one determination of a serum ALT level more than 100 IU/L; (xiii) the number of HIV-positive patients whose transmission routes were classified as "others"; (xiv) the number of HBsAg-positive patients among (xiii); and (xv) the number of patients among (xiv) who were determined at least once to have a serum ALT level higher than 100 IU/L.

The questionnaire was sent to the 372 hospitals belonging to the HIV/AIDS Network of Japan by mail. Answers were mostly returned by mail and in some cases by fax. The list of the hospitals in the HIV/AIDS Network of Japan can be viewed at http://www.acc.go.jp/mLhw/mLhw_frame.htm.

RESULTS

THE QUESTIONNAIRE WAS sent to all 372 hospitals that were on the list of the hospitals in the HIV/AIDS Network of Japan in January 2006. Two hundred and seven hospitals (55.6%) responded within the indicated period. In total, 5998 patients were reported to be HIV positive. The collection rate of 55.6% was higher than that (47.8%) for a questionnaire HIV–HCV co-infection study carried out in 2003.[15] It may appear rather low, particularly considering the number of reported HIV-positive people in 2006, which was approximately 14 000, according to the AIDS National Survey in Japan.[14] However, not all of the HIV-positive people were going to hospitals, and the answers to the questionnaire were obtained from most of the major hospitals in the HIV/AIDS Network in big cities around Japan. This suggests that not all, but a majority of HIV-positive Japanese patients were enrolled in the study.

Among the 5998 patients reported to be HIV positive, 377 (6.3%) patients were positive for HBsAg (Table 1). Of these 377 patients, 122 (32.4%) had elevated serum ALT levels at least one time during the 1-year observation period.

The HBV prevalence rates, when fractionated by the routes of transmission, were as follows: among the 508 HIV-positive patients who contracted HIV from blood products, such as unheated concentrated coagulation factors, only 30 (5.9%) were HBsAg positive, which shows a marked contrast to the prevalence of HCV in this cohort (Fig. 1).[16] Among the 23 intravenous drug users, three (13.0%) were HBsAg positive. Among the 3213 HIV-positive patients who were homosexual men, 267 (8.3%) were HBsAg positive. In the remaining 2254 patients who were HIV-positive and whose route of HIV transmission was classified as "others", most contracted HIV heterosexually. This number (2254) showed a substantial increase from the 1316 obtained in the questionnaire for the HIV–HCV co-infection study in 2003, while the total number of HIV-positive patients increased from 4877 to 5998.[16] Among these, 77 (3.4%)

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Table 1 Prevalence rates of hepatitis B virus infection among HIV-positive patients

Routes of transmission	No. patients	HBsAg positive (% in HIV positive according to route)	ALT >100 IU/L (% in HBsAg positive according to route)
Blood products	508 (5.9%)	30 (40.0%)	12
Homosexual men	3213 (8.3%)	267 (32.2%)	86
Drug addicts	23 (13.0%)	3 (66.7%)	2
Others (heterosexual etc.)	2254 (3.4%)	77 (28.6%)	22
Total	5998	377 (6.3%)	122 (32.4%)

ALT, serum alanine aminotransferase; HBsAg, hepatitis B surface antigen.

were HBsAg positive. In terms of the route of HIV infection, 267 (70.8%) of the 377 patients were homosexual men among the HIV–HBV co-infected patients. This shows a contrast to the status of HIV–HCV co-infection, in which the majority of HIV–HCV co-infected Japanese patients contracted both viruses from blood products.[16]

There were one or more HIV-positive patients in 154 (74.4%) of the 207 hospitals in the HIV/AIDS Network of Japan (Table 2). Twenty four (11.6%) of 207 hospitals had 20–49 HIV-positive patients, and 16 (7.7%) hospitals had 50 or more HIV-positive patients. There were one or more patients who were co-infected with HIV and HBV in 64 (30.9%) of the 207 hospitals. There were 10 or more HIV–HBV co-infected patients in nine (4.3%) hospitals, all of which had 50 or more HIV-positive patients (Table 2). HIV–HBV co-infected patients were concentrated in specific hospitals in big cities around Japan. In particular, in the Kanto area, HIV–HBV co-infected patients were concentrated in the HIV/AIDS Network hospitals in the Tokyo city area.

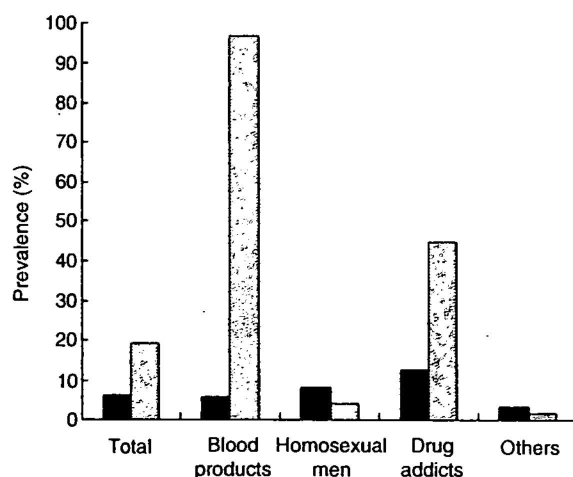

Figure 1 Prevalence rates of persistent hepatitis B virus and hepatitis C virus infections in the HIV-positive population sorted by the HIV risk group. (■), HBsAg, hepatitis B surface antigen; (□), anti-HCV, antibody to hepatitis C virus. *Prevalence rates of anti-HCV are obtained from Koike K *et al.*[16]

DISCUSSION

ALONG WITH THE increase in the number of HIV-infected patients in Japan, co-infection with HIV and hepatitis viruses has become a major medical issue. HBV infection of HIV-positive patients raises several difficult problems: HBV infection tends to develop into persistent infection, even in adults; some NRTI used in HAART also have inhibitory effects on the replication of HBV, the improper administration, or discontinuation of which may lead to drug resistance; and HIV–HBV co-infected patients on HAART have liver injuries more frequently than HIV-monoinfected patients. It is important to determine the status of HBV infection in HIV-positive patients.

According to the statistics of the Ministry of Health, Labor, and Welfare of Japan, the number of reported HIV-positive people was slightly over 14 000 in 2006.[14] In the present study, 6.4% of HIV-positive patients were positive for HBsAg, the most reliable marker for ongoing HBV infection. It might have been advantageous if

Table 2 Number of hospitals categorized according to the number of patients infected with HIV and those co-infected with HIV and hepatitis B virus (HBV)

No. HIV (+)/ HBV (+)	No. HIV(+)				
	0	1–19	20–49	50+	Total
0	53	76	13	1	143
1–9	0	38	11	6	55
10+	0	0	0	9	9
Total	53	114	24	16	207

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serum HBV-DNA levels were determined, but unfortunately, HBV-DNA level determination was not a routine laboratory test in most hospitals. In addition, considering that the antibody to the hepatitis B core antigen might be the only marker of ongoing HBV infection in some immuno-compromised patients, it would also be advantageous if this viral marker were available. These issues should be investigated in future studies. Comments from hospitals to the questionnaire included one indicating that not all HIV-positive patients underwent a test for serum HBsAg, suggesting the actual prevalence of HBsAg in HIV-infected patients might be higher than 6.4%.

In a previous questionnaire study of HIV-HCV co-infection, the prevalence of HCV infection among HIV-infected patients was 19.2%;[16] the prevalence of HBV infection (6.4%), is one-third of it. The lower positivity for HBsAg than for the anti-HCV antibody among those who contracted HIV through blood products accounts for this difference: almost all (96.9%) of the patients who contracted HIV through blood products were also anti-HCV antibody positive.[16] It should be noted that among the homosexual male patients who were HIV positive, 8.3% were HBsAg positive, which is twice as high as that of the anti-HCV antibody in these populations. A higher prevalence of HBV infection as a sexually transmitted infection than that of HCV[17] may explain the high prevalence of HBV infection in HIV-positive homosexual men. Similarly, a HBV prevalence of 3.4% in heterosexually transmitted HIV-positive patients is higher than that of the general Japanese population of the same age.[15]

Of the 377 patients who were HBsAg positive, 122 (32.4%) had elevated serum ALT levels at least once in the 1-year observation period. In this type of study using a questionnaire, it is difficult to obtain the details of patients' data, including age, body weight, and the degrees of liver injuries and fibrosis. If detailed items were included in the questionnaire, then the collection rate would be low. This time, to obtain a high collection rate, we asked whether the patients with HBsAg showed an elevated ALT level higher than 100 IU/L at least once during the 1-year observation period. We thereby do not have details on liver disease in HIV-HBV co-infected patients in the current study. Nonetheless, one-third of HIV-HBV co-infected patients have moderate liver injuries, either chronic hepatitis B or adverse effects of drugs, and are waiting for an aid for the amelioration of liver disease. A detailed analysis of the progression and activity of liver disease in HIV-HBV co-infected patients is expected.

The collection rate of the present questionnaire from the hospitals belonging to the HIV/AIDS Network was 55.6% (207 of 372). This was higher than that (47.8%) in the HIV-HCV co-infection questionnaire study carried out in 2003. The reason for this increase is not clear, but presumably the questionnaire conducted in 2003 has raised awareness among hospital staff regarding the relevance of hepatitis virus and HIV co-infection in clinical practice.

In the current study, both Japanese patients and those of other nationalities/ethnicities were included in the study. Although the ratio of newly diagnosed HIV-positive foreign people has been declining to approximately 10% in 2006, the one in total HIV positive still accounts for approximately 25% in Japan. Because the rates of the HBV carrier are different among countries, it is ideal to analyze the HBV prevalence separately according to the nationalities/ethnicities. However, in the current survey to the hospitals in HIV/AIDS Network of Japan, nationality/ethnicity was not itemized in order to make the questionnaire simple. If we would attempt to obtain such data under the approval of the ethical committee in each hospital, the response rate to questionnaire would be extremely lowered.

To establish measures that decrease the morbidity and mortality of HIV-HBV co-infected patients, it is essential to determine the current status of co-infection. In the present study, the number and transmission routes of HIV-HBV co-infected patients in Japan were determined for the first time, although detailed information on the severity and progression of liver disease in HIV-HBV co-infected patients has not been obtained yet. Undoubtedly, this will be the first step towards improving the prognosis and quality of life of Japanese patients co-infected with HIV and HBV.

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DYSFUNCTION OF ENERGY METABOLISM IN HEPATIC CARCINOGENESIS

Hepatitis C virus contributes to hepatocarcinogenesis by modulating metabolic and intracellular signaling pathways

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Key words
hepatitis C virus, hepatocarcinogenesis, intracellular signaling transduction, oxidative stress, transgenic mouse.

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Abstract

Persistent infection with hepatitis C virus (HCV) is a major risk factor for development of hepatocellular carcinoma (HCC). However, it remains controversial in the pathogenesis of HCC associated with HCV as to whether the virus plays a direct or an indirect role. The studies using transgenic mouse models, in which the core protein of HCV has an oncogenic potential, indicate that HCV is directly involved in hepatocarcinogenesis, albeit other factors such as continued cell death and regeneration associated with inflammation would also play a role. The downstream events of the core protein are segregated into two components. One is the augmented production of oxidative stress along with the activation of scavenging system, including catalase and glutathione, in the putative pre-neoplastic stage with steatosis in the liver. Thus, oxidative stress production in the absence of inflammation by the core protein would partly contribute to the development of HCC. The generation of oxidative stress is estimated to originate from mitochondrial dysfunction in hepatocytes by HCV infection. The other component is the alteration of intracellular signaling cascade of mitogen-activated protein kinase and activating factor (AP)-1, leading to the activation of cell cycle control. The combination of these pathways, collective with HCV-associated alterations in lipid and glucose metabolism, would lead to the frequent development of HCC in persistent HCV infection. These results suggest that there would be a mechanism for hepatocarcinogenesis in persistent HCV infection that is distinct from those for the other cancers. Similar to the pathogenesis of other cancers, the accumulation of a set of genetic aberrations may also be necessary for a multistage development of HCC. However, HCV core protein, to which an oncogenic potential is ascribed, may allow some of the multiple steps to be bypassed in hepatocarcinogenesis. Therefore unlike for other cancers, HCV infection may be able to cause HCC in the absence of a complete set of genetic aberrations. Such a scenario, 'non-Vogelstein-type' carcinogenesis, would explain the rare feature of hepatocarcinogenesis in HCV infection, the extraordinarily high incidence and the multicentric nature of HCC development.

Introduction

Hepatitis C virus (HCV) infects hundreds of millions of people persistently, and induces a spectrum of chronic liver disease worldwide.[1] It impacts on society in a number of domains including the medical, sociological and economic. Hepatocellular carcinoma (HCC) has become the major cause of death in individuals persistently infected with HCV. In particular, HCV has been given increasing attention because of its wide and deep penetration in the community, coupled with a very high incidence of HCC in persistent HCV infection. Once liver cirrhosis is established in hosts infected with HCV, HCC develops at a yearly rate of 5–7%.[2] Knowledge on the mechanism of HCC development in chronic HCV infection therefore is urgently required for the prevention of HCC.

Hepatocellular carcinoma frequently develops in persistent HCV infection

How HCV induces HCC is not clear yet, despite the fact that more than 70% of patients with HCC in Japan are infected with HCV.[1,3,4] Hepatitis C virus infection is also common in patients with HCC in other countries albeit to a lesser extent. These lines of evidence obligate hepatologists to the considerable task of determining the role of HCV in hepatocarcinogenesis. Inflammation induced by HCV, manifesting in various forms of hepatitis, should be considered in a study on the carcinogenic capacity of hepatitis viruses. It has been proposed repeatedly that the necrosis of hepatocytes due to chronic inflammation and ensuing regeneration enhances mutagenesis in host cells, the accumulation of which culminates in HCC. This theory presupposes an indirect involvement of hepatitis

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viruses in HCC via hepatic inflammation. However, this leaves specialists in hepatology with a serious question: can inflammation per se result in the development of HCC in such a high incidence or multicentric nature in HCV infection? The secondary role of HCV would have to be weighed against an extremely rare occurrence of HCC in patients with autoimmune hepatitis in whom severe inflammation in the liver persists indefinitely.

This background and reasoning led to the suggestion that HCC may be induced, at least in part, by viral proteins. This possibility has been evaluated by introducing genes of HCV into hepatocytes in culture with little success. One of the difficulties in using cultured cells is the carcinogenic capacity of HCV, if any, which would be weak and would take a long time to manifest itself. It takes 30–40 years for HCC to develop in individuals infected with HCV. Another constraint common to studies on carcinogenesis is the development of HCC by transformed cells that might have gone out of growth control and escaped surveillance of the host. Should this be the case, the analysis of transformed cells would not be sufficient for solving the mystery of carcinogenesis. On the basis of these points, we chose to investigate carcinogenesis in chronic viral hepatitis using transgenic mouse technology.

HCV core protein has an oncogenic activity in transgenic mouse

Transgenic mouse lines with sections of the HCV genome were engineered by introducing genes excised from the cDNA of the HCV genome of genotype 1b.[5,6] The mouse lines were from a C57BL/6 strain, which is known for a rare spontaneous occurrence of HCC.[7] Three different transgenic mouse lines have been established, which carry the core gene, envelope genes or non-structural genes (Fig. 1), respectively, under the same transcriptional control element. Among these mouse lines, only the transgenic mice carrying the core gene develop HCC in two independent lineages.[6] The envelope gene transgenic mice do not develop HCC, despite high expression levels of both E1 and E2 proteins.[8,9] The transgenic mice carrying the entire non-structural genes have developed no HCC.

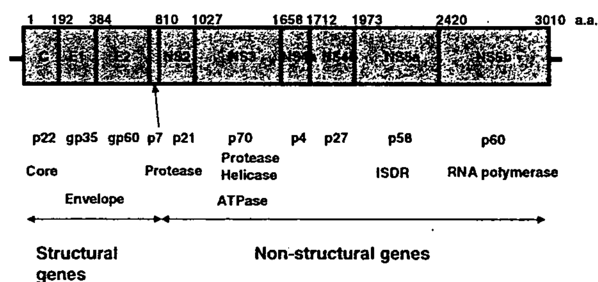

Figure 1 Structure of the hepatitis C virus (HCV) genome. The HCV genome consists of structural and non-structural regions. The structural region consists of the core, envelope and p7 genes. The non-structural region cones enzyme proteins of NS3 to NS5B. Among the three different transgenic mouse lines established, which carry the core, envelope and non-structural region, respectively, only the transgenic mice carrying the HCV core gene develop hepatocellular carcinoma (HCC) after an early phase with hepatic steatosis in two independent lineages. The mice transgenic for the envelope genes or non-structural genes do not develop HCC. ISDR, interferon-sensitivity determining region.

The transgenic mice carry the core gene and express the core protein of an expected size, approximately 21 kDa, the level of which in the liver is similar to that in the liver of chronic hepatitis C patients. Early in life, these mice develop hepatic steatosis, which is one of the histologic characteristics of chronic hepatitis C, along with lymphoid follicle formation and bile duct damage.[10] Thus, the core gene transgenic mouse model well reproduces this feature of chronic hepatitis C. Of note, significant inflammation is not observed in the liver of this animal model. Late in life, these transgenic mice develop HCC. Most hepatic nodules have a pathology characterized by 'nodule in nodule', and HCC with a low degree of differentiation develops within adenoma as well as within HCC with a higher degree of differentiation.[6] Although numerous lipid droplets are found in cells forming adenoma, as in non-tumorous cells, they are rarely observed in HCC cells. These histological features closely resemble those observed in HCC developing in chronic hepatitis C patients, in whom prominent lipid droplets are found in small, well-differentiated HCC and its precursors; poorly differentiated HCC without lipid droplets develops from within differentiated HCC.[6] Notably, the development of steatosis and HCC has been reproduced in other HCV transgenic mouse lines, which harbor the entire HCV genome or structural genes including the core gene.[11] These outcomes indicate that the core protein per se of HCV has an oncogenic potential when expressed *in vivo*.

Sequence to the core protein expression in the liver

It is difficult to clarify the mechanism of carcinogenesis even for our simple model in which only the core protein is expressed in otherwise normal liver tissues. There is a notable feature of the localization of the core protein in hepatocytes: although the core protein predominantly exists in the cytoplasm associated with lipid droplets, it is also present in the mitochondria and nuclei.[6,12] On the basis of this finding, the pathways related to these two organelles, the mitochondria and nuclei, were analyzed.

One activity of the core protein is an increased production of oxidative stress in the liver. We note that the production of oxidative stress is increased in our transgenic mouse model in the absence of inflammation in the liver (hepatitis). This reflects overproduction of reactive oxygen species (ROS) in the liver, or predisposition to it, which is staged by the HCV core protein without any intervening inflammation.[13,14] The overproduction of oxidative stress results in the generation of deletions in the mitochondrial DNA, an indicator of genetic damage. Thus, the core protein induces oxidative stress overproduction in the absence of inflammation, and may, at least in part, contribute to hepatocarcinogenesis in HCV infection (Fig. 2). If inflammation is induced in the liver with the HCV core protein, the production of oxidative stress is escalated to an extent that cannot be scavenged by a physiological antagonistic system. This indicates that the inflammation in chronic HCV infection would be different to that produced in other