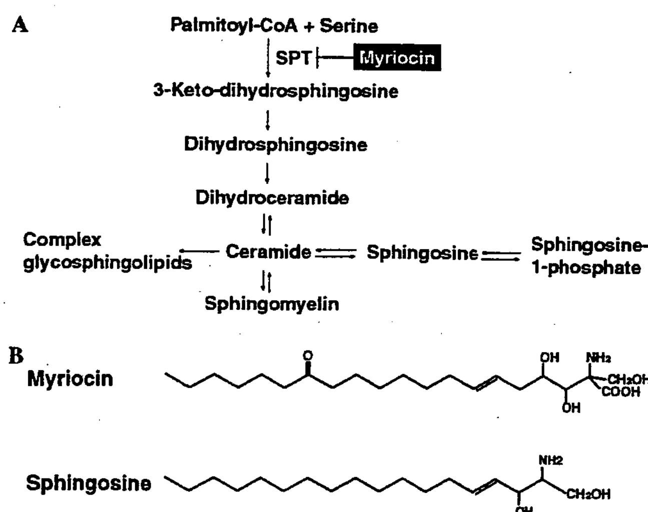

Fig. 1. Sphingolipid biosynthetic pathway. (A) Biosynthesis pathway of sphingolipid. (B) Structure of myriocin and sphingosine.

Previously, we isolated the compound NA255, which suppresses replication of the HCV subgenomic replicon [12]. NA255 is structurally similar to myriocin and inhibits the enzymatic activity of SPT, resulting in suppression of replication without affecting the enzyme activities of HCV NS3 (protease and helicase) or NS5B (RNA-dependent RNA polymerase). Thus, replication of the HCV subgenomic replicon was suppressed by NA255 in response to the decrease in amount of sphingolipid, ceramide, and sphingomyelin. These findings suggest that NA255 disrupts assembly of the lipid raft associated with HCV NS proteins. In the present study, we performed comparative and concomitant trials of one SPT inhibitor, myriocin, and PEG-IFN in chimeric mice with humanized liver (chimeric mice) infected with intact HCV. The results demonstrate for the first time that suppression of SPT inhibits replication of intact HCV in vivo.

Materials and methods

Inhibition assay of replication in HCV replicon cells by myriocin. Myriocin (Sigma, St. Louis, MO, USA) was added in the growth medium of HCV subgenomic replicon cells FLR3-1 (genotype 1b, Con-1; [12]) at a final concentration of 0.2, 1.0, 3.9, 15.6 or 62.5 nM. After 72 h incubation, we performed luciferase assays using the Bright-Glo luciferase assay kit (Promega, Madison, WI, USA).

Measurement of cell viability using the Tetra Color One (WST-8) assay. Myriocin was added to FLR3-1 cells as described above. After 72 h incubation, cell viability was measured using the Tetra Color One kit (Seikagakukougyo, Tokyo, Japan) according to the manufacturer's instructions.

Immunoblotting analysis. Cells were harvested and lysed in lysis buffer (PBS containing 0.5% Triton X-100 and 0.5 mM PMSF), and then 5 μg of protein separated by 12% SDS–PAGE and electro-blotted onto a nitrocellulose membrane (Shleicher & Schuell, Dassel, Germany). A rabbit polyclonal anti-NS3 antibody [12] and anti-actin (20–33) antibody (Sigma, St. Louis, MO, USA) were used as the primary antibodies. The proteins were then detected by an anti-rabbit antibody HRP-linked IgG (Cell Signaling Technology, Beverly, MA, USA).

Immunofluorescent staining of HCV replicon cells. After treatment of 250 nM myriocin for 72 h, FLR3-1 cells were probed with a primary antibody, an anti-NS3 polyclonal antibody, after blocking with TNB blocking buffer (Perkin-Elmer, Wellesley, MA, USA). Next, an anti-rabbit IgG-Alexa-488 conjugate (Invitrogen, Carlsbad, CA, USA) was applied as the secondary antibody.

TLC analysis. Cells were incubated for 2 h with [^{14}C] serine (0.5 μCi/ml) in Opti-MEM (Invitrogen). After the cells were lysed with 0.1% SDS, and total lipids were extracted with chloroform/methanol (1:2 v/v). The extracts were spotted onto Silica Gel 60 thin-layer chromatography (TLC) plates (Merck, Darmstadt, Germany) and chromatographed with methyl acetate/1-propanol/chloroform/methanol/0.25% KCl (25:25:25:10:9, v/v). Radioactive spots were detected by BAS 2000 (Fuji Film, Kanagawa, Japan).

Complementation of sphingolipid intermediates. FLR3-1 cells were incubated with 1 or 2.5 μM of sphingolipid intermediates (dihydrosphingosine, sphingosine, or sphingosine-1-phosphate) and sequentially diluted myriocin then added. After 72 h, the IC_{50} of each combination was measured by the luciferase assay.

Infection of HCV genotype 1a and 1b in chimeric mice. Chimeric mice were purchased from PhenixBio Co., Ltd. (Hiroshima, Japan). The mice were generated by transplanting human primary hepatocytes into SCID mice carrying the urokinase plasminogen activator transgene controlled by an albumin promoter (*Alb-uPA*) [13–15]. Overexpression of this transgene induces a profoundly hypofibrinogenemic state and accelerated hepatocyte death. HCG9 (genotype 1a) and HCR6 (genotype 1b, Accession No: AY045702), originally from patient serum, were intravenously injected at 10^6 copies/mouse at about 40 days after transplantation of human hepatocytes. After 4 weeks, the HCV 1a and 1b RNA levels had reached ~10^8 copies/ml and ~10^7 copies/ml, respectively, in the mice serum.

Administration of myriocin and/or PEG-IFN into chimeric mice infected with HCV 1b. Injections of myriocin or PEG-IFN (Chugai, Tokyo, Japan) or both were administered to HCV genotype 1b (HCR6) infected mice and blood then collected according to the protocol in Table 2.

Quantification of HCV RNA by real-time PCR. Total RNA was purified from 1 μl of serum or 50 μg of liver tissue from chimeric mice using the AGPC method. HCV RNA was quantified by real-time PCR as previously reported [16].

Measurement of human albumin in the serum. Human albumin concentration was measured in 2 μl of serum using the Alb-II kit (Eiken Chemical, Tokyo, Japan) according to the manufacturer's instructions.

Detection of core protein in live tissue. We used chimeric mice with a high RNA levels of HCV genotype 1a (HCG9) in the serum to easily detect HCV RNA and core protein in liver tissue. We administered 2 mg/kg myriocin daily for 6 days to a chimeric 1a-4 mouse and extirpated the liver. For comparison, the livers of non-treated (1a-1), non-infected (1a-2), and PEG-IFN treated (1a-3) mice were also extirpated. The liver tissues were homogenized in RIPA buffer and 100 μg of total protein was used for the detection of core protein using the Ortho HCV core protein ELISA kit (Eiken Chemical).

Immunofluorescent and histological staining of chimeric mouse liver tissue. Liver sections from 1a-1 and 1a-4 mice were probed by biotinylated anti-HCV core protein monoclonal antibody, and human hepatocyte monoclonal antibody (Dako, Glostrup, Denmark) as the primary antibodies, followed by streptavidin-Alexa-488 (Invitrogen) and anti-mouse-IgG-Alexa-546 (Invitrogen). The nuclei were stained using DAPI. Biotinylated normal mouse IgG (Ancell, Bayport, MN, USA) was used as the negative control. For histological analysis, liver sections from 1a-1 and 1a-4 mice were stained by hematoxylin-eosin (H&E staining).

Results

Anti-HCV effect of the SPT inhibitor, myriocin

We examined the anti-HCV effect and cell toxicity of myriocin in the HCV subgenomic replicon cells FLR3-1. Luciferase activity was greatly decreased by myriocin in a dose-dependent manner without affecting cell viability

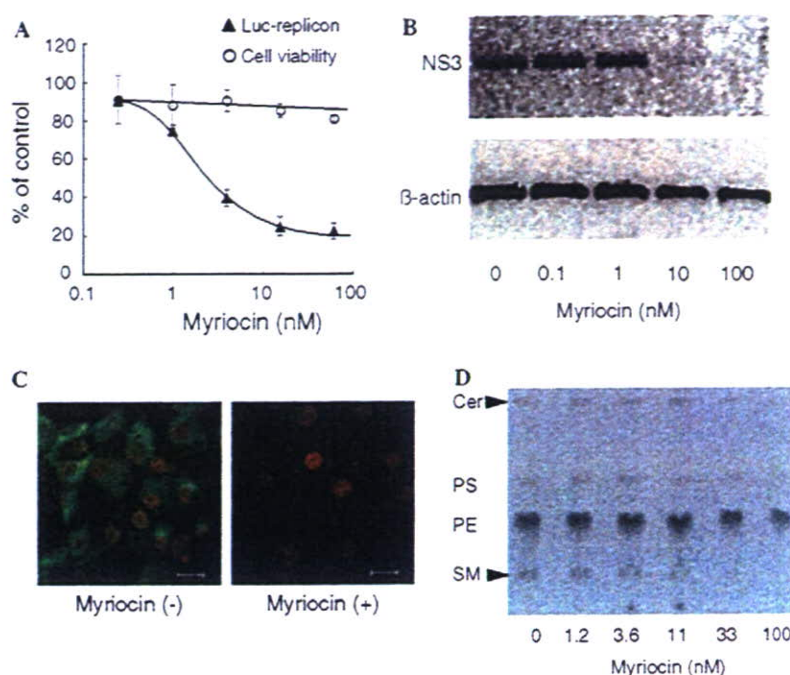

Fig. 2. Anti-HCV effect of myriocin in HCV replicon cells. (A) Luciferase activity and cell viability of FLR3-1 replicon cells in the presence of myriocin. Experiments were conducted independently at least three times. (B) Detection of NS3 protein by immnoblotting analysis. (C) Detection of NS3 protein by immunofluorescent staining in FLR3-1 cells. HCV NS3 protein and nucleus are shown in green and red, respectively. Scale bar: 20 μm. (D) De novo sphingolipid biosynthesis in the presence of myriocin was monitored by TLC. Cer, ceramide; PS, phosphatidylserine; PE, phosphatidylethanolamine; and SM, sphingomyelin.

Table 1
IC_{50} values of myriocin in the presence of sphingolipids

Supplement (μM)		IC_{50} of myriocin (nM)
Absence	0	5.8
Dihydrosphingosine	1.0	77.7
	2.5	>1000
Sphingosine	1.0	22.4
	2.5	>1000
Sphingosine-1-phosphate	1.0	14.7
	2.5	>1000

IC_{50} values of myriocin measured in the presence of 1. and 2.5 μM dihydrosphingosine, sphingosine or sphingosine-1-phosphate, show suppression of HCV replicon replication by myriocin.

(Fig. 2A) or cell growth (data not shown). The maximum inhibition rate was about 80% in the presence of over 62.5 nM myriocin (Fig. 2A), while the 50% inhibitory concentration (IC_{50}) was about 5.8 nM (Table 1). Reduction of NS3 protein, which plays a key role in HCV replication, was also observed by immunoblotting analysis and staining (Figs. 2B and C), suggesting that myriocin has a potent anti-HCV effect.

Relationship between sphingolipid metabolites and HCV replication

To examine the relationship between sphingolipid metabolites and HCV replication, we monitored de novo sphingolipid biosynthesis by FLR3-1 cells in the presence of myriocin. The production of both ceramide and sphingomyelin was inhibited in a dose-dependent manner, whereas production of the phosphatidylethanolamine and phosphatidylserine, metabolites of sphingosine, was unaffected (Fig. 2D). To confirm whether suppression of HCV subgenomic replicon replication was caused by sphingolipid depletion, we examined the anti-HCV effect of myriocin in the presence of three sphingolipids, dihydrosphingosine, sphingosine, and sphingosine-1-phosphate, which are intermediates in the sphingolipid biosynthesis pathway (Fig. 1A). Replication of the HCV replicon was recovered by complementation of the intermediate molecules of sphingolipid biosynthesis (Table 1). These results indicate that suppression of replication by myriocin was due to a reduction in sphingolipid biosynthesis.

Anti-HCV effects of myriocin and PEG-IFN in chimeric mice infected with HCV

The inhibitory ability of myriocin was investigated using chimeric mice infected with HCR6 (genotype 1b). We administered myriocin or PEG-IFN via intraperitoneal or subcutaneous injection, as shown in Table 2. In the myriocin-treated group, the HCV RNA levels were reduced in the serum from 3×10^6–1×10^7 copies/ml to 6×10^5–1×10^4 copies/ml over 8 days (an approximately 1/10–1/100 reduction). The same level of reduction was

Table 2
Administration schedule into chimeric mice infected with HCV genotype 1b

Day	−1	0	1	2	3	4	5	6	7	8
Collection of blood	B		B			B				B
PEG-IFN		I			I				I	
Myriocin		M	M	M	M	M	$M_{1/2}$	$M_{1/2}$	$M_{1/2}$	
Myriocin + PEG-IFN		M/I	M	M	M/I	$M_{1/2}$	$M_{1/2}$		I	

B, I or M indicates that each manipulation was performed as required, and administration of reagents was started from day zero. PEG-IFN was subcutaneously injected at 30 μg/kg. The amount of myriocin intraperitoneally injected was adjusted according to the body weight of the mice. Doses began at 1 mg/kg, and at a 10% reduction in body weight, the dose was reduced to 0.5 mg/kg ($M_{1/2}$). At 20% reduction, administration was discontinued.

observed in the PEG-IFN-treated group, which was injected with a 10-fold larger amount of PEG-IFN than that used in clinical treatment (30 μg/kg body weight). Moreover, combined treatment with myriocin and PEG-IFN reduced the HCV RNA levels to less than 1/1000 of the control levels, and in 2 (1b-7 and 1b-9) of 3 mice HCV RNA was not detected at all on day 8 (Fig. 3A). Concurrently, we monitored the concentration of human albumin (h-Alb) and found slight reductions only in the combined treatment group (Fig. 3B). These results indicate that myriocin suppressed replication of intact HCV without interfering with h-Alb expressed from humanized liver, whereas the combination of myriocin and PEG-IFN synergistically suppressed HCV replication with slight liver damage.

Detection of HCV RNA and core protein in the liver of chimeric mice

To clarify whether HCV was reduced by myriocin from the humanized liver, we examined the livers of the chimeric mice infected with another HCV genotype, 1a (HCG9). The RNA level of HCV 1a in serum reached approximately 1×10^8 copies/ml, which is 10-fold higher than that of HCV 1b (HCR6). Thus, we speculated that HCV core protein in hepatocytes would be easily detected by immunofluorescent staining. The liver of a 1a-4 mouse was extirpated after daily administration of 2 mg/kg of myriocin for 6 days. Following treatment, the HCV RNA level in the serum of the 1a-4 mouse fell to 1×10^5 copies/ml (Fig. 4A). The amount of HCV 1a RNA and core protein

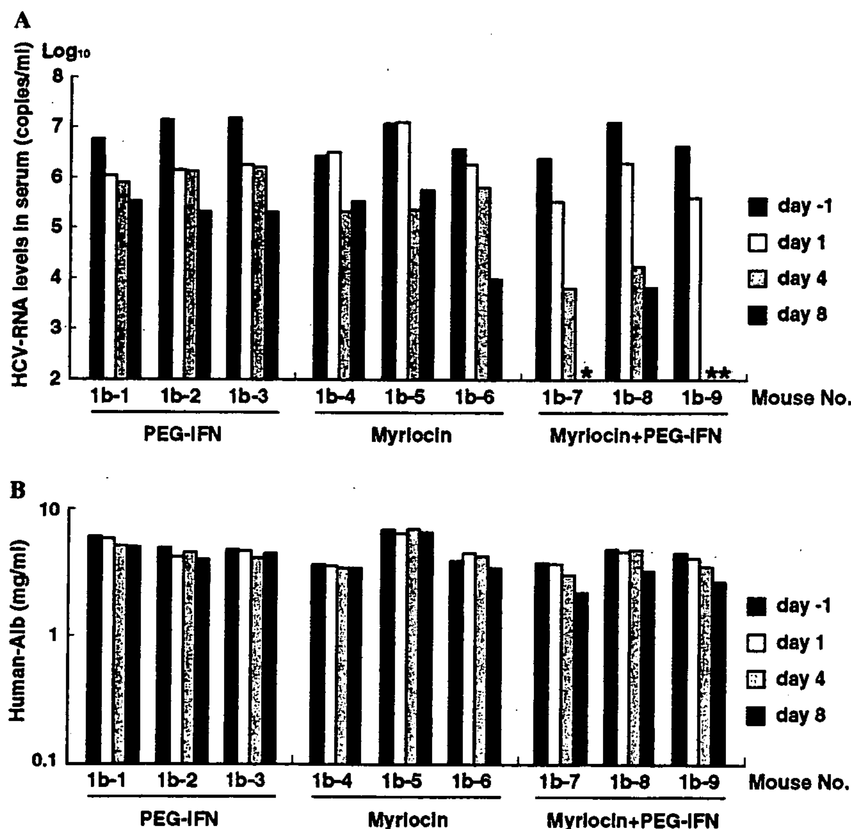

Fig. 3. Anti-HCV effect of myriocin in chimeric mice infected with HCV genotype 1b. (A) HCV RNA levels in the serum of chimeric mice. Asterisks indicate no HCV RNA was detected. (B) Human albumin concentrations in serum of chimeric mice.

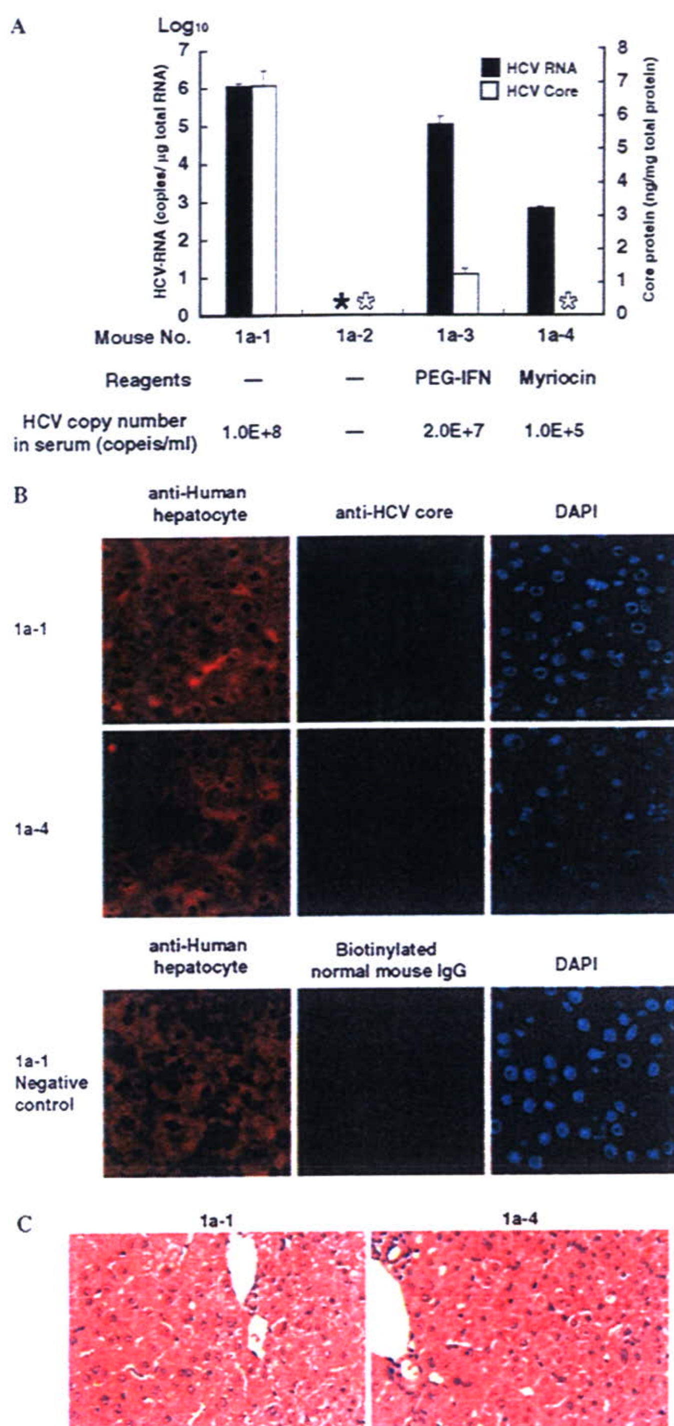

Fig. 4. Analysis of liver tissue from chimeric mice. HCV RNA and core protein were detected in the liver of chimeric mice infected with HCV genotype 1a. 1a-1, non-treated; 1a-2, non-treated and non-infected; 1a-3, PEG-IFN treated; and 1a-4, myriocin treated mouse. (A) Copy number of HCV RNA per 1 μg total RNA, and expression levels of HCV core proteins per 1 mg total protein. Asterisks indicate no HCV RNA or core proteins were detected. (B) Immunofluorescent staining of HCV core protein and human hepatocytes in chimeric mice liver. Human hepatocytes, HCV core protein, and nucleus are shown in red, green, and light blue, respectively. As a negative control of anti-core monoclonal antibody staining, the liver of 1a-1 was also stained with biotinylated normal mouse IgG. (C) H&E staining of liver tissue from chimeric mice 1a-1 and 1a-4. Primary human hepatocytes were observed in these mice and these hepatocytes displayed no significant morphological changes.

in the liver was quantified and had also reduced, as well as that in serum (Fig. 4A). Immunofluorescent staining revealed that the core protein of the non-treated mouse, 1a-1, which had 1×10^8 copies/ml serum (Fig. 4A), expressed a human hepatocyte moiety, whereas the core protein of the 1a-4 mouse disappeared (Fig. 4B). These results indicate that myriocin causes a decrease not only in HCV genotype 1b but also in genotype 1a, and eliminates HCV from the liver.

H&E staining of chimeric mouse liver

We performed histological analysis of the livers from non-treated (1a-1) and myriocin-treated (1a-4) mice (Fig. 4C). No significant morphological differences were observed between the tissues of the 1a-1 and 1a-4 mice. Thus, myriocin did not induce hepatocyte damage in chimeric mice to any biologically significant degree.

Discussion

In the present study, the SPT inhibitor myriocin was shown to inhibit replication of intact HCV in vivo. We initially investigated the fundamental inhibitory effects and mechanisms of myriocin against replication of the HCV replicon and found that inhibition of HCV replicon replication is compatible with a decrease in ceramide and sphingomyelin in the cells. The inhibitory effect of myriocin on replication of the HCV replicon differed slightly from that on de novo biosynthesis of ceramide and sphingomyelin. It has been previously reported that the membranous web formed in HCV replicon cells is the site of viral RNA synthesis and is not observed in naive HuH-7 cells [17]. Furthermore, HCV replication is known to occur on the lipid raft assembly [7]. The lipid raft associated with HCV replicase exists as an aberrant structure that forms a membranous web, and thus the lipid raft formed by HCV differs from the one in healthy cells. Therefore, we speculate that the above difference indicates that the lipid raft associated with HCV replicase is sensitively disrupted by myriocin.

Furthermore, HCV replication inhibition was complemented in the presence of the sphingomyelin biosynthetic intermediates dihydrosphingosine, sphingosine, and sphingosine-1-phosphate. These results indicate that depletion of sphingolipids induces disruption of the lipid raft assembly, resulting in suppression of replication of the HCV replicon. Disruption of the lipid raft assembly would lead to failure of HCV replicase to associate with the raft, and thus interrupt genome replication. This result is consistent with a previous study [12]. In order to demonstrate whether inhibition of SPT can be linked to anti-HCV therapy in vivo, we used a chimeric mouse model.

Recently, Mercer et al. developed a chimeric mouse containing human hepatocytes in which infection and replication of intact HCV occurs [14]. We examined the anti-HCV effect of myriocin in chimeric mice infected with HCV

genotypes 1a and 1b, and succeeded in the effective elimination of both HCV genotypes in their serum. Myriocin did not induce significant liver damage or interfere with the amount of human albumin in the chimeric mice or the ALT levels in Balb/c mice (data not shown), and thus the reduction in HCV RNA levels by myriocin is not due to induction of human hepatocyte damage. In the analysis of liver tissue infected with HCV genotype 1a, both HCV RNA and core protein levels were reduced by myriocin more effectively than by PEG-IFN. Thus, myriocin appears to be able to directly suppress replication of intact HCV in human hepatocytes, regardless of the HCV genotype. This is the first report of an SPT inhibitor suppressing intact HCV replication in vivo. Furthermore, combined treatment was more effective than myriocin or PEG-IFN alone, with HCV RNA levels reduced to less than 1/1000 of the controls, suggesting that myriocin with PEG-IFN cooperatively and synergistically inhibits the replication and proliferation of HCV.

Cholesterol is another major component of the lipid raft assembly, in addition to the sphingolipids [11]. The cholesterol biosynthetic pathway has also been a target for disruption of lipid raft assembly. However, recent studies have shown that the protein associated with geranylgeranylation, rather than cholesterol, is important for HCV replication [7,18,19]. Thus, it appears likely that disruption of lipid raft assembly is effectively caused by inhibition of sphingolipid biosynthesis rather than inhibition of cholesterol biosynthesis.

Myriocin is a known immunosuppressant [9], and mainly inhibits generation of cytotoxic T lymphocytes and T-cell dependent antibody production via inhibition of SPT activity in vivo. In chimeric mice deficient in both T and B cells (SCID), the immunosuppressant effect of myriocin does not cause a reduction in HCV replication but simply causes disruption of sphingolipid biosynthesis. In addition, the inhibitory mechanism in vivo, as in vitro analysis has shown, is likely to be disrupted by myriocin of the lipid raft assembly. Whether the sphingolipid level in liver in vivo is disrupted is currently unknown and the focus of future study. We continually monitored HCV for 14 days after the administration regimen and detected the same level of HCV as before the administration among all groups. Thus, to eliminate HCV completely, it will be necessary to adjust the dosage of myriocin and PEG-IFN and further extend the duration of administration.

In conclusion, we elucidated the mechanism of myriocin inhibition of HCV replication in vitro and determined that myriocin inhibits HCV replication in a chimeric mouse model with humanized liver. Although the toxicity of myriocin renders it unsuitable for use as an anti-HCV drug in human patients, our results suggest that SPT may be an effective target of drugs designed to inhibit HCV replication, and that SPT inhibitors such as myriocin are good candidates on which to base the development of new anti-HCV drugs.

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Clinical Studies

Measurement of hepatitis B virus core-related antigen is valuable for identifying patients who are at low risk of lamivudine resistance

Tanaka E, Matsumoto A, Suzuki F, Kobayashi M, Mizokami M, Tanaka Y, Okanoue T, Minami M, Chayama K, Imamura M, Yatsuhashi H, Nagaoka S, Yotsuyanagi H, Kawata S, Kimura T, Maki N, Iino S, Kiyosawa K, HBV Core-Related Antigen Study Group. Measurement of hepatitis B virus core-related antigen is valuable for identifying patients who are at low risk of lamivudine resistance.
Liver International 2006: 26: 90–96. © Blackwell Munksgaard 2005

Abstract: *Objective:* The clinical usefulness of hepatitis B virus core-related antigen (HBVcrAg) assay was compared with that of HBV DNA assay in predicting the occurrence of lamivudine resistance in patients with chronic hepatitis B. *Patients:* Of a total of 81 patients who were treated with lamivudine, 25 (31%) developed lamivudine resistance during a median follow-up period of 19.3 months. *Results:* The pretreatment positive rate of HBe antigen, or pretreatment levels of HBVcrAg or HBV DNA did not differ between patients with and without lamivudine resistance. Levels of both HBVcrAg and HBV DNA decreased after the initiation of lamivudine administration; however, the level of HBVcrAg decreased significantly more slowly than that of HBV DNA. The occurrence of lamivudine resistance was significantly less frequent in the 56 patients whose HBV DNA level was less than 2.6 log copy/ml at 6 months of treatment than in the remaining 25 patients. The cumulative rate of lamivudine resistance was as high as 70% within 2 years in the latter group, while it was only 28% in the former group. Lamivudine resistance did not occur during the follow-up period in the 19 patients whose HBVcrAg level was less than 4.6 log U/ml at 6 months of treatment, while it did occur in 50% of the remaining patients within 2 years. *Conclusion:* These results suggest that measurement of HBV DNA is valuable for identifying patients who are at high risk of developing lamivudine resistance, and that, conversely, measurement of HBVcrAg is valuable for identifying those who are at low risk of lamivudine resistance.

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Key words: chronic hepatitis B – HBV core-related antigen – HBV DNA – lamivudine resistance

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Lamivudine, a nucleoside analogue that inhibits reverse transcriptases, was first developed as an anti-viral agent against human immunodeficiency virus (HIV). It was later also found to be effective against hepatitis B virus (HBV) because HBV is a member of the Hepadnaviridae family of viruses, which use reverse transcriptases in their replication process (1, 2). Lamivudine was found to inhibit the replication of HBV, reduce hepatitis, and improve histological findings of the liver in long-term treatment (3–5). Furthermore, it has been shown that lamivudine treatment improves the long-term outcome of patients with chronic hepatitis B (6, 7). However, there are a number of problems with lamivudine therapy, such as relapse of hepatitis because of the appearance of YMDD mutant viruses and the reactivation of hepatitis after discontinuation of the treatment (8–11).

The concentration of HBV DNA in serum decreases and usually becomes undetectable during lamivudine administration, but it rapidly increases when HBV becomes resistant to lamivudine. Thus, the measurement of HBV DNA is useful for monitoring the anti-viral effects of lamivudine. However, a negative result of HBV DNA in serum does not necessarily indicate a good outcome of lamivudine therapy, because lamivudine resistance may occur even if HBV DNA levels remain undetectable during therapy (11–13). Recently, a chemiluminescence enzyme immunoassay (CLEIA) was developed in our laboratory for the detection of hepatitis B virus core-related antigen (HBVcrAg) (14, 15). The assay reflects the viral load of HBV in a similar manner to that used in assays, which detect HBV DNA. HBVcrAg consists of HBV core and e antigens; both proteins are transcribed from the precore/core gene and their first 149 amino acids are identical (16–18). The HBVcrAg CLEIA simultaneously measures the serum levels of hepatitis B core (HBc) and e (HBe) antigens, using monoclonal antibodies, which recognize common epitopes of these two denatured antigens. In the present study, we analyzed the clinical significance of the HBVcrAg assay in monitoring the anti-viral effects of lamivudine treatment.

Patients and methods

Patients

A total of 81 patients with chronic hepatitis B, who received lamivudine therapy, were enrolled in the present study. These were 58 men and 23 women with a median age of 49 years (range 24–79 years). The 81 patients were selected retrospectively from six medical institutions in Japan (Shinshu University Hospital, Toranomon Hospital, Nagoya City University Hospital, Kyoto Prefectural University Hospital, Hiroshima University Hospital, National Nagasaki Medical Center). Eight to 25 patients who met the following three criteria were selected consecutively in each institution: the first, a daily dose of 100 mg lamivudine was administered for at least 6 months in a period from 1999 to 2004; the second, histologically confirmed for chronic hepatitis without liver cirrhosis; and the third, serum samples at several time points available for testing. All patients were naïve for lamivudine therapy. Chronic hepatitis B was defined as positive hepatitis B surface (HBs) antigen for more than 6 months with elevated levels of serum transaminases. The HBV genotype was A in two patients, B in three and C in 76. Serum HBV DNA was detectable in all patients, and HBe antigen was positive in 51 (63%) of the 81 patients just before lamivudine administration. The median follow-up period was 19 months with a range from 6 to 50 months. Follow-up of patients ended when lamivudine administration was discontinued. Written informed consent was obtained from each patient.

The occurrence of lamivudine resistance was defined as a rapid increase in serum HBV DNA levels with the appearance of the YMDD mutations during lamivudine administration. Using this criteria, resistance appeared in 27 (33%) of the 81 patients. The median period from the start of lamivudine administration to the occurrence of resistance was 12 months with a range from 4 to 37 months.

Serological markers for HBV

HBs antigen, HBe antigen and anti-HBe antibody were tested using commercially available enzyme immunoassay kits (Abbott Japan Co., Ltd., Tokyo, Japan). Six major genotypes (A–F) of HBV can be detected using the method reported by Mizokami et al. (19), in which the surface gene sequence amplified by polymerase chain reaction (PCR) is analyzed by restriction fragment length polymorphism. The YMDD motif, that is, lamivudine resistant mutations in the active site of HBV polymerase, was detected with an enzyme-linked mini-sequence assay kit (HBV YMDD Mutation Detection Kit, Genome Science Laboratories Co., Ltd., Tokyo, Japan) (20).

Serum concentration of HBV DNA was determined using Amplicor HBV monitor kit (Roche, Tokyo, Japan), which had quantitative range from 2.6 to 7.6 log copy/ml. Sera containing

over 7.0 log copy/ml HBV DNA were diluted 10- or 100-fold with normal human serum and re-tested to obtain the end titer.

Serum concentrations of HBVcrAg were measured using the CLEIA method reported previously (10, 11). Briefly, 100 µL serum was mixed with 50 µL pretreatment solution containing 15% sodium dodecylsulfate and 2% Tween 60. After incubation at 70 °C for 30 min, 50 µL pretreated serum was added to a well coated with monoclonal antibodies against denatured HBc and HBe antigens (HB44, HB61 and HB114) and filled with 100 µL assay buffer. The mixture was incubated for 2 h at room temperature and the wells were then washed with buffer. Alkaline phosphatase-labeled monoclonal antibodies against denatured HBc and HBe antigens (HB91 and HB110) were added to the well, and the mixture was incubated for 1 h at room temperature. After washing, CDP-Star with Emerald II (Applied Biosystems, Bedford, MA) was added and the plate was incubated for 20 min at room temperature. The relative chemiluminescence intensity was measured, and the HBVcrAg concentration was determined by comparison with a standard curve generated using recombinant pro-HBe antigen (amino acids, 10–183 of the precore/core gene product). The HBVcrAg concentration was expressed as units/ml (U/ml) and the immunoreactivity of recombinant pro-HBe antigen at 10 fg/ml was defined as 1 U/ml. In the present study, the cutoff value was tentatively set at 3.0 log U/ml. Sera containing over 7.0 log U/ml HBVcrAg were diluted 10- or 100-fold in normal human serum and re-tested to obtain the end titer.

Statistical analysis

The Mann–Whitney *U*-test and Wilcoxon signed-ranks test were utilized to analyze quantitative data, and Fisher's exact test was used for qualitative data. A log-rank test was used to compare the occurrence of lamivudine resistance. Statistical analyses were performed using the SPSS 5.0 statistical software package (SPSS, Inc., Chicago, IL). A *P*-value of less than 0.05 was considered to be statistically significant.

Results

Table 1 shows a comparison of the clinical and virological backgrounds of the 27 patients who showed lamivudine resistance and the 54 patients who did not. Median age, gender distribution and median follow-up period did not differ between the two groups, and the positive rate of HBe antigen was similar. Both HBV DNA and HBVcrAg levels at the beginning of lamivudine administration were similar between the two groups; however, both HBV DNA and HBVcrAg levels at 6 months after the start of lamivudine administration were significantly lower in the lamivudine resistance negative group than in the positive group. ALT level was normal at the beginning in eight (15%) of the 54 patients without lamivudine resistance and in two (7%) of the 27 patients with it ($P > 0.2$).

Table 1. Comparison of the clinical and virological backgrounds of patients who showed lamivudine resistance and those who did not

	Appearance of lamivudine resistance		
Characteristics	Negative ($n = 54$)	Positive ($n = 27$)	*P*
Age (years)*	47.0 (24–79)	50.6 (34–67)	0.140†
Gender (male %)	74%	67%	>0.2‡
Follow-up period (months)*	16 (6–50)	21 (9–43)	>0.2†
HBV genotype (A/B/C)	2/2/50	0/1/26	>0.2‡
HBe antigen (positive %)	59%	70%	>0.2‡
ALT (IU/ml)*			
Initial	85 (22–713)	95 (20–1140)	>0.2†
At 6 months	27 (11–115)	30 (15–92)	>0.2†
HBV DNA (log copy/ml)*			
Initial	7.0 (3.5–9.1)	7.3 (4.2–9.2)	>0.2†
At 6 months	<2.6 (<2.6–4.8)	3.3 (<2.6–6.6)	<0.001†
HBVcrAg (log U/ml)*			
Initial	6.2 (<3.0–8.8)	7.3 (4.4–9.1)	0.073†
At 6 months	5.2 (<3.0–6.7)	5.8 (4.7–8.4)	<0.001†

HBe antigen, hepatitis B e antigen; HBV, hepatitis B virus; ALT, alanine aminotransferase; HBVcrAg, HBV core-related antigen. *Data are expressed as median (range). †Mann–Whitney *U* test. ‡χ^2-test.

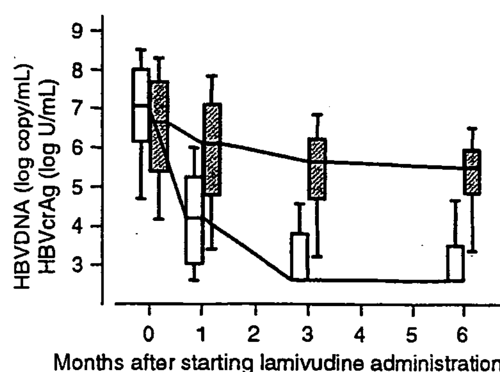

Fig. 1. Changes in the median levels of hepatitis B virus core-related antigen (HBVcrAg) and hepatitis B virus (HBV) DNA during lamivudine administration. The box plots show the 10th, 25th, 50th, 75th and 90th percentiles, with the open boxes indicating HBV DNA and shaded boxes indicating HBVcrAg. The median amount of decrease from the baseline in HBVcrAg levels was significantly smaller (Wilcoxon signed-ranks test) than that in HBV DNA level at 1 (2.80 log copy/ml vs. 0.27 log U/ml, $P < 0.001$), 3 (3.60 log copy/ml vs. 0.83 log U/ml, $P < 0.001$) and 6 months (3.90 log copy/ml vs. 1.15 log U/ml, $P < 0.001$) after the initiation of lamivudine administration.

Figure 1 shows changes in HBV DNA and HBVcrAg levels during lamivudine treatment in all patients. The level of HBV DNA decreased rapidly and became undetectable at 3 months after treatment was initiated. On the other hand, although HBVcrAg levels decreased continuously, the median amount of decrease from the base-line was significantly lower than that in HBV DNA levels at 1, 3 and 6 months after starting lamivudine administration (Wilcoxon signed-ranks test, $P<0.001$ at all analyzed points in time).

Changes in HBV DNA and HBVcrAg levels during lamivudine administration are compared in Fig. 2 between the 27 patients who showed lamivudine resistance and the 54 patients who did not. Serum HBV DNA levels were found to decrease rapidly and become undetectable within 6 months in 45 (83%) of the 54 patients without lamivudine resistance. On the other hand, only 11 (41%) of the 27 patients with lamivudine resistance showed a similar rapid decrease, and the HBV DNA levels of the remaining patients stayed above the detection limit during the follow-up period. HBVcrAg levels decreased but did not reach levels lower than 4.7 log U/ml (5000 U/ml) in the 27 patients with lamivudine resistance. In 19 (35%) of the 54 patients without lamivudine resistance, on the other hand, the levels decreased to levels below 4.7 log U/ml within 6 months after the start of lamivudine administration. The level of HBVcrAg increased rapidly as did the level of HBV DNA when lamivudine resistance occurred.

The occurrence of lamivudine resistance was significantly less frequent in the 56 patients whose HBV DNA level was less than 2.6 log copy/ml at 6 months after the initiation of treatment than in the remaining 25 patients (Fig. 3). The cumulative occurrence of lamivudine resistance was as high as 70% within 2 years in the latter group, while it was only 28% in the former group. There was no occurrence of lamivudine resistance during the follow-up period in the 19 patients whose HBVcrAg levels were less than 4.6 log U/ml at 6 months after the initiation of lamivudine therapy (Fig. 3). On the other hand, lamivudine resistance occurred in 50% of the remaining patients within 2 years.

Discussion

The HBVcrAg assay is a unique assay, which measures the amounts of e and core antigens

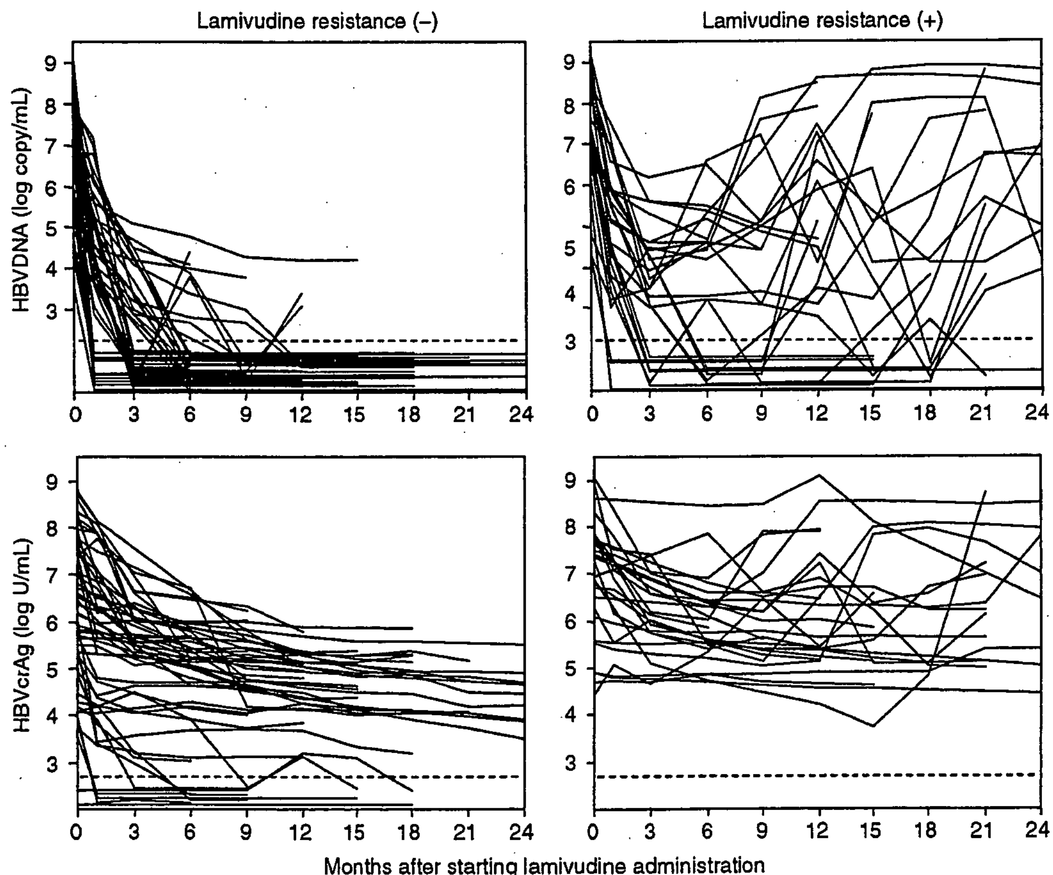

Fig. 2. Comparison of changes in serum hepatitis B virus (HBV) DNA and serum HBV core-related antigen (HBVcrAg) levels between patients who showed lamivudine resistance and those who did not. The broken lines indicate the detection limit of each assay.

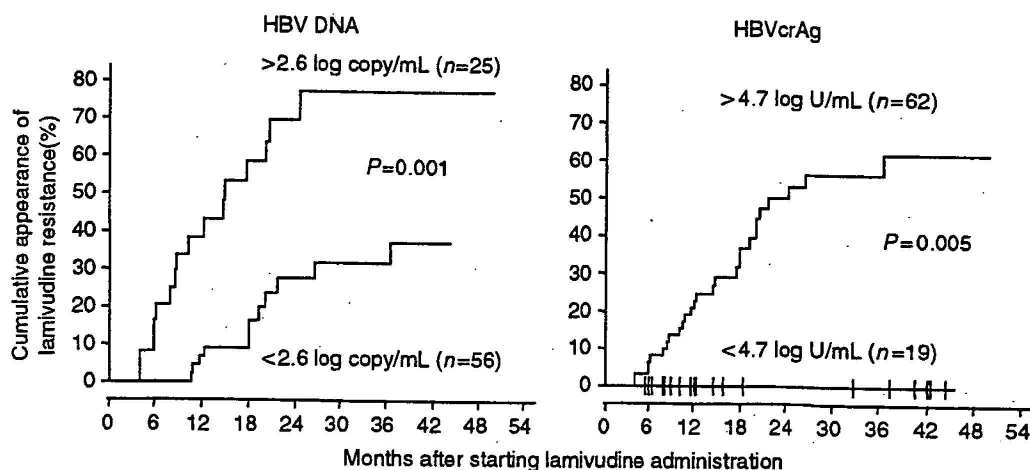

Fig. 3. Comparison of the cumulative occurrence of lamivudine resistance between patients who showed hepatitis B virus (HBV) DNA levels of less than the detection limit (2.6 log copy/ml) at 6 months after starting lamivudine administration and those who did not (left figure), and similarly between patients who showed HBV core-related antigen (HBVcrAg) levels of less than 4.7 log U/ml and those who did not (right figure).

coded by the core gene of the HBV genome with high sensitivity and a wide quantitative range. Serum HBVcrAg levels reflect the viral load in the natural course because these levels correlate linearly with those of HBV DNA (14, 15). On the other hand, the character of HBVcrAg is somewhat different from that of HBV DNA in patients undergoing anti-viral therapies such as lamivudine. That is, HBVcrAg levels decrease significantly more slowly than those of HBV DNA after the initiation of lamivudine administration.

HBV is an enveloped DNA virus containing a relaxed circular DNA genome, which is converted into a covalently closed circular DNA (cccDNA) episome in the nucleus of infected cells (18, 21–23). The cccDNA molecules serve as the transcriptional template for the production of viral RNAs that encode viral structural and non-structural proteins. Reverse transcription of the viral pregenomic RNA and second-strand DNA synthesis occur in the cytoplasm within viral capsids formed by the HBV core protein. Because lamivudine, a nucleoside analogue, inhibits reverse transcription of the pregenomic RNA, it directly suppresses the production of HBV virion. Thus, serum HBV DNA levels decrease rapidly after the initiation of lamivudine administration. On the other hand, the production of viral proteins is not suppressed by lamivudine because the production process does not include reverse transcription. Furthermore, it has been reported that the amount of cccDNA, which serves as a template for mRNA, decreases quite slowly after starting the administration of nucleoside analogues (24–26). Thus, it is reasonable that serum HBVcrAg levels decrease much more slowly than HBV DNA levels after the initiation of lamivudine therapy.

Significant markers that can predict the presence or absence of lamivudine resistance are clinically valuable because the emergence of this resistance and the subsequent recurrence of hepatitis are fundamental problems in lamivudine therapy. Serum markers that reflect the activity of HBV replication have been reported to be associated with the occurrence of lamivudine resistance (11, 12, 27, 28). However, neither the pretreatment existence of HBe antigen nor pretreatment levels of HBV DNA or HBVcrAg were found to be significant markers in the present study. These results may reflect a weak association between the pretreatment activity of HBV replication and the occurrence of lamivudine resistance (13, 29). Changes in HBV DNA and HBVcrAg levels after starting lamivudine administration clearly differed between patients with and without lamivudine resistance. Thus, HBV DNA and HBVcrAg levels at 6 months after starting lamivudine administration were analyzed to determine whether these levels might serve as predictive markers; both were found to be significantly lower in patients without lamivudine resistance at the tested point in time. Furthermore, patients who showed higher levels of HBV DNA and HBVcrAg at 6 months after the initiation of treatment were significantly more likely to develop lamivudine resistance than those who showed lower levels.

We believe that the measurement of HBV DNA levels is useful to identify patients who are at high risk for lamivudine resistance because as many as 70% of patients who were positive for HBV DNA at 6 months after starting lamivudine

administration developed lamivudine resistance within 2 years. However, a negative result of HBV DNA at 6 months does not necessarily guarantee the absence of lamivudine resistance because nearly 30% of such patients developed resistance within 2 years. On the other hand, HBVcrAg levels of less than 4.7 log U/ml at 6 months are a useful indicator of patients who are unlikely to develop lamivudine resistance, because no such patients developed resistance during the follow-up period in the present study. Lower serum HBVcrAg levels may reflect lower levels of cccDNA in hepatocytes because the mRNAs of HBVcrAg are transcribed from the cccDNA (18, 22, 23). This possibility may explain our finding that patients whose HBVcrAg levels decreased sufficiently were unlikely to develop lamivudine resistance, because cccDNA provides the templates for viral and pregenomic messenger RNA (18, 22, 23), which may be a source of lamivudine-resistant strains.

In conclusion, our results suggest that measurement not only of HBV DNA but also of HBVcrAg is useful for predicting the occurrence of lamivudine resistance. HBV DNA measurement is valuable for identifying patients who are at high risk of developing this resistance and HBcrAg measurement is valuable for identifying those who are at low risk.

Acknowledgements

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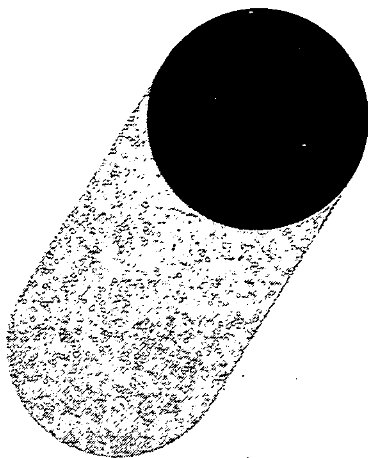

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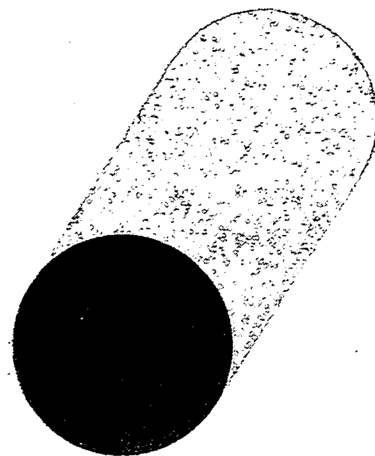

ヒト肝細胞キメラマウス

再生医療，創薬などの動物モデルとして有用．様々な病態モデルの開発にも期待

組織の再生力を利用して，損傷組織の治療を行なう新しい医療技術が注目されている．生体組織は，分化細胞と未分化細胞によって構成されている．前者は，組織の構造形成と機能の発現に寄与している．後者は，前者に比べてその数が著しく少なく，組織の特殊な部位（ニッチ）に存在し，分裂せず静止期（G_0）に留まっているが，組織が損傷すると，分裂を開始し，失われた細胞を補給する．このような細胞は組織幹細胞と呼ばれている．

筆者らは，肝臓の再生に関する研究を行なっている．肝臓は，終生，高い再生能力を有する器官として知られているが，その実質組織を構成する肝細胞の細胞周期時間がおよそ1年と長いため，その幹細胞（肝臓幹細胞）の性質に関しては不明なことが多い．幹細胞の性質を調べるためには，これを増殖させ分化させるための実験系が必要である．そのような実験系として，*in vivo* および *in vitro* の2つの実験系がある．前者は，生体組織に傷害を与え，目的とする細胞を適当な方法で標識後，その組織に移植し，増殖分化させるものである．後者は，生体外に分離し，これを培養し，増殖分化させるものである．通常は，この2つの方法を併用して，組織幹細胞の性質に関する情報を得ている．本稿では，ヒト肝細胞を *in vivo* で増殖分化させる方法を紹介する．

筆者は，成人肝細胞をマウスに移植し，ホストの肝細胞のほとんどをヒト肝細胞で置換させる方法を開発した．このようなマウスをヒト肝細胞キメラマウスと呼ぶ．このキメラマウスは，ヒト肝細胞で構成されている肝臓を有するため，実験研究として利用することが不可能な人体肝臓の代わりに，ヒト細胞の性質を動物実験的に研究することを可能にしてくれるばかりでなく，医学的あるいは薬学的研究の動物モデルとしても価値が出てきた．また，大量のヒト肝細胞を得ることが可能なので，これを医療用に利用する可能性も秘めている．

マウスにヒト肝細胞を移植してこれを増殖させるためには，ホストは免疫不全と肝不全の2つの条件を満たす必要がある．免疫不全マウスとしては，スキッド（SCID, severe combined immunodeficiency）マウス（SCIDマウス）を利用した．SCIDマウスは，BおよびTリンパ細胞を欠如している．NK細胞，マクロファージおよび補体系は有している．肝不全マウスとしては，アルブミンエンハンサープロモーターの下流にウロキナーゼ型線溶活性化酵素（プラスミノーゲンアクチベータ）（uPA）を連結した遺伝子を導入したマウス（uPA-トランスジェニックマウス，以下，uPAマウス）[1]を利用した．PAはプラスミン（線溶酵素）の不活性前駆体であるプラスミノーゲンをプラスミンに変換させる酵素である．プラスミンは止血栓（フィブリン fibrin）の分解作用ばかりでなく，細胞外基質の分解や肝細胞増殖因子（HGF）の不活性前駆体（proHGF）を活性HGFに変換させる作用もある[2]．このマウス肝臓は，出生時から急性/亜急性肝障害に似た症状を示す．その色は，正常肝の赤色ではなく，詳しい理由は不明であるが，脂肪顆粒を多く含むため白い色をしている．

uPA遺伝子のヘテロ接合体トランスジェニックマウスでは，導入遺伝子の欠失を起こした細胞が野生型の表

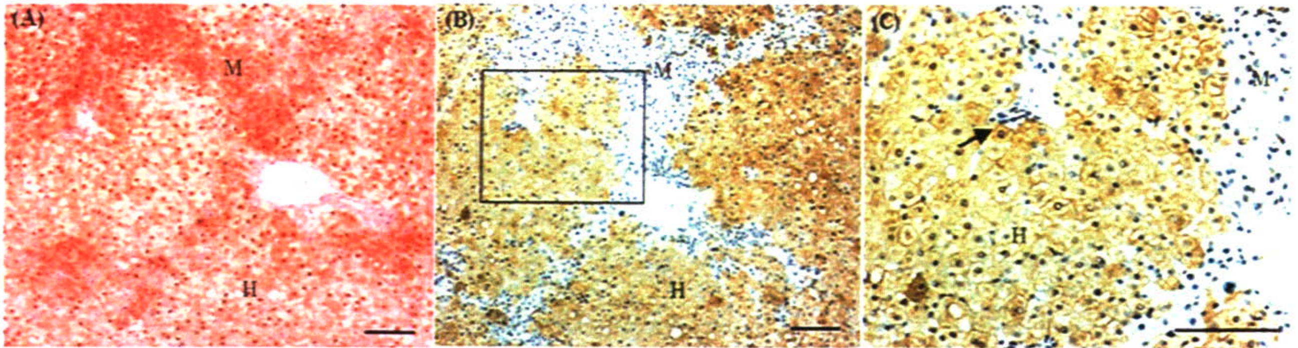

図1■マウス肝臓のヒト肝細胞による置換

(A) 9ヵ月男児の肝細胞を移植した．置換率67%．ヒトゲノムDNAプローブによる *in situ* ハイブリダイゼーション．H：ヒト肝細胞領域（核が茶色に染まっている領域），M：マウス肝細胞領域．(B) (A) のキメラマウス肝臓切片の隣接切片をヒトサイトケラチン8/18を特異的に染色する抗体で反応させた．Hは茶色に染まっているヒト領域．(C) (B) の図中の長方形で囲んだ部位の拡大写真．矢印は胆管細胞を示しているが，抗体に反応していない．バーは100 μm.

現型を示すため正常肝細胞となり，増殖を開始し，正常肝細胞集落（赤いコロニー）を形成する．この集落はいわば再生コロニー（regenerative nodule）である．白い障害肝の中で赤い再生コロニーは次第に大きくなり，またコロニーどうしが融合して，8週齢までには完全に正常肝が構築される．したがって，この肝臓は障害肝の再生過程の研究のモデルとして利用されている．障害領域では，肝細胞がTGF-β1やp53などのアポトーシス誘導因子を合成分泌し，そのオートクライン作用でアポトーシスを起こす．一方，非実質細胞は，HGFなどの肝細胞増殖因子を活発に合成分泌し，隣接する再生コロニーの肝細胞の増殖をパラクライン作用で誘導する[3]．

uPAマウスとSCIDマウスを交配させることによって，肝障害で免疫不全マウス（uPA/SCIDマウス）を得ることができる．このマウスに，正常ヒト肝細胞を移植する．マウスの肝門脈は径が細いため，通常，脾臓に移植する．移植細胞は，血管を流れ，肝臓に到達し，肝細胞索に侵入し，そこに生着する．このヒト肝細胞が増殖する仕組みは，上述のヘテロ接合体肝臓における再生コロニーの出現と増大の仕組みと同じであると考えられる．ホストの障害肝細胞はアポトーシスを起こす一方，ホストの非実質細胞はHGFなどの肝細胞増殖促進因子を分泌し，ヒト肝細胞の増殖を促す．ホスト肝細胞から分泌されるuPAは，増殖しようとしているドナー肝細胞の周辺の細胞外基質を分解するであろう．これによって生じたスペースを利用してドナー肝細胞はその数を増やす．この過程を繰り返しながら，ドナーのヒト肝細胞は次第に正常肝を構築していくと考えられる．この置換の様子は，キメラマウスの肝臓切片を作製して，ヘマトキシリンによる一般組織染色とヒト特異的抗原染色を行なうことにより（図1），あるいは，肝臓のゲノムDNAに対して，ヒトおよびマウス細胞に特異的配列をPCR法で増幅して見ることができる．

ところが，ヒト肝細胞が分泌する補体系因子がホストの腎臓などの器官を攻撃して壊死させるためと思われるが，ヒト肝細胞がある程度以上増殖するとホストが死ぬ．筆者らは，移植後，ホストに補体因子の活性阻害物質を投与することによってこの問題を解決することができた[4]．高置換率のヒト肝細胞キメラマウスを得ることは，種々の要因が関係しており，かなり困難であるが，現在，筆者らは，70%以上の置換率をもつキメラマウスを50%の効率で作製可能な方法を確立できている．

以上の研究の結果，ヒト肝細胞は，異種動物の体内環境でも増殖して正常な肝臓組織を構築することができることが示された．このようなヒト肝細胞は，はたしてヒト体内環境で表現している正常な機能を発現しているのであろうか．この疑問に答えるために，筆者らは，薬物の代謝機能と肝炎ウイルス被感染性を調べた．薬物代謝に関与するチトクロームP450 (CYP) のうち代表的な6種類のサブメンバーのヒト遺伝子の発現パターンを調べたところ，移植に利用したドナーの発現パターンに酷似していた[4]．また，キメラマウスをリファンピシンあるいは3-メチルコラントレンで処理したところ，前者ではCYP3A4，後者ではCYP1A1とCYP1A2の遺伝子が特異的にその発現を高めていた[4]．これらのことから，キメラマウスでのヒト肝細胞は化合物特異的に正常な代謝

反応を行なっていると考えられる．また，BおよびC型ウイルス性肝炎の患者血清をキメラマウスの鎖骨下静脈から注入すると，マウス血清中のこれらウイルスタイターが増加すること，このとき，既知の抗ウイルス剤の投与を開始するとタイターが減少することがわかった(5)（一部未発表）．肝炎ウイルスは培養ヒト肝細胞では感染増殖しないので，これらの結果は，キメラマウスのヒト肝細胞の正常性を示している．

今後，ヒト肝細胞キメラマウスはヒト肝臓を有するモデル動物として，生物学，医学，医療，薬学，および創薬などの分野で利用価値が増大するものと考えられる．特に，次のような研究に関してはすでに活発な活動が始まっている．

①障害肝臓を，正常ヒト肝細胞を移植して治療することを目指す再生医療の方法開発と理論の構築のためのモデル研究

②医薬品候補化合物の代謝パターンの解析

③抗肝炎ウイルス剤の開発

筆者らは，今後，キメラマウスを肝炎，肝硬変，肝癌などの有効な病態モデル動物として利用するための研究を行なう予定である．

1) J. L. Heckel *et al.* : *Cell*, **62**, 447 (1990).
2) T. H. Kim *et al.* : *Hepatology*, **26**, 896 (1997).
3) S. Locaputo *et al.* : *Hepatology*, **29**, 1106 (1999).
4) C. Tateno *et al.* : *Am. J. Pathol.*, **165**, 901 (2004).
5) M. Tsuge *et al.* : *Hepatology*, **42**, 1046 (2005).

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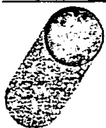

重要農作物を荒らす疫病菌の繁殖ホルモンをつきとめた

遺伝子多様化のチャンスを逃さない疫病菌の性の仕組み

疫病菌とは，植物に感染してしばしば甚大な被害を与える*Phytophthora*属の病原糸状菌の総称である．*Phytophthora*という属名はギリシャ語のphyton（植物）とphthora（破壊者）に由来することからも，たちの悪さが伺える．疫病菌は60種以上が知られており，その多くが植物病原性である．なかでもジャガイモとトマトを宿主にする*P. infestans*は，1840年代中ごろジャガイモを主食としていたアイルランドで蔓延し，約100万人の餓死者と100〜150万人のアメリカ移民を出す「アイルランド飢饉」をひき起こした(1)．このときのアメリカ移民の中にケネディ家の祖先も含まれていたといわれる．ジャガイモ疫病による経済的損失は世界で年間数十億ドルにのぼり，膨大な量の農薬が防除に用いられるため環境への影響が懸念されている．最近では，新種の疫病菌*P. ramorum*の感染による樹木の大規模な立枯れ（sudden oak death）が欧米で広がりつつある(2)．

疫病菌は，最近まで五界説でいう菌界（Fungi）に分類されていたが，カビやキノコとは異なる点が多く，今では原生生物界（Protista）に分類されている．菌糸状の外観（図1）からは想像しがたいが，珪藻や褐藻に近いらしい．疫病菌は，後で述べるように有性生殖において耐久性の「卵胞子」を形成することから卵菌類と呼ばれ，金魚の死骸や水中のスルメなどに生えるミズカビと同類である．

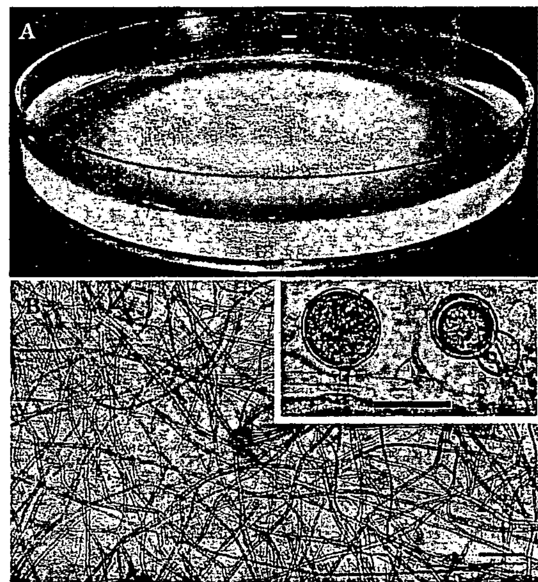

図1■疫病菌のすがた
(A) 寒天培地上のコロニー．(B) 菌糸および無性胞子（囲み左）と卵胞子（囲み右）．卵胞子は二重膜構造で小型球状の造精器を伴う．バーは50 μm

さて，疫病菌には植物病原菌という恐ろしい特徴に加え，有性生殖を営むという興味深い側面がある．有性生殖では菌糸から分化した造卵器と造精器が融合し，受精

【プロテオミクス研究とそれに注目した動機及びその後の発展について】

Proteomics: encounter and research progress with mass-spectrometer

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Oofusa　Ken　Yoshizato　Katsutoshi

Keywords
MS, 2D-PAGE, STAP

要旨

プロテオーム用質量分析装置の登場により，少量のタンパク質の同定作業が可能になったことで，2次元電気泳動の再評価が行われ，HPLCとならんでプロテオーム解析を支える柱の一つとなった。最初に導入した質量分析装置は場所取りで，ペプチドマスフィンガープリンティング(PMF)解析がメインであったが，基礎生物学の立場からタンパク質に焦点を当てながら研究するための強力なツールであった。その後の質量分析装置の改良に加えて，ヒトゲノムの解析完了につづき，マウスゲノムの解析も進行しており，医歯薬分野における質量分析装置を用いたプロテオーム解析プラットホームは確立された。これにより従来のタンパク質化学的解析手法が汎用化され，医療応用を可能にするブレークスルーを生み出すきっかけとなった。これにより，医療に関与する研究者によるタンパク質解析が広く行われ，今後の成果が期待されている。

1．タンパク質解析用質量分析装置登場以前

タンパク質解析用質量分析装置登場以前には，2次元電気泳動により発現するタンパク質の差異を解析しても，小さいスポットでは，エドマンシーケンサによるアミノ酸配列が決定できず，抗体を得ることも難しく，限定された用途にしか使われていなかった。このため生命現象の原因遺伝子あるいは遺伝子産物の解明は，相同・相似塩基配列を持つヌクレオチドプローブを用いたλgt10ライブラリのスクリーニング，あるいは，原因となるタンパク質を精製し抗体を得て，λgt11ライブラリ（発現ライブラリ）の抗体スクリーニングによってクローニングされてきた。しかし，これらのアプローチにおいても，入手できたプローブと目的遺伝子の配列類似性が低く擬陽性プラークが多いケースや，精製に手間と時間がかかるため原因となるタンパク質をcDNAが得られるより前の段階で，1つに絞り込む必要があった。そのため，真の原因タンパク質である確証が無い状態で研究資源を投入すべきか悩むケースが多かった。このような状況下では，精製されたタンパク質が目的遺伝子の産物かどうかも確認できない場合もあった。

2. プロテオーム用質量分析装置の実用化と2次元電気泳動の復権

プロテオーム用質量分析装置の登場により，少量のタンパク質の同定作業が可能になったことで，1970年代に既に確立されていた古い技術である2次元電気泳動の再評価が行われ，HPLCとならんでプロテオーム解析を支える柱の一つとなった。さらに，2次元電気泳動の技術も合成両性電解質とそれを利用した固定化pH勾配を用いたプレキャストゲル（IPGストリップ）の開発，実用

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化によって，再現性（繰り返し再現性と研究室間における再現性）が大きく改善されたことも再評価に大きく寄与している。

3. 筆者のラボで活躍した質量分析装置

筆者らの研究グループが最初に導入した質量分析装置はBruker Daltonics社のreflex Ⅲであった。セットアップのためにドイツから来訪した技術者は，理論上はデータベースに依存せず，内部アミノ酸配列情報が得られる「de novo sequence」も可能であるとの説明を行ったが，運用を始めると同装置で可能な解析は，ペプチドマスフィンガープリンティング（PMF)解析が主であった。また，当時はデータベースが充実しておらず，同定できないタンパク質も多くあった。さらに，設置室が居室と兼用になったため部屋が狭くなり動作音も大きく悩まされたスタッフも多かった。しかし，発生生物学，再生学，細胞生物学などの研究プロジェクトをタンパク質に着目して推進するためのきわめて有力なツールであることには偽りがなかった。

続いて，筆者らは，Micromass社のQ-Tofを運用する機会に恵まれた。この装置は，Q-TrapとTofを組み合わせることにより，MS/MSモードでの運用が可能であり，2次元電気泳動後のゲルより抽出したタンパク質のトリプシン消化ペプチドのアミノ酸配列をde novo sequencingによって決定することが可能であった。これにより，我々は多くのタンパク質の同定に成功した。その中には，肝星細胞の活性化に伴って増加するスポットに由来するタンパク質も含まれており，当時はこの配列がデータベースに収載されていなかったためSTAP(stellate cell activation-associated protein)と命名した。STAPは，酸素結合能を持つ第四のグロビンとして注目を浴びている。その生物学的機能は現在のところ不明であるが，臓器の線維化や酸素濃度のセンサーとして発生過程など種々の生命現象で重要な役割を果たしている可能性が高い。

4. プロテオーム解析プラットホームの確立

質量分析装置の改良と進歩に加えて，ゲノム解析の進行により，高感度にタンパク質同定が可能となり，研究対象の組織・細胞で発現しているタンパク質を網羅的に解析対象とする「プロテオミクス」が脚光を浴びるようになった。感度と速度の向上により，2次元電気泳動によってゲル上に分離展開された1000～数千のスポットの解析が可能になり，Protein-omicsつまり，定義に即したプロテオミクス解析が現実のものとなった。

ヒトゲノムの解析完了につづき，マウスゲノムの解析も進行しており，医歯薬分野における質量分析装置を用いたプロテオーム解析プラットホームは確立したと言って良く，同定作業はPMFで行える環境が整備されつつあるため，医用応用分野においては，感度よりコスト，操作性，あるいは解析速度を優先した質量分析装置が用いられるケースも多い。もちろん，ゲノムデータベースが整備されていない生物種を対象とした基礎生物学分野においては，PMF解析では同定が行えず，de novo sequencingによる同定作業が要求される場面も多く残されており，高感度化への要求も高く，超伝導状態においた高感度で広い質量レンジに対応したディテクター（検出器）の開発が行われている。

5. 網羅的解析から標的を絞った解析へ

タンパク質について，網羅的解析を突き詰めていくと，ゲノム情報に行き着く部分と，ゲノム情報には含まれていない部分が見えてくる。ゲノム情報に行き着く部分については，ゲノムDNAあるいはその翻訳産物であるRNAの解析が容易であり，かつハイスループットである。しかし，設計図であるDNAから中間体であるRNAを経て作られた部品としてのタンパク質は，転写と翻訳の過程を経た後に，酵素として働く他のタンパク質の作用によって完成品となるものがある。このプロセスは，翻訳後修飾と呼ばれ，リン酸化，酸化，糖鎖付加，及び脂質付加などが知られている。この中で特にリン酸化では，多くのプロテインキナ

ーゼやプロテインホスファターゼなどの酵素によって生じるタンパク質のリン酸化状態の変化が，細胞の分裂，分化，及びガン化などに深く関わっていることが知られており，多くの研究者の研究対象となっている。この翻訳後修飾に関して，ゲノム解析のみから得られる情報はきわめて限られており，実際のタンパク質の修飾状態を確認していく必要があり，研究者の期待を集めている解析手法である。

このニーズを満たすために，翻訳後修飾を解析するための機構が付加された質量分析装置が開発・販売されている。とくに，トリプシン消化ペプチドの翻訳後修飾部位を残したままでイオンを分離し，質量を計測するための新しい電場型トラップ（目的の質量数を持つイオンだけを一時的にためておくユニット）を用いたMSnが開発され，翻訳後修飾解析に力を発揮している。

プロテオーム用質量分析装置の登場は，従前のタンパク質化学的解析手法を汎用化し，医用応用を可能にするブレークスルーを生み出すきっかけとなった。これにより，本特集においても紹介するとおり，医療に関与する研究者によるタンパク質解析が広く行われ，その成果も得られている。今後のタンパク質解析のさらなる発展とその応用が社会にもたらす果実に期待したい。

<細　胞ニュース>

第14回　日本消化器関連学会週間（DDW-Japan）

下記日程で日本消化器関連学会（第48回日本消化器病学会大会　第72回日本消化器内視鏡学会総会
第10回日本肝臓学会大会　第44回日本消化器癌検診学会大会　第37回日本消化吸収学会総会）
が開催されます。
2006年10月11日（水）～14日（土）
会場：札幌コンベンションセンター・道立総合体育センター

シンポジウム（抜粋）
1.炎症と消化器発がん
2.消化管腫瘍の分子診断と標的治療
3.大腸癌に対する抗癌剤のシンポとQOL
4.肝移植後のウイルス肝炎再発対策
5.内視鏡でみた胃炎・十二指腸炎と腹部症状の関連
6.膵がん診療におけるトランスレーショナルリサーチの現状と展望　　他

招待講演（抜粋）
1.Multiple Pathways to Colorectal Cancer
2.Endoscopic diagnosis of esophageal columnar metaplasia or Barrett's esophagus
3.電子内視鏡時代の胃炎粘膜拡大分類
4.Function of Neonatal Fc receptor for IgG in mucosal immunity
5.消化器管腫瘍内視鏡 -現在と未来-
6.IBDにおける内視鏡の役割り -特にDALMの発見、治療について-　　他

特別講演（抜粋）
1.幹細胞生物学と再生医療への展開
2.診療情報のセキュリティと相互利用性 －デジタル内視鏡画像の真正性確保を中心に－
3.Helicobacter pyloriと胃癌；The final answer
4.私の内視鏡人脈記　－デジタルカメラ論をふくめて－
5.これからの日本のがん対策のあり方について
6.ニュートリゲノミクスを展望する　－食品の消化系調節機能の解析例を含めて－　　他

ワークショップ（抜粋）
1.ペグインターフェロン・リバビリン併用療法無効例の特徴と対策
2.トランスレーショナルリサーチの現況と問題点
3.肝疾患における酸化ストレス
4.思い出の症例：臨床の原点
5.膵胆道の早期癌：発見と治療のための戦略 8.胆膵画像診断の最先端
6.大腸がんの発育進展と治療　　他

表　題

著者名

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