7.4], 140 mM NaCl, and 0.5% (v/v) NP-40) followed by centrifugation for 2 min at 15,000*g*. The core-associated HBV genome was immunoprecipitated by mouse anti-core monoclonal antibody 2A21 (Institute of Immunology, Tokyo) and subjected to Southern blot analysis after SDS/proteinase K digestion followed by phenol extraction and ethanol precipitation. Quantitative analysis was performed by real-time PCR with cyber green using Light Cycler. The HBV-specific primers used for amplification were 5′-TTTGGGCATGGACATTGAC-3′ and 5′-GGTGAACAATGTTCCGGAGAC-3′. The amplification conditions included initial denaturation at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 15 sec, annealing at 58°C for 5 sec, and extension at 72°C for 6 sec. The lower detection limit of this assay was 300 copies.

Statistical Analysis

Data are expressed as mean SD. Group comparisons were performed using the Student's *t*-test. A *P*-value of less than 0.05 was considered statistically significant.

RESULTS

Patient's Profile

An ETV-resistant strain of HBV was isolated from a 44-year-old Japanese woman with hepatitis B e antigen-positive chronic HBV infection (Fig. 1A). In this patient, LAM successfully reduced the HBV at the initial stage of treatment. However, viral breakthrough was observed at 11 months after the beginning of LAM therapy and the HBV viral load reached up to 7.5 log copies/ml. After 17 months of LAM, interferon was added to LAM therapy for 6 months. However, after withdrawal of IFN, the viral load and ALT rebounded. Thus, the patient was switched to 0.5 mg of ETV. This resulted in reduction of HBV-DNA and normalization of ALT. After 12 months of ETV therapy, the viral load rebounded, and following 12 more months of ETV, breakthrough hepatitis was observed. After stopping ETV, because of the inadequate effect of IFN monotherapy for one month, the patient was switched to 10 mg of ADV. This treatment reduced both the viral load and ALT level to acceptable levels (Fig. 1).

treatment		month	ALT (IU/L)	HBV-DNA (log copies/ml)
		-3	246	7.2
LAM		0	46	5.2
		5	28	3.7
		11	33	4.1
	IFN	17	72	7.5
		18	1184	5.6
		20	39	3.9
		23	34	3.4
		27	117	7.1
ETV		31	112	7.2
		39	40	2.9
		43	28	4.2
	IFN	56	140	6.8
ADV		57	313	6.8
		60	38	4
	LAM	71	24	3.3
		75	19	3.1

Fig. 1. Clinical course of a patient who developed entecavir resistant mutant.

Isolation of a Multiple Drug-Resistant Hepatitis Strain

Isolates from this patient were analyzed for substitutions in HBV reverse transcriptase (RT). Comparison of the nucleotide sequences by the direct sequence method obtained throughout the clinical course showed three amino acid substitutions in the RT domain of the polymerase (Table I). At the baseline of LAM, all three substitutions were of the wild-type by direct sequence analysis and clonal analysis (Table II). After breakthrough hepatitis induced by LAM, direct sequence analysis showed mixed type (YIDD and YVDD) mutant strain. The rtM204V mutant was detected in 65% of HBV clones and the rest were all the YIDD type. Importantly, at this point, there was no amino acid substitution at rt202. After 12 months of ETV therapy when the viral load was slightly increased, the rtL180M + M204V + S202G mutant was detected in 45% of the HBV clones, followed by decrease of the YIDD and YVDD mutants without substitution at rtS202G. Finally, after 24 months of ETV therapy, when the breakthrough hepatitis occurred, the rtL180M + M204V + S202G mutant was detected in 92% of the HBV clones and the rest were rtL180M + M204V mutants without substitution at rtS202G. Interestingly, the rtM204I + S202G strain never appeared during nucleotide therapy.

Susceptibility of Mutants to Entecavir In Vitro

To analyze the role of the rtL180M, rtG202S, and rtM204V substitutions in ETV resistance, four patient-specific strains were transfected into HepG2 cells (Table III). ETV was added after 24 hr to the culture plate containing the cells, and harvested after 5 days. The core-associated HBV genome was extracted from cells and quantified by real-time PCR. The double amino acid substitutions rtL180M + M204V, which is related to LAM resistance, displayed a 38-fold decrease in susceptibility to ETV compared with the wild-type. Moreover, triple amino acid substitutions rtL180M + M204V + S202G, isolated from the patient

TABLE I. Direct Sequence Analysis of Samples From Our Patient With Entecavir (ETV) Resistance

	rt L180	rt S202	rt M204
(1) At the beginning of LMV	—	—	—
(2) At the beginning of ETV	L/M	—	I/V
(3) One year after ETV	M	G/S	V
(4) Two years after ETV	M	G	V

LMV, lamivudine.

who developed breakthrough hepatitis during ETV therapy, induced 198 times greater resistance than the wild-type. In agreement with the above data, the appearance of the rtS202G substitution in the rtL180M + M204V mutant strain resulted in a fivefold decrease in ETV susceptibility. On the other hand, only a single amino acid substitution rtS202G, which was artificial and did not truly exist, had little effect on the susceptibility to ETV (Table III, Fig. 3).

Susceptibility of Mutants to Lamivudine and Adefovir In Vitro

The susceptibility of the rtL180M + M204V and rtL180M + M204V + S202G mutants to LAM was also analyzed using transient transfection assay with HepG2 cells. Both strains displayed strong resistance to LAM (>1,000-fold). We also examined whether ADV was as effective against the rtL180M + M204V + S202G mutant strain as the wild-type. The IC_{50} values of the mutant strain and wild-type for adefovir were almost the same, which displayed the same result in vivo (Fig. 2, Table IV).

DISCUSSION

The present study describes the identification of an ETV-resistant strain of HBV after prolonged ETV therapy in a patient who was resistant to LAM therapy. Using direct sequencing and clonal analysis, the results demonstrated that the addition of rtS202G mutation to the LAM-resistant mutant strain correlated with the ETV-resistance. To our knowledge, this is the first report of a patient who developed not only virologic breakthrough but also biochemical breakthrough, followed by successful treatment with ADV (Fig. 1).

Clonal analysis showed mixed type of LAM-resistant strains at the commencement of ETV treatment. All of the rtM204V mutant strains were accompanied by rtL180M mutation, but none of the rtM204I mutant did. After 1 year of ETV therapy, the rtL180M + M204V + S202G mutant emerged in 45% of the HBV clones. Furthermore, almost all clones became the rtL180M + M204V + S202G variant 2 years after ETV therapy. These results suggest two important things. Firstly, the addition of the rtS202G mutant to the rtM204V mutant induced the ETV resistance. Secondly, the S202G was induced only in the mutant strains with rtM204V not in the rtM204I.

The in vitro study described in this article demonstrated that the rtL180M + M204V mutation reduced the susceptibility to ETV by 38-fold compared with wild-type (Table III). Furthermore, the addition of the rtS202G substitution to the rtL180M + M204V mutant strain resulted in a fivefold decrease in ETV susceptibility. Interestingly, the single S202G substitution did not induce ETV resistance in vitro. Thus, it appears that the rtS202G substitution never reduced the susceptibility to ETV in the absence of rtM204V substitution. The amino acid substitutions rtS202G have been reported to emerge with resistance against ETV [Yim et al., 2006; Tenney et al., 2007; Villet et al., 2007]. In all previous studies, the rtS202G mutation was accompanied by rtM204V substitution and our results are similar to those of the reported in vitro studies. It is known that other amino acid substitutions, rtT184 and rtM250 in the RT domain are associated with ETV resistance and they also need the substitution at rt204 to achieve such resistance. Tenney et al. [2004] reported that the rates of T184, S202, and M250 mutations in LAM-resistant patients before ETV treatment were 5.2%, 1.2%, and 1.8%, respectively. Moreover, these ETV-resistance-related residues emerged in 6% more patients by 1-year ETV therapy and 8% more patients by 2-year therapy.

TABLE II. Clonal Analysis of Samples From the Patient With Entecavir (ETV) Resistance

	Relative rate (%) of clones (no. of clones/total)			
	Wild	M204I	L180M + M204V	L180M + M204V + S202G
(1) At the beginning of LMV	100 (6/6)	0	0	0
(2) At the beginning of ETV	0	35 (7/20)	65 (13/20)	0
(3) 12 months after ETV	0	14 (3/22)	41 (9/22)	45 (10/22)
(4) 24 months after ETV	0	0	8 (1/13)	92 (12/13)

LMV, lamivudine.

TABLE III. In Vitro Susceptibility of rtL180/rtM204/rtS202 Mutants to Entecavir

				ETV	
	rt L180	rt M204	rt S202	IC_{50} (μM)	Resistance (fold)
Wild	—	—	—	0.00081	1
S202G	—	—	G	0.00054	0.67[a]
L180M + M204V	M	V	—	0.031	38**
L180M + M204V + S202G	M	V	G	0.16	198**

Experiments were performed in triplicates.
[a]NS, not significant.
**$P < 0.001$ compared with the wild-type.

In the present study, clonal analysis showed the rtS202G substitution was induced only in the mutant strains with rtM204V but not in the rtM204I, as described recently [Yim et al., 2006; Tenney et al., 2007; Villet et al., 2007]. A recent study demonstrated similar results; all 16 patients with virologic rebounds with ETV resistance had the rtM204V substitution, either alone or in combination with rtM204I substitution [Tenney et al., 2007]. Ono et al. [2001] reported that the clinical frequency of LAM-resistant mutants was 18.6% for the rtM204I, 1.4% for the rtM204V, 11.4% for the rtL180M + M204I, and 64.3% for the rtL180M + M204V. In other words, most of the YVDD mutants were accompanied with rtL180M mutation. On the other hand, only about one-third of YIDD mutants were accompanied with rtL180M. Previous in vitro studies demonstrated that both the rtM204I and rtL180M + rtM204V substitutions had incomplete cross-resistance to ETV, and reported that the rtL180M + rtM204V mutant was more susceptible than the rtM204I mutant. The replication capacity of the rtL180M + rtM204V was four-times larger than the rtM204I mutant [Ono et al., 2001]. Thus, it was considered that the addition of rtS202G substitution to the rtL180M + rtM204V mutant could strengthen the replication ability, or could reduce susceptibility to ETV more strongly than the rtM204I mutant. Further studies are needed to confirm the above hypothesis.

There is no consensus regarding the management of patients with ETV resistance. There are few reports of successful treatment of ETV resistant viruses in vivo. Villet et al. [2007] reported that ADV was clinically effective for virological breakthrough caused by ETV-resistant HBV variant. However, different from the previous report, the present study demonstrated the emergence of biochemical breakthrough after viral rebound caused by ETV resistance. Moreover, it was confirmed that ADV was effective in not only viral breakthrough but also biochemical breakthrough. Our in vitro study also indicated that the rtL180M + M204V + S202G mutant had no resistance against ADV. This result is compatible with the response in vivo. In this regard, recent studies demonstrated that ADV and tenofovir are effective for ETV-resistance in vitro and that ADV was definitely effective against other ETV-related amino acid substitutions S184 and M250 in vitro [Tenney et al., 2007; Villet et al., 2007]. However, the clinical effect has never been reported.

In conclusion, the present study showed that virological and biochemical breakthrough due to ETV could occur in patients infected with LAM-resistant HBV. It was confirmed that the addition of rtS202G substitution to the rtM204V mutant strain is responsible for ETV resistance and the resistant mutant could be treated successfully. While ETV resistance is rare in treatment-naïve patients, the amino acid substitution associated with ETV resistance is similar to the substitution seen in patients with LAM-resistance. Thus, it is considered that the successful salvage therapy described in this study could be a potentially helpful for similar events during ETV therapy. The possibility of emergence of novel mutants resistant to

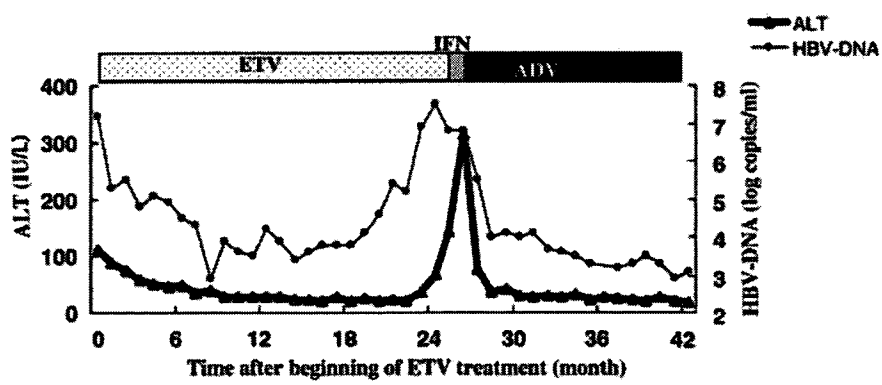

Fig. 2. Clinical course of a patient who developed breakthrough during entecavir therapy.

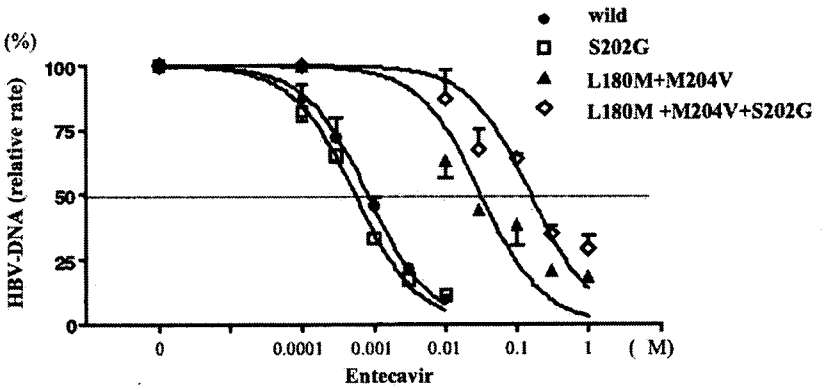

Fig. 3. In vitro analyses of susceptibilities of wild-type HBV and three mutants (rtS202G, rtL180M + M204V, rtL180M + M204V + S202G) to entecavir (ETV) after transient transfection into HepG2 cells. Cells were transiently transfected with plasmids containing 1.4 genome lengths HBV and treated with the indicated amount of entecavir. Data are the dose–response curves of the four HBV strains against entecavir. The strains were used to estimate the entecavir IC_{50} values for each HBV strains. Values are relative to no entecavir treatment controls for each strain. Experiments were performed in triplicates.

TABLE IV. In Vitro Susceptibility of rtS202/rtM204 Mutant to Lamivudine (LAM) and Adefovir (ADV)

	LAM		ADV	
	IC_{50} (μM)	Fold resistance	IC_{50} (μM)	Fold resistance
Wild	0.1	1	0.39	1
L180M + M204V	>100	>1,000**	—	—
L180M + M204V + S202G	>100	>1,000**	0.32	0.82[a]

Experiments were performed in triplicates.
[a]NS, not significant.
**$P < 0.001$ compared with the wild-type.

multiple anti-HBV drugs is real. Therefore, further studies are necessary to develop safes and more useful treatment strategies.

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REFERENCES

Allen MI, Deslauriers M, Andrews CW, Tipples GA, Walters KA, Tyrrell DL, Brown N, Condreay LD. 1998. Identification and characterization of mutations in hepatitis B virus resistant to lamivudine. Lamivudine Clinical Investigation Group. Hepatology 27:1670–1677.

Bruix J, Llovet JM. 2003. Hepatitis B virus and hepatocellular carcinoma. J Hepatol 39:S59–S63.

Colonno RJ, Rose R, Baldick CJ, Leveine S, Pokornowski K, Yu CF, Walsh A, Fang J, Hsu M, Mazzucco C, Eggers B, Zhang S, Plym M, Klesczewski K, Tenney DJ. 2006. Entecavir resistance is rare in nucleoside naive patients with hepatitis B. Hepatology 44:1656–1665.

Delaney WE, Yang H, Westland CE, Das K, Arnold E, Gibbs CS, Miller MD, Xiong S. 2003. The hepatitis B virus polymerase mutation rtV173L is selected during lamivudine therapy and enhances viral replication in vitro. J Virol 77:11833–11841.

Ganem D, Prince AM. 2004. Hepatitis B virus infection–natural history and clinical consequences. N Engl J Med 350:1118–1129.

Gunther S, Sommer G, Von Breunig F, Iwanska A, Kalinina T, Sterneck M, Will H. 1998. Amplification of full-length hepatitis B virus genomes from samples from patients with low levels of viremia: Frequency and functional consequences of PCR-introduced mutations. J Clin Microbiol 36:531–538.

Lai CL, Chien RN, Leung NW, Chang TT, Guan R, Tai DI, Ng KY, Wu PC, Dent JC, Barber J, Stephenson SL, Gray DF. 1998. A one-year trial of lamivudine for chronic hepatitis B. Asia Hepatitis Lamivudine Study Group. N Engl J Med 339:61–68.

Lai CL, Dienstag J, Schiff E, Leung NW, Atkins M, Hunt C, Brown N, Woessner M, Boehme R, Condreay L. 2003. Prevalence and clinical correlates of YMDD variants during lamivudine therapy for patients with chronic hepatitis B. Clin Infect Dis 36:687–696.

Lee YS, Suh DJ, Lim YS, Jung SW, Lee HC, Chung YH, Lee YS, Yoo W, Kim SO. 2006. Increased risk of adefovir resistance in patients with lamivudine-resistant chronic hepatitis B after 48 weeks of adefovir dipivoxil monotherapy. Hepatology 43:1385–1391.

Levine SD, Hernandez G, Yamanaka S, Zhang R, Rose R, Weinheimer S, Colonno RJ. 2002. Efficacies of entecavir against lamivudine-resistant hepatitis B virus replication and recombinant polymerases in vitro. Antimicrob Agents Chemother 46:2525–2532.

Maecellin P, Asselah T. 2005. Resistance to adefovir: A new challenge in the treatment of chronic hepatitis B. J Hepatol 43:920–923.

Nevens F, Main J, Honkoop P, Tyrrell DL, Barber J, Sullivan MT, Fevery J, De Man RA, Thomas HC. 1997. Lamivudine therapy for chronic hepatitis B: A six-month randomized dose-ranging study. Gastroenterology 113:1258–1263.

Ono SK, Kato N, Shiratori Y, Kato J, Goto T, Schinazi RF, Carrilho FJ, Omata M. 2001. The polymerase L528M mutation cooperates

with nucleotide binding-site mutations, increasing hepatitis B virus replication and drug resistance. J Clin Invest 107:449–455.

Rapti I, Dimou E, Mitsoula P, Hadziyannis SJ. 2007. Adding-on versus switching-to adefovir therapy in lamivudine-resistant HBeAg-negative chronic hepatitis B. Hepatology 45:307–313.

Sherman MC, Yurdaydin C, Sollano J, Silva M, Liaw YF, Cianciara J, Boron-Kaczmarska, A, Martin P, Goodman Z, Colonno R, Cross A, Denisky G, Kreter B, Hindes R. 2006. Entecavir for treatment of lamivudine-refractory, HBeAg-positive chronic hepatitis B. Gastroenterology 130:2039–2049.

Suzuki Y, Kumada H, Ikeda K, Chayama K, Arase Y, Saitoh S, Tsubota A, Kobayashi M, Koike M, Ogawa N, Tanikawa K. 1999. Histological changes in liver biopsies after one year of lamivudine treatment in patients with chronic hepatitis B infection. J Hepatol 30:743–748.

Tenney DJ, Levine SM, Rose RE, Walsh AW, Weinheimer SP, Discotto L, Plym M, Pokornowski K, Yu CF, Angus P, Ayres A, Bartholomeusz A, Sievert W, Thompson G, Warner N, Locarnini S, Colonno RJ. 2004. Clinical emergence of entecavir-resistant hepatitis B virus requires additional substitutions in virus already resistant to Lamivudine. Antimicrob. Agents Chemother. 48:3498–3507.

Tenney DJ, Rose RE, Baldick CJ, Levine SM, Pokornowski KA, Walsh AW, Yu CF, Zhang S, Mazzucco CE, Eggers B, Hsu M, Plym MJ, Poundstone P, Yang J, Colonno RJ. 2007. Two-year assessment of entecavir resistance in Lamivudine-refractory hepatitis B virus patients reveals different clinical outcomes depending on the resistance substitutions present. Antimicrob Agents Chemother 51:902–911.

Villet S, Ollivet A, Pichoud C, Barraud L, Villeneuve JP, Trepo C, Zoulim F. 2007. Stepwise process for the development of entecavir resistance in a chronic hepatitis B virus infected patient. J Hepatol 46:531–538.

Yim HJ, Hussain M, Liu Y, Wong SN, Fung SK, Lok SF. 2006. Evolution of multi-drug resistant hepatitis B virus during sequential therapy. Hepatology 44:703–712.

Original article

Earthworm fibrinolytic enzyme: anti-tumor activity on human hepatoma cells *in vitro* and *in vivo*

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Keywords: earthworm; fibrinolysin; liver neoplasm; mice, nude; apoptosis,

Background The earthworm fibrinolytic enzyme (EFE) is a complex protein enzyme that is widely distributed in the earthworm's digestive cavity. Possessing strong protein hydrolysis activity, EFE not only has a direct effect on fibrin, but also can activate plasminogen. Its therapeutic and preventative effects on thrombosis-related disease have been confirmed clinically. Recently, there has been increased interest in the anti-tumor activity of EFE. In this study, the anti-tumor activity of EFE, isolated from *Eisenia foetida*, on human hepatoma cells was evaluated *in vitro* and *in vivo*. The potential mechanisms involved were also studied.

Methods *In vitro* experiments were performed in four human hepatoma cell lines: HLE, Huh7, PLC/PRF/5 and HepG2. After treatment with EFE in various concentrations, the inhibition of the rate of cell proliferation was measured. For the *in vivo* studies, tumor-bearing models xenografted with Huh7 cells were developed in nude mice, and then the mice were fed with EFE once a day for 4 weeks, and the control group received only saline. An inhibitory effect on tumor growth was observed. Also, apoptosis was observed with flow cytometric assay and fluorescent dye staining with acridine orange and ethidium bromide (AO/EB). The expression of matrix metalloproteinase 2 (MMP-2) were detected by Western blotting assay.

Results After treatment with various concentrations of EFE, the proliferation of all hepatoma cell lines was suppressed to varying degrees *in vitro*. The IC_{50} for HLE, Huh7, PLC/PCF/5 and HepG2 were 2.11, 5.87, 25.29 and 17.30 uku/ml, respectively. After administration of EFE orally for 4 weeks, the growth of tumor xenograft of Huh7 cells in nude mice was significantly inhibited *in vivo*. The tumor inhibitory rates in the EFE 500 uku/(kg·d) and 1000 uku/(kg·d) groups were 46.08% (compared with control group, P=0.026) and 57.52% (compared with control group, P=0.002) respectively. Meanwhile, the average weight of body, spleen or thymus did not show any remarkable differences among the various groups. The population in sub-G_1 stage was more in the EFE treated groups than in the control group according to flow cytometric assay. After treatment with EFE 0, 5, 10 uku/ml for 72 hours, the apoptotic rates were 3.5%, 10.9% and 12.3% in HLE cells, and 5.0%, 24.7% and 34.5% in Huh7 cells respectively. Under fluorescent staining with AO/EB, the apoptotic morphological changes could be detected more significantly in the EFE treated groups than in the untreated groups. After treatment with EFE in doses of 0, 5, 10 uku/ml for 72 hours, the apoptotic rates were 3.02%, 8.76%, 10.54% in HLE cells, and 3.95%, 18.27%, 30.89% in Huh7 cells respectively. The apoptosis-inducing effects of EFE occurred in a dose dependent manner. Western blotting assay showed that, after treatment with EFE, the secretions of MMP-2 were significantly inhibited in HLE and Huh7 cells.

Conclusions EFE showed significant anti-tumor activity in hepatoma cells both *in vitro* and *in vivo*, which may be because EFE could induce apoptosis of hepatoma cells and inhibit the expression of MMP-2. This suggests that EFE has a potential role in the treatment of hepatoma.

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Hepatocellular carcinoma (HCC) is the fifth most common cancer and the third leading cause of cancer related mortality worldwide.[1,2] With recent advances in medical techniques and imaging modalities, hepatologists can detect small HCC less than 2 cm in diameter and treat the disease with local ablation modalities, such as percutaneous ethanol injection, microwave coagulation therapy, and radio frequency ablation. In advanced cases for which non-surgical treatments are indicated, transarterial chemoembolization (TACE) is an effective therapeutic modality. Although these various therapeutic procedures have decreased the mortality rate of this disease, more effective therapies are still warranted, especially for cases of metastatic disease. The chemotherapeutic regimens that are currently in use are either not effective enough to destroy the

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cancer cells or, alternatively, result in significant side effects, such as bone marrow suppression, gastrointestinal mucosal damage and neuropathy. Thus, it is necessary to find new promising therapeutic agents. .

Earthworms have been widely used in traditional Chinese medicine for thousands of years. However, it is only during the past few decades, with the development of biochemical technologies, that research on the pharmaceutical effects of earthworms has been initiated. Fibrinolytic enzymes were first isolated from earthworms in 1980's,[3-5] and, since that time, the medical value of earthworms has been given much more consideration. It was found that earthworm extracts could significantly diminish the coagulation of platelets and promote the dissolution of thrombi in the blood. Its therapeutic and preventive effects for thrombosis-related disease have been confirmed clinically. In fact, earthworm extracts had been clinically made into medications for the treatment of thrombosis-related disease in China. Recently earthworm extract was found to have an anti-tumor effect.[6-10] In our previous studies, we found that a relatively crude protein preparation extracted from whole earthworm tissue of *Eisenia foetida*, named No.2 of earthworm extraction (EE2), could suppress tumor growth in mice inoculated with sarcoma 180 and Ehrlich ascites tumor.[11] Subsequent studies then demonstrated that earthworm fibrinolytic enzyme (EFE) was the main factor responsible for the observed anti-tumor activity.[12-14] EFE is a complex protein enzyme that is widely distributed in the earthworm's digestive cavity. Possessing strong proteinase activity, EFE not only has a direct effect on fibrin, but also can activate plasminogen. In this study, we evaluated the anti-tumor activity of EFE on the hepatoma cells both *in vitro* and *in vivo*.

METHODS

Test product

The sample of earthworm fibrinolytic enzyme (EFE) was provided by the Institute of Immunology and Biochemistry, Nanjing Agricultural University, China. The earthworm (*Eisenia foetida*) was washed and homogenized, then extracted by normal saline and salted out by low concentrations of $(NH_4)_2SO_4$. The super extracted solution was ultrafiltrated to remove the low molecules and concentrate. Then gel chromatography was performed in Sephadex G75, and the second peak with fibrinolytic activity was collected and ultrafiltrated. The concentrated protein solution was subjected to column chromatography by use of DEAE-Cellulose and eluted by the solution of sodium chloride with a graduated electric conductivity. Five active peaks were obtained. The fibrinolytic active peaks were collected, ultrafiltrated and freeze-dried. The sample of EFE was white freeze-dried powder with fibrinolytic activity 320 uku (urokinase unit)/mg, and stored at 4°C for further use. Before use in the subsequent *in vitro* or *vivo* experiments, the EFE solutions were filtered through syringe filter.

Tumor cell lines

Three human HCC cell lines HLE, Huh7, and PLC/PRF/5, and one human hepatoblastoma cell line (HepG2) were purchased from Health Science Research Resources Bank (Osaka, Japan). The cells were cultured as monolayers in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, USA) supplemented with 10% fetal bovine serum (FBS, Sigma, USA), 100 U/ml penicillin and 0.1 mg/ml streptomycin in a humidified atmosphere of 5% CO_2 at 37°C. Cells were subcultured using 0.25% trypsin (Sigma, USA) twice a week.

Nude mice

Male BALB/c AnNCrj-nu nude mice, 5 weeks old, were provided by and maintained in the Institute of Laboratory Science of Hiroshima University, Japan. The mice were kept in a specific pathogen-free facility with well-controlled light-dark cycles, temperature and humidity. The cages, bedding, feed, and water were autoclaved prior to use. Housing was in groups of 5 mice per cage. The study received prior institutional approval and the number of license was F04070025.

Cell proliferation assay

Cells were seeded into 96-well plates (Falcon, Becton Dickinson, USA) at 5×10^4 for HLE, Huh7 and 5×10^5 for PLC/PRF/5 and HepG2 respectively. After incubation for 24 hours, the medium was changed with new medium containing various concentrations of EFE. The final concentrations of EFE in the medium ranged from 1.25 uku/ml to 40.0 uku/ml. Four wells were prepared for each concentration and an additional four wells without EFE were used as controls. The plates were incubated in 5% CO_2 incubator at 37°C for 72 hours. Then, a cell proliferation assay kit (Chemicon, USA) was used to analyze the growth of cells. After the WST-1/ECS solution was added into each well, cells were incubated for an additional 2 hours. Subsequently the absorbance of the medium in each well were measured with a spectrophotometer at 450 nm.

Tumor xenograft in nude mice

Totally 30 nude mice were used for the experiment. Human HCC cells Huh7 cultured *in vitro* were harvested and resuspended in DMEM and 1×10^7 cells in 0.2 ml of DMEM were inoculated subcutaneously into the backside of right hind limb. Thirty inoculated mice were randomly divided into three groups: low-dose EFE, high-dose EFE and a negative control group. The next day after inoculation, EFE was administered orally at a dose of 500 uku/(kg·d) or 1000 uku/(kg·d) in the low-dose and high-dose group, once a day, for 4 weeks, while the mice in negative control group received only saline. During this period, growth time and tumor size were measured once a week by determining two perpendicular dimensions. Then the volume of the tumor was calculated by the formula, $volume=1/2\times length\times width^2$.[15] Twenty-four hours after the final administration of EFE on the 28th day of the experiment, all the mice were

sacrificed under anesthesia induced by ether and the tumor tissues were removed in their entirety. The weights of tumor, spleen and thymus were recorded. The inhibitory rates of tumor growth were calculated as: Inhibitory rate=(1 − average tumor weights in the experimental group / average tumor weights of control group) ×100%. Tumor tissues were kept in formalin or at −80°C for further analysis.

Flow cytometric assay

For HCC cell lines HLE and Huh7, after being subcultured and allowed to adhere by incubating for 24 hours, the medium were changed to a medium containing EFE, and then cells were treated for 72 hours. The final concentrations of EFE were 10 uku/ml, 5 uku/ml respectively and 0 uku/ml for the control group. Two dishes were prepared for each concentration. The cells were incubated in 5% CO_2 at 37°C. After 72 hours, the adhesive cells and suspended cells were harvested, pooled and pelleted. After washing with PBS, cells were treated with 70% ethanol for 1 hour. Then, after again washing with PBS, cells were incubated with staining buffer containing RNase A and propidium iodide (PI) for 1 hour. Cell cycle analyses were carried out with FACS Calibur (Becton Dickinson, USA). The tests were repeated three times.

Fluorescent dye staining

Acridine orange is a cell-permeable dye that intercalates into DNA and results in a green color change. Ethidium bromide enters cells with disrupted membrane integrity and intercalates into RNA and double-stranded DNA to appear orange. Thus, differential uptake and binding of these dyes allows us to identify cells in the early and late stages of apoptosis and necrosis. Under a fluorescent microscope, early apoptotic cells showed the presence of green patches of fragmented and condensed chromatin, and late apoptotic cells showed the presence of orange patches of fragmented and condensed chromatin. By comparison, viable cells were uniformly green and necrotic cells were uniformly orange. HCC cell lines HLE and Huh7 were exposed to 10 uku/ml, 5 uku/ml and 0 uku/ml of EFE for 72 hours as above. The adhesive cells and suspended cells were harvested, pooled, pelleted and resuspended in 200 μl medium. Then 8 μl of a mixture of fluorescent dyes containing 100 μg/ml acridine orange and 100 μg/ml ethidium bromide (AO/EB) was added to the cells and mixed gently. A drop of the mixture was placed on a microscope slide and covered with a coverslip. The cells were visualized under a fluorescent microscope (Nikon, Japan), using a blue filter. At least 200 cells were randomly counted in various fields. The percentage of apoptotic cells was calculated by following formula: The apoptotic rate (%)=(numbers of early apoptotic cells+numbers of late apoptotic cells)/ numbers of all the cells counted.[16,17] The tests were repeated three times.

Western blot analysis

HLE and Huh7 cells were starved in serum-free medium for 16 hours to minimize nonspecific induction of MMP. They were treated with EFE for 72 hours, and the medium was collected. Medium was concentrated by an ultrafiltration centrifugal concentrator (30-kD cutoff; Centricon-0; Amicon Beverly, MA) and adjusted to a final concentration ratio of 10:1. Equivalent volumes (40 μl) of medium were used for Western blot analysis (loaded onto 10% SDS-polyacrylamide gels followed by transfer to a nitrocellulose membrane). Then the metalloproteinase-2 (MMP-2, 72 kDa) was detected with a rabbit anti-MMP2 polyclonal antibody (diluted 1:1000; Biomol, USA). Horseradish peroxidase (HRP)-linked donkey-anti-rabbit antibody (diluted 1:1000; Amersham Biosciences, UK) was used as a secondary antibody. Protein-antibody complexes were detected by chemiluminescence and exposed to chemiluminescent detection films. Glyceraldehyde-3-phosphate dehydrogenase (GADPH) was used as an internal control.

Statistical analysis

Data were expressed as mean ± standard deviation (SD). Independent-sample *t* test was carried out using SPSS 11.0 for Windows software. $P<0.05$ was considered statistically significant.

RESULTS

The proliferation of liver cancer cell lines

In order to analyze the effect of EFE on hepatoma cells' growth, cell proliferation assays were performed for HLE, Huh7, PLC/PRF/5 and HepG2. Seventy two hours after EFE treatment in several concentrations, the inhibition of cell proliferation was recognized in all of the 4 types of hepatic cell lines (Fig. 1). The inhibition of cell growth was increased in proportion to the concentration of EFE, although the sensitivity to EFE was different for each cell line. For two of the four types of cells tested, the growth of HLE and Huh7 were inhibited significantly. As the concentration of EFE increased, the rate of cell proliferation decreased as a result of increased inhibition efficiency on cell growth. The IC_{50} for HLE and Huh7 were 2.11 uku/ml and 5.87 uku/ml respectively. While in other two types of HCC cell lines, PLC/PCF/5 and HepG2, the inhibitory effects were not as significant as that observed in HLE and Huh7, and the IC_{50} were 25.29 uku/ml and 17.30 uku/ml. For this reason, HLE or Huh7 cells were selected for further study.

The growth of liver cancer cell in nude mice

After the inoculation of HCC cells Huh7, the mice developed palpable tumors within 10 days and the tumors grew well in all of the nude mice during the experiment. The mass appeared as being elliptic or round. Two weeks after administration of EFE, the differences in the size of xenograft had already become apparent to some extent between EFE groups and controls (Fig. 2). With further administration of EFE, the differences became more notable. Table 1 presents the mean weights of tumors derived from the mice in each group four weeks after the

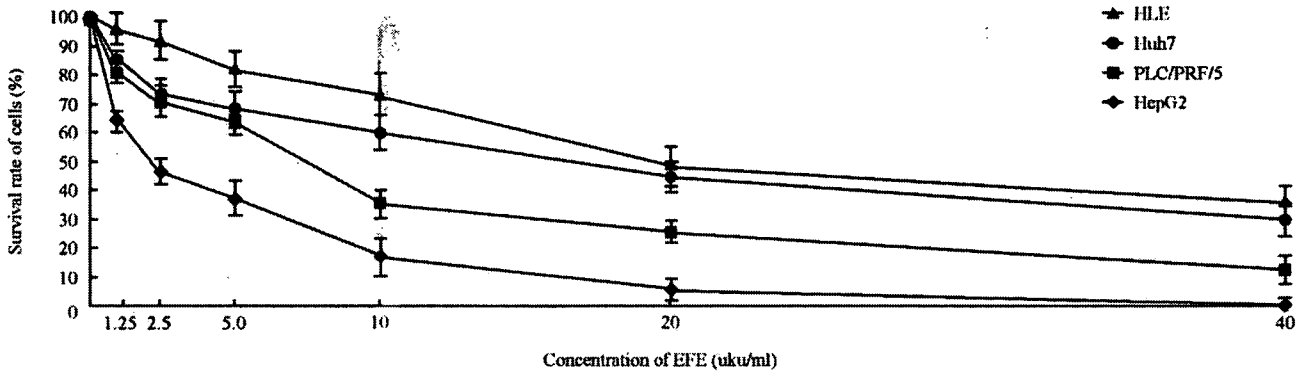

Fig. 1. Survival rate of hepatoma cells 72 hours after treatment with various concentrations of EFE. EFE could inhibit cell proliferation of all kinds of examined hepatoma cell lines in a dose-dependent manner.

Table 1. Inhibition of EFE on the growth of HCC cell lines Huh7 xenograft in nude mice

Groups	*n*		Weight of body (g)		Weight of tumor (g)	Inhibition rate of tumor growth (%)	Weight of spleen (mg)	Weight of thymus (mg)
	Initial	End	Initial	End				
Control	10	8	19.5±1.62	22.9±3.83	1.13±0.44	–	39.12±4.3	13.78±2.7
EFE 500 uku/(kg·d)	10	8	19.0±1.83	22.4±3.05	0.61±0.40 *	46.08	40.45±5.6 △	13.39±1.4 ▲
EFE 1000 uku/(kg·d)	10	8	19.7±1.79	23.2±2.48	0.48±0.24 #	57.52	38.63±4.8 ☆	14.21±2.1 ★

Compared with control group, * P=0.026; # P=0.002; △P=0.371; ☆P=0.494; ▲ P=0.562; ★ P=0.823.

administration of EFE. The growth of tumor was significantly suppressed in EFE group compared to the control group. The tumor inhibitory rates in EFE group at a dose of 500 uku/(kg·d) and 1000 uku/(kg·d) were 46.08% (compared with control group, P=0.026) and 57.52% (compared with control group, P=0.002), respectively.

During the experiment, the nude mice tolerated the treatments well. There were no significant differences in feed consumption, daily activity or increase of body weight among mice in the different groups. Furthermore, at the end of the experiment, the weight of spleen and thymus also did not shown significant differences among the 3 groups. These results indicated that EFE did not have remarkable side effects on either growth or the immune system.

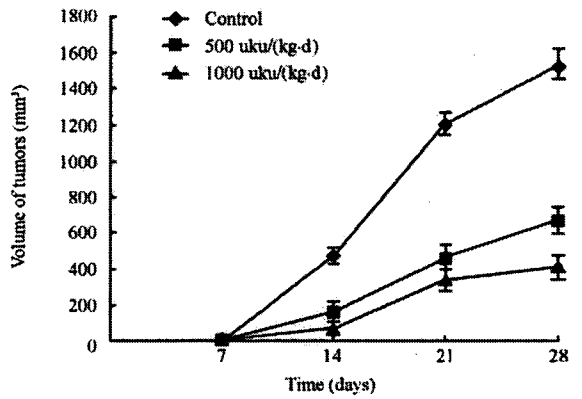

Fig. 2. Inhibitory effect of EFE on HCC cell lines Huh7 xenograft in nude mice. Two weeks after treatment with EFE, the differences of tumor mass volume between the negative control group and the EFE group were detectable at both concentrations. Four weeks after treatment, the differences were more remarkable. The inhibitory effects were dose-dependent.

Table 2. Cell cycles of Huh7 and HLE after treatment with EFE for 72 hours

Cells	Groups	Cell cycle (%)			
		sub-G_1	G_1	S	G_2/M
Huh7	Control	5.0	76.0	4.2	14.3
	EFE 5 uku/ml	24.7	61.5	4.8	8.8
	EFE 10 uku/ml	34.5	58.3	2.3	4.1
HLE	Control	3.5	46.5	3.6	41.4
	EFE 5 uku/ml	10.9	44.9	4.7	39.6
	EFE 10 uku/ml	12.3	41.7	3.4	40.4

Flow cytometric assay

Seventy-two hours after treatment with EFE, hepatoma cells were examined with flow cytometry to check the cell cycle status. The results showed that the proportion of cells in sub-G_1 stage, representing the apoptotic cells, was more in the EFE treated group than in the control group (Table 2). This indicated that EFE could induce HCC cell lines to undergo apoptosis. In Huh7 cells, the effect of EFE was significant. The proportion of cells in sub-G_1 stage was increased from 5.0% to 34.5% after treatment with 10 uku/ml of EFE. Meanwhile, the impact of EFE in HLE was not as strong; however, the proportion of sub-G_1 was raised from 3.5% to 12.3% after treatment with 10 uku/ml of EFE. The data represent a duplicate analysis reproduced three times.

Fluorescent dye staining

Cellular morphological changes were also investigated by AO/EB staining using fluorescence microscopy. Morphology was defined according to descriptions in references 16 and 17. After treatment with EFE for 72 hours, the cells were stained by AO/EB. The results demonstrated that apoptotic morphological changes could be detected in all groups (Fig. 3). Calculated by the formula given above, with doses of 0, 5, 10 uku/ml EFE treatment for 72 hours, the apoptotic ratio were 3.02%,

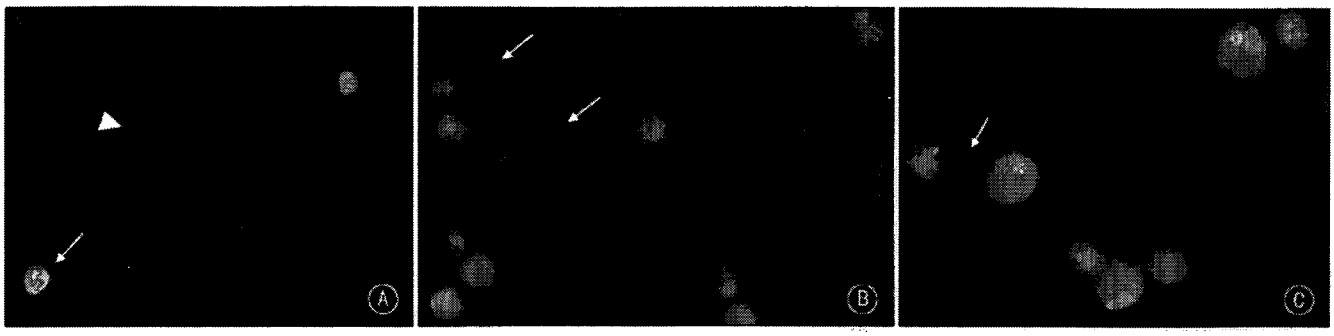

Fig. 3. Representative photomicrographs showing apoptosis of HLE cells under AO/EB staining. **A:** Early apoptotic cell (arrow), showing the presence of green patches of fragmented and condensed chromatin. Viable cells were uniformly green (arrowhead). **B:** Late apoptotic cells fluorescing orange (arrow). As the plasma membrane lost its integrity, EB entered the cell and intercalated into fragmented DNA, staining the cell red. **C:** Necrotic cell (arrow) that had lost its selective permeability, allowing EB to intercalate into DNA, produced a uniform red color. The Huh7 cell lines showed similar morphological changes (Original magnification ×20).

8.76%, 10.54% in HLE cell line, and 3.95%, 18.27%, 30.89% in Huh7 cell line, respectively. This result was corresponded with the results of flow cytometric assay. It could be seen clearly that there were increasing apoptosis-inducing effects with increases in the EFE concentration. This indicates that EFE induces apoptosis in a dose-dependent manner.

The expression of MMP-2

The expression levels of MMP-2 were detected by Western blotting analysis. The results showed that both of HLE and Huh7 cells could express MMP-2 protein. After treated with 5 uku/ml of EFE for 72 hours, the expression of MMP-2 decreased to an almost undetectable level in both cell lines (Fig. 4). This result suggests that EFE could significantly inhibit the expression of MMP-2 in HLE and Huh7 cells.

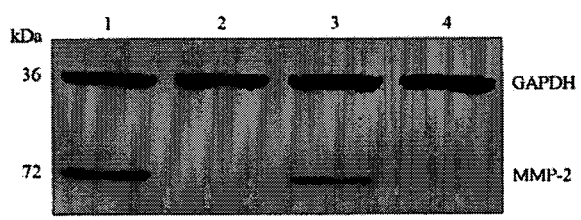

Fig. 4. Western blot result of MMP-2 secreted from HLE and Huh7 cells after treated with EFE for 72 hours. Lane 1: HLE cell, control; Lane 2: HLE cell, 5 uku/ml EFE. Lane 3: Huh7 cell, control; Lane 4: Huh7 cell, 5 uku/ml EFE. MMP-2 protein was visualized in both the control group of HLE and Huh7 cells. In the presence of EFE, MMP-2 secreted from both cell groups decreased to an almost undetectable level.

DISCUSSION

Animals have been used as medicinal resources for the treatment and relief of a myriad of illnesses and diseases in practically every human culture.[18] In China, the medicinal uses of earthworms have a history of thousands of years. *Compendium of Materia Medica*, written by Li Shi-zhen in 1578 AD, recorded the medicinal uses of earthworm. According to traditional Chinese medicine, earthworms possess antipyretic, antispasmodic, diuretic, antihypertensive, antiallergic, antiasthmatic, detoxifying, and spermatocidal effects.

In recent years, the pharmaceutical effects of earthworm medicines has received much more attention both in China and several other countries.[19-26] Apart from the anticoagulatory and fibrinolytic activities of earthworm medicines, studies also indicated that the coelomic fluid of earthworms exhibits other biological functions, including bacteriostatic, proteolytic, cytolytic (hemolytic) and mitogenic activities.[25,26] It was found that certain molecules from the coelomic fluid inhibited bacterial growth and lyse different mammalian red blood cells.[22-24] Lately, attention has focused on the earthworm's anti-tumor activity. There have been numerous articles demonstrating that certain components extracted from earthworms possess anti-tumor effects.[6-10,19-21] However, due to the different extracting methods used by various researchers, the ingredients derived from earthworm varied significantly in different studies. The protein component responsible for the observed anti-tumor effect remains unknown.

However, our previous research indicated that EE2, isolated from whole earthworm tissue of *Eisenia foetida*, could suppress tumor growth *in vivo*.[11] After administration of EE2 in doses of 2000 uku/(kg·d) and 4000 uku/(kg·d) for 7 days, the inhibitory rates in the xenograft of Sarcoma 180 (S180) in mice were 43.08% and 50.33%, respectively; the life span for Ehrlich ascites tumor-bearing mice increased by 30.30% and 42.42% respectively. By separating EE2 into 3 different components and examining each component separately, it was determined that EFE was responsible for the anti-tumor effect. Using the MTT assay, it was found that EFE could inhibit the proliferation of several cancer cell lines *in vitro*, including gastric cancer cell line SCG7901, esophagus cancer cell line Eca-109, cervical cancer cell line Hella, leukemia cell line K562, among others.[12,13] The results suggested that the anti-tumor spectrum of EFE was relatively wide.

In this study, using a cell proliferation assay, a more sensitive and convenient method than MTT for calculating the cell proliferation rate *in vitro*, it was found that EFE could also inhibit the proliferation of hepatoma cells, including human HCC cell lines HLE, Huh7, PLC/PRF/5 and human hepatoblastoma cell line HepG2. For the *in vivo* study, after inoculating Huh7 cells to nude mice and administering EFE at doses of 500 uku/(kg·d) or 1000 uku/(kg·d) for 4 weeks, the inhibitory rate of tumor growth were increased 46.08% and 57.52% respectively compared to the control group. Furthermore, there were no remarkable differences in the body weight, spleen weight and thymus weight among the various groups at the end of experiment. The results showed that EFE possesses significant anti-tumor effects in hepatoma cells; the side effect was not remarkable.

The mechanisms of the anti-tumor effects of earthworm extracts are still unknown. Some studies found that it could significantly enhance the function of phagocytic function of macrophages in abdominal cavity of tumor-bearing mice. Therefore, one possible mechanism may relate to enhancement in macrophage activity.[7] Studies also found that earthworm extracts could activate the catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) in mice serum.[9] It is possible that the anti-tumor effects of earthworm extracts may relate to anti-oxidative benefits and the scavenging of oxygen free radicals. In our experiments, through flow cytometric assay and fluorescent dye staining, we found that EFE could induce HCC cells to undergo apoptosis. We considered that this is another important mechanism underlying the anti-tumor activity of EFE.

The ability to invade surrounding tissues and metastasize from the primary site to one or more distant sites through blood vessels is an important characteristic of cancerous cells. It has been widely believed that the adhesion of cancerous cells to the basement membrane, the degradation of extracellular matrix (ECM) and the transmission of cells in the blood are three crucial steps in the progress of tumor invasion and metastases. MMPs have been closely linked with the invasive and metastatic phenotype of cancer cells. MMPs are a family of zinc-dependent endopeptidases that selectively degrade or remodel most of the extracellular matrix (ECM) components of tumor tissues including collagen and other structural molecules.[27] The enzyme activity is regulated extracellularly and its regulation is mainly based on the balance between pro-enzyme activation and inhibition by tissue inhibitors of MMPs (TIMPs). MMPs, specifically MMP-2 (gelatinase A) and MMP-9 (gelatinase B) are responsible for degradation of type IV and V collagen, elastin and fibronectin. MMP-2 is constitutively expressed and participates in the remodeling of the ECM, promoting the angiogenesis in the tumor tissue and facilitates the invasion and metastasis. Our study indicated that EFE could inhibit the expression of MMP-2 of HLE and Huh7 cells, so it is reasonable to assume that EFE may inhibit the invasion and metastasis of hepatoma.

In summary, our studies demonstrated that EFE possesses a significant anti-tumor activity in some of hepatoma cells both *in vitro* and *in vivo*, and could induce these cells to undergo apoptosis. It also results in decreased secretion of MMP-2 from these cells. The results indicate that EFE, which is readily available, can be extracted at a minimal cost and has almost negligible side effects, is a promising pharmacological agent.

It has been apparent for many years that the coagulation system augments metastasis. The inhibition of coagulation could drastically reduce metastasis.[28-30] As an anti-coagulation medicine, the possible relationship of EFE and anti-metastasis merits further study.

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REFERENCES

1. Ahn J, Flamm SL. Hepatocellular carcinoma. Dis Mon 2004; 50: 556-573.
2. Sherman M, Takayama Y. Screening and treatment for hepatocellular carcinoma. Gastroenterol Clin North Am 2004; 33: 671-691.
3. Mihara H, Sumi H, Akazawa T, Yineda T, Mazumoto H. Fibrinolytic enzyme extracted from the earthworm. Thromb Haemostas 1983; 50: 258-263.
4. Lu YH, Jin RC, Wu YW. Separation and purification of fibrinolytic enzyme from earthworm. J Biochem (Chin) 1988; 4: 166-172.
5. Lin SQ, Yu P, Lan RF. Application of affinity chromatography for purification of fibrinolytic enzyme from earthworm. Pharm Biotech (Chin) 2000; 7: 229-233.
6. Xie JB, He WG, Weng N, Guo ZQ, Yu MM, Liu N, et al. Extraction and isolation of the anti-tumor protein components from earthworm (eisenia fetida Andrei) and the anti-tumor activity. Chin J Bio Mol Biol 2003; 19: 359-366.
7. Hu YL, Xu M, Zhang SQ, Huang WH, Kang SH, Wang QX. Study of earthworm extracts on the immunologic activity and anti-tumor effects. Biotechnology (Chin) 2002; 12: 9-10
8. Yuan L, Xu JM, Zhou YC. Effects of the purified earthworm extracts on various hemal tumor cells. Clin Med J China (Chin) 2004; 11: 177-179
9. Lin SQ, Zhou KH. Affecting tumorigenic mouse immune function and antioxidase of QY-I from Earthworm. Strait Pharm J (Chin) 2002; 14: 9-11.
10. Zhao R, Ji JG, Tong YP, Chen Q, Pu H, Ru BG. Isolation and identification of proteins with anti-tumor and fibrinolysogen kinase activities from *Eisenia foetida*. Acta Biochim Biophys Sin (Chin) 2002; 34: 576-582.
11. Chen H. Anti-tumor effect of Earthworm Extracts EE2. Chin Clin Oncol (Chin) 2001; 6: 349-350.
12. He DW, Zhou F. Study on the anti-tumor effects of earthworm extract. J Yangtze Univ (Nat Sci Edit) (Chin) 2005; 18: 225-228.

13. He DW, Chen H, YE YY. Study on the anti-tumor effects of earthworm extracts both *in vivo* and *in vitro*. Chin J Biochem Pharma (Chin) 2005; 26: 353-355.
14. Li HY, Liu Y, Zhang FR, Huang WH, Li Y, Chen XG. Antitumor activity of earchworm fibrinolytic enzyme. Chin Pharma Bull 2004; 20: 908-910.
15. Nishimura G, Yanoma S, Mizuno H, Kawakami K, Tsukuda M. An antioxidant, probucol, induces anti-angiogenesis and apoptosis in athymic nude mouse xenografted human head and neck squamous carcinoma cells. Jpn J Cancer Res 1999; 90: 1224-1230.
16. Kern JC, Kehrer JP. Acrolein-induced cell death: a caspase-influenced decision between apoptosis and oncosis/necrosis. Chem Biol Interact 2002; 139: 79-95.
17. Zhang GS, Zhou GB, Dai CW. Upregulation and activation of caspase-3 or caspase-8 and elevation of intracellular free calcium mediated apoptosis of indomethacin-induced K562 cells. Chin Med J 2004; 117: 978-984.
18. Costa-Neto EM.. Animal-based medicines: biological prospection and the sustainable use of zootherapeutic resources. An Acad Bras Cienc. 2005; 77: 33-43.
19. Popovic M, Hrcenjak TM, Babic T, Kos J, Grdisa M. Effect of earthworm (G-90) extract on formation and lysis of clots originated from venous blood of dogs with cardiopathies and with malignant tumors. Pathol Oncol Res 2001; 7: 197-202.
20. Cooper EL, Hrzenjak TM, Grdisa M. Alternative sources of fibrinolytic, anticoagulative, antimicrobial and anticancer molecules. Int J Immunopathol Pharmacol 2004; 17: 237-244.
21. Engelmann P, Kiss J, Csongei V, Cooper EL, Nemeth P. Earthworm leukocytes kill HeLa, HEp-2, PC-12 and PA317 cells *in vitro*. J Biochem Biophys Methods 2004; 61(1-2): 215-227.
22. Mohrig W, Eue I, Kauschke E, Hennicke F. Crossreactivity of hemolytic and hemagglutinating proteins in the coelomic fluid of Lumbricidae (Annelida). Comp Biochem Physiol 1996; 116A: 19-30.
23. Pan W, Liu X, Ge F, Zheng T. Reconfirmation of antimicrobial activity in the coelomic fluid of the earthworm Eisenia fetida andrei by colorimetric assay. J Biosci 2003; 28: 723-731.
24. Kohlerova P, Beschin A, Silerova M, De Baetselier P, Bilej M. Effect of experimental microbial challenge on the expression of defense molecules in *Eisenia foetida* earthworm. Dev Comp Immunol 2004; 28(7-8): 701-711.
25. Cooper EL, Kauschke E, Cossarizza A. Digging for innate immunity since Darwin and Metchnikoff. Bioessays 2002; 24: 319-333.
26. Cooper EL, Ru B, Weng N. Earthworms: sources of antimicrobial and anticancer molecules. Adv Exp Med Biol 2004; 546: 359-389.
27. Egeblad M, Werb Z. New functions for the matrix metalloproteinases in cancer progression. Nat Rev Cancer 2002; 161-174.
28. Im JH, Fu W, Wang H, Bhatia SK, Hammer DA, Kowalska MA. Coagulation facilitates tumor cell spreading in the pulmonary vasculature during early metastatic colony formation. Cancer Res 2004; 64: 8613-8619.
29. Ren Y, Houghton P, Hider RC. Relevant activities of extracts and constituents of animals used in traditional Chinese medicine for central nervous system effects associated with Alzheimer's disease. J Pharm Pharmacol 2006; 58: 989-996.
30. Wang J, Chen C, Li B, Yu H, Zhao Y, Sun J, et al. Antioxidative function and biodistribution of [Gd@C82(OH)22] nanoparticles in tumor-bearing mice. Biochem Pharmacol 2006; 71:872-881.

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Short Communication

Low serum level of hepatitis B core-related antigen indicates unlikely reactivation of hepatitis after cessation of lamivudine therapy

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Aim: The clinical significance of hepatitis B virus (HBV) core-related antigen (HBcrAg) in predicting the reactivation of hepatitis after halting lamivudine administration was analyzed.

Methods: A total of 34 patients with chronic hepatitis B were enrolled. Lamivudine was administered for at least 6 months before cessation, and reactivation of hepatitis was defined as elevation of alanine aminotransferase levels to more than 80 IU/L within 12 months of cessation.

Results: In total, 20 (59%) patients experienced hepatitis reactivation. Although concentrations of HBV DNA and HBcrAg in serum did not differ between the two groups of patients at the onset of lamivudine administration, HBcrAg serum levels were significantly higher ($P = 0.009$) in the reactivation patients (median 4.9, 25–75% range 4.7–5.9 log unit/mL) than the non-reactivation patients (median 3.2, 25–75% range <3.0–4.5 log unit/mL) post-lamivudine treatment. The concentration of HBV DNA did not differ between the two groups (median <3.7, 25–75% range <3.7–<3.7 log copy/mL in the reactivation group vs. median <3.7, 25–75% range <3.7–<3.7 log copy/mL in the non-reactivation group). Receiver operating characteristic analysis of HBcrAg concentration showed an area under the curve of 0.764 in predicting patients without reactivation of hepatitis.

Conclusion: HBcrAg can be a useful marker to identify patients who are not at risk of reactivation of severe hepatitis after discontinuation of lamivudine administration.

Key words: chronic hepatitis B, hepatitis B virus core-related antigen, hepatitis B virus DNA, hepatitis reactivation, lamivudine

INTRODUCTION

LAMIVUDINE, A NUCLEOSIDE analog that inhibits reverse transcriptase, has been found to inhibit the replication of hepatitis B virus (HBV), reduce hepatitis, and improve histological findings of the liver in long-term treatment.[1,2] Furthermore, it has been shown that lamivudine treatment improves the long-term outcome of patients with chronic hepatitis B.[3,4] However, there are a number of problems with lamivudine therapy, including hepatitis relapse due to the appearance of YMDD mutant viruses and the reactivation of hepatitis after its discontinuation.[5,6]

During lamivudine administration, the concentration of serum HBV DNA decreases, and usually becomes undetectable to even high sensitivity HBV DNA assays. However, this undetectable level is an inadequate indicator for safely discontinuing lamivudine

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administration as active hepatitis often recurs in patients post-treatment.

Previously, a chemiluminescence enzyme immunoassay (CLEIA) was developed by our laboratory to detect of hepatitis B core-related antigen (HBcrAg).[7,8] This HBcrAg 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 because both proteins are transcribed from the precore/core gene and their first 149 amino acids are identical.[9–11] Although this assay reflects the viral load of HBV in a similar manner to HBV DNA assays during disease progression, HBcrAg CLEIA shows characteristics different from HBV DNA assays under lamivudine administration since HBcrAg levels decrease more slowly than HBV DNA after treatment begins.[12] In the present study, we analyzed the clinical significance of the HBcrAg assay in predicting the likelihood of non-reactivation of hepatitis after discontinuing lamivudine administration in HBV treatment.

METHODS

Patients

A TOTAL OF 34 patients with chronic hepatitis B who were treated with lamivudine for at least 6 months were enrolled in the present study. The patients comprised 20 men and 14 women with a median age of 46 years (range 23–65 years), and were selected retrospectively from five medical institutions in Japan (Shinshu University Hospital, Kyoto Prefectural University Hospital, National Nagasaki Medical Center, Toranomon Hospital, and Hiroshima University Hospital). Written informed consent was obtained from each patient.

Of the 27 patients whose HBV genotype was determined, 25 (93%) were genotype C and the remaining two (7%) were genotype B. Serum HBV DNA was detectable in all patients, and HBe antigen was positive in 16 (47%) of the 34 patients before lamivudine administration.

For treatment of HBV infection, daily doses of 100 mg lamivudine were administered for at least 6 months. Lamivudine administration was stopped when alanine aminotransferase (ALT) levels were reduced to 40 IU/L or less in at least three separate tests. Serum samples were taken at several time points during and after lamivudine administration, and patients were seen at least once a month for at least 12 months after cessation of lamivudine. Estimated duration of HBV DNA level <3.7 log copy/mL before stopping lamivudine was a median 10 months (range 0–29 months).

Reactivation of hepatitis was defined as elevation of ALT to more than 80 IU/L within 12 months of stopping lamivudine treatment.

Serological markers for HBV

Serum hepatitis B surface antigen, HBe antigen, and anti-HBe antibody were measured by commercially available CLEIA kits (Fujirebio, Tokyo, Japan). Six major genotypes (A–F) of HBV are detectable using the method reported by Mizokami *et al.*[13] in which the surface gene sequence is amplified by polymerase chain reaction (PCR) and analyzed by restriction fragment length polymorphism. Serum concentration of HBV DNA was determined using a transcription mediated amplification (TMA) assay kit (Chugai Diagnostics Science, Tokyo, Japan) which has a quantitative range of 3.7–8.7 log copy/mL.

Serum concentration of HBcrAg was measured using a CLEIA developed by Fujirebio, as described previously.[7] Briefly, 150 μL of serum was incubated with 150 μL of pretreatment solution containing 15% sodium dodecylsulfate at 60°C for 30 min. After incubation, 120 μL of pretreated specimen was added to a ferrite microparticle solution in an assay tube. Ferrite microparticles were coated with monoclonal antibodies (HB44, HB61, HB114) against denatured HBc and HBe antigens. After washing, two other monoclonal antibodies against denatured HBcAg and HBeAg (HB91 and HB110) labeled with alkaline phosphatase were added as secondary antibodies. After further washing, 200 μL of AMPPD (3-(2'-spiroadamantan)-4-methoxy-4-(3''-phosphoryloxy) phenyl-1, 2-dioxetane disodium salt; Applied Biosystems, Bedford, MA) solution was added as substrate, and the assay tube was incubated for 5 min at 37°C.

From this, the relative chemiluminescence intensity was measured, and HBcrAg 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 HBcrAg concentration was expressed as units/mL (U/mL) and a immunoreactivity of recombinant pro-HBe antigen of 10 fg/mL was defined as 1 U/mL. In the present study, the cutoff value of HBcrAg concentration was set at 3.0 log U/mL.

Statistical analysis

The Mann–Whitney *U*-test was used to analyze quantitative data, and Fisher's exact test was used for

qualitative data. Receiver operating characteristic (ROC) curve analysis was used to analyze cut-off levels of HBcrAg concentration for prospective recurrence of hepatitis. Statistical analyses were performed using the SPSS 14.0 J statistical software package (SPSS, Chicago, IL, USA), and a *P*-value of less than 0.05 was considered to be statistically significant.

RESULTS

TWENTY (59%) OF the 34 patients enrolled in the present study showed reactivation of hepatitis within 12 months after discontinuing lamivudine administration, with 15 (75%) showing reactivation within 6 months. The peak serum ALT levels in the 20 reactivation patients ranged from 103 to 1019 IU/L, with a median of 308 IU/L. After lamivudine cessation, the maximum serum HBV DNA was significantly higher ($P < 0.001$) in the reactivation patients (median 7.8, 25–75% range 7.4–8.1 log copy/mL) than in the non-reactivation patients (median 4.8, 25–75% range 4.1–5.9 log copy/mL).

Table 1 shows a comparison of the clinical backgrounds at the onset and completion of lamivudine administration between the two groups of patients. Although backgrounds were similar between the two groups just prior to lamivudine administration, HBcrAg levels were significantly higher in the reactivation patients after treatment. Both HBV DNA levels and positive rates of HBe antigen were similarly low between the two groups. The duration of undetectable HBV DNA before stopping lamivudine administration was also similar ($P > 0.2$) between the two groups (reactivation patients, median 11 months, 25–75% range 8–13 months vs. non-reactivation patients, median 6 months, 25–75% range 5–13 months).

In 23 patients who were negative for HBe antigen after treatment, HBcrAg levels were significantly higher ($P = 0.011$) in the reactivation patients ($n = 12$, median 4.8 log U/mL, 25–75% range 4.0–5.0 log U/mL) than in non-reactivation patients ($n = 11$, median 3.0 log U/mL, 25–75% range 2.5–4.4 log U/mL). In contrast, levels were similar ($P > 0.2$) between the two groups in 11 patients who were positive for HBe antigen after treatment (reactivation patients $n = 8$, median 5.9 log U/mL, 25–75% range 5.1–6.1 log U/mL vs. non-reactivation patients $n = 3$, median 5.6 log U/mL, 25–75% range 2.5–8.0 log U/mL).

The ability of HBcrAg concentration to predict non-recurrence of hepatitis was analyzed using a ROC curve (Fig. 1), and the area under the curve was as wide as 0.764. The point at which specificity was 0.8 and sensi-

Table 1 Comparison of clinical characteristics at the onset and cessation of lamivudine administration between patients with and without reactivation of hepatitis

Characteristics	Reactivation of hepatitis		*P*-value†
	Positive ($n = 20$)	Negative ($n = 14$)	
Demographics			
Age (years)	44 (38–51)	50 (35–59)	NS
Sex (male/female)	13/7	7/7	NS
HBV genotype (B/C)	0/16	2/9	NS
At onset of lamivudine administration			
ALT (IU/mL)	103 (57–234)	211 (76–515)	NS
HBeAg (positive)	12 (60%)	4 (29%)	NS
HBV DNA (log copy/mL)	7.1 (6.1–8.1)	6.0 (5.3–7.4)	NS
HBcrAg (log unit/mL)	6.2 (5.6–7.7)	6.4 (5.0–6.6)	NS
At cessation of lamivudine administration			
Duration of lamivudine (months)	12.7 (10.4–16.3)	10.3 (6.4–17)	NS
ALT (IU/mL)	30 (15–36)	21 (15–24)	NS
HBeAg (positive)	8 (40%)	3 (21%)	NS
HBV DNA (log copy/mL)	<3.7 (<3.7–<3.7)	<3.7 (<3.7–<3.7)	NS
HBcrAg (log unit/mL)	4.9 (4.7–5.9)	3.2 (<3.0–4.5)	0.009

†Analysis of continuous variables performed using Mann–Whitney *U*-test; analysis of dichotomous variables performed using Fisher's exact test. Values shown as median (25–75% range) or *n* (%).
ALT, alanine aminotransferase; HBcrAg, hepatitis B core-related antigen; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; NS, not significant.

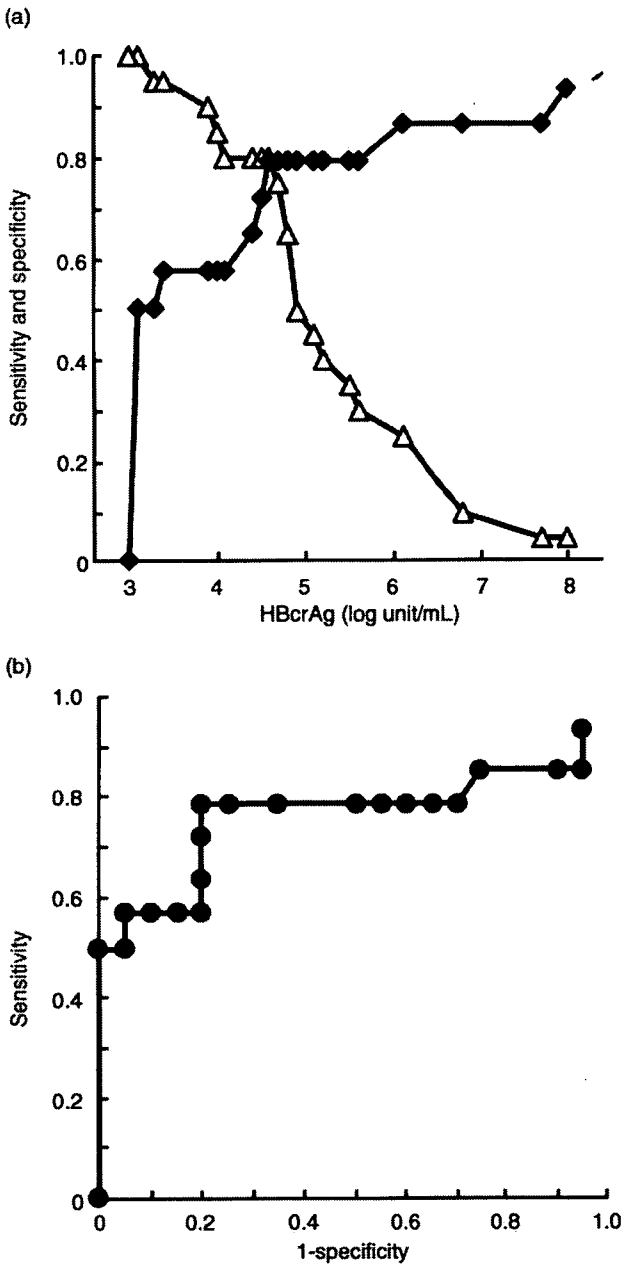

Figure 1 Receiver-operator characteristic (ROC) analysis of hepatitis B core-related antigen (HBcrAg) concentration for predicting patients without risk of reactivation of hepatitis within 12 months after halting lamivudine administration. (a) Sensitivity (■) and specificity (△) curves according to concentration of HBcrAg. (b) The ROC curve with the area under curve of 0.764.

tivity approximately 0.8 was deemed best for halting treatment without the risk of hepatitis recurrence. This point corresponds to an HBcrAg concentration of 4.1–4.6 log unit/mL.

DISCUSSION

THE REACTIVATION OF hepatitis following lamivudine administration was defined in the present study as an elevation of serum ALT level to more than 80 IU/L because we sought to find a more reliable indicator for safer discontinuation of lamivudine administration. Under these conditions, the majority (20/34) of patients showed reactivation of hepatitis within 12 months, as has been previously reported.[5,6] HBV DNA levels at the time of discontinuing lamivudine were similarly low between the two groups of patients, which is understandable as an undetectable reading typically indicates HBV remission following lamivudine therapy. However, HBcrAg levels were significantly higher in reactivation patients, implying that HBcrAg level is a better marker than HBV DNA level for predicting non-reactivation of hepatitis after discontinuing lamivudine administration especially in patients without HBe antigen.

In this study, ROC curve analyses showed a wide area under the curve of 0.764 in predicting the non-reactivation of HBV with HBcrAg level. If the corresponding cutoff is set at 4.5 logU/mL, then both specificity and sensitivity are as high as approximately 0.8. To obtain a higher specificity of 0.9, the cutoff value of HBcrAg concentration should be set at 4.0 log unit/mL. In this case, the sensitivity would still be nearly 0.6. The cutoff value of HBcrAg for predicting the non-relapse of hepatitis in our study is a little higher than that reported by Shinkai *et al.* (3.4 logU/mL).[14] Because numbers of patients analyzed were small in both studies, further studies are required to confirm the most appropriate cutoff value. It is noteworthy that this cutoff value may also differ among genotypes, which have been reported to be correlated with outcome of chronic HBV infection.[15] However, as over 90% of the patients had genotype C in this study, reactivation could not be analyzed in relation to HBV genotypes.

The 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 and serves as transcriptional template for the production of viral RNA.[11,16,17] Reverse transcription of pregenomic RNA and second-strand DNA synthesis then occur in the cytoplasm within viral

capsids formed by the HBV core protein. Because lamivudine inhibits reverse transcription of pregenomic RNA, it directly suppresses production of HBV virions, and serum HBV DNA levels decrease rapidly after the initiation of lamivudine administration. However, the production of viral proteins is not suppressed by lamivudine as this process does not include reverse transcription. Furthermore, it has been reported that the amount of cccDNA, which also serves as a template for mRNAs, decreases quite slowly after commencement of administration of nucleoside analogs.[18,19] Thus, it is possible that serum HBcrAg levels reflect the cccDNA level in hepatocytes more accurately than serum HBV DNA. High levels of cccDNA are considered to be associated with hepatitis reactivation because they preceed reactivation of viral replication and consequent elevation of HBV DNA level in serum.

Lamivudine has already been eliminated from first line therapy in naïve chronic hepatitis B patients due to a higher incidence of developing resistant mutations than new antiviral agents, such as adefovir dipivoxil and entecavir.[20] However, the distinct characteristic of the HBcrAg assay under lamivudine therapy that is different from other HBV DNA assays is that lamivudine suppresses production of HBV virions by inhibiting reverse transcription of pregenomic RNA, but does not suppress the production of viral proteins, in which reverse transcription is unnecessary. Thus, it is possible that the HBcrAg assay may also useful for patients undergoing entecavir or adefovir dipivoxil administration because the main mechanism of suppressing HBV replication is similar between lamivudine and other antiviral agents. As a considerable number of patients who started lamivudine administration in the past are still taking this treatment now, the present study may be valuable for such patients when they consider changing therapies in the future. Additionally, further studies are required to determine whether the HBcrAg assay is indeed applicable to antiviral agents other than lamivudine.

In conclusion, significant markers that can predict reactivation of hepatitis after discontinuing lamivudine administration are clinically valuable because the reactivation of hepatitis is a fundamental problem in lamivudine therapy. Our results suggest that patients with an HBcrAg level of less than 4.5 log unit/mL may stop lamivudine administration with a lower risk of reactivation. The present study is a preliminary one because the patients enrolled were selected retrospectively without standardized criteria for stopping lamivudine and the number of patients enrolled was not large; however, the results may be valuable for patients with hepatitis B undergoing lamivudine therapy as such a diagnostic marker has rarely been reported. Further studies are required to establish the clinical significance of the HBcrAg assay in the treatment of hepatitis B.

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REFERENCES

1 Dienstag JL, Goldin RD, Heathcote EJ *et al.* Histological outcome during long-term lamivudine therapy. *Gastroenterology* 2003; 124: 105–17.
2 Lai CL, Chien RN, Leung NW *et al.* A one-year trial of lamivudine for chronic hepatitis B. Asia Hepatitis Lamivudine Study Group. *N Engl J Med* 1998; 339: 61–8.
3 Liaw YF, Sung JJ, Chow WC *et al.* Lamivudine for patients with chronic hepatitis B and advanced liver disease. *N Engl J Med* 2004; 351: 1521–31.
4 Matsumoto A, Tanaka E, Rokuhara A *et al.* Efficacy of lamivudine for preventing hepatocellular carcinoma in chronic hepatitis B. A multicenter retrospective study of 2795 patients. *Hepatol Res* 2005; 32: 173–84.
5 Liaw YF, Chien RN, Yeh CT, Tsai SL, Chu CM. Acute exacerbation and hepatitis B virus clearance after emergence of YMDD motif mutation during lamivudine therapy. *Hepatology* 1999; 30: 567–72.
6 Lok AS, Lai CL, Leung N *et al.* Long-term safety of lamivudine treatment in patients with chronic hepatitis B. *Gastroenterology* 2003; 125: 1714–22.
7 Kimura T, Rokuhara A, Sakamoto Y *et al.* Sensitive enzyme immunoassay for hepatitis B virus core-related antigens and their correlation to virus load. *J Clin Microbiol* 2002; 40: 439–45.
8 Rokuhara A, Tanaka E, Matsumoto A *et al.* Clinical evaluation of a new enzyme immunoassay for hepatitis B virus core-related antigen; a marker distinct from viral DNA for monitoring lamivudine treatment. *J Viral Hepat* 2003; 10: 324–30.
9 Bruss V, Gerlich WH. Formation of transmembraneous hepatitis B e-antigen by cotranslational *in vitro* processing of the viral precore protein. *Virology* 1988; 163: 268–75.
10 Garcia PD, Ou JH, Rutter WJ, Walter P. Targeting of the hepatitis B virus precore protein to the endoplasmic reticulum membrane: after signal peptide cleavage translocation can be aborted and the product released into the cytoplasm. *J Cell Biol* 1988; 106: 1093–104.
11 Lee WM. Hepatitis B virus infection. *N Engl J Med* 1997; 337: 1733–45.
12 Tanaka E, Matsumoto A, Suzuki F *et al.* Measurement of hepatitis B virus core-related antigen is valuable for iden-

tifying patients who are at low risk of lamivudine resistance. *Liver Int* 2006; 26: 90–6.
13 Mizokami M, Nakano T, Orito E *et al.* Hepatitis B virus genotype assignment using restriction fragment length polymorphism patterns. *FEBS Lett* 1999; 450: 66–71.
14 Shinkai N, Tanaka Y, Orito E *et al.* Measurement of hepatitis B virus core-related antigen as predicting factor for relapse after cessation of lamivudine therapy for chronic hepatitis B virus infection. *Hepatol Res* 2006; 36: 272–6.
15 Fung SK, Lok AS. Hepatitis B virus genotypes: do they play a role in the outcome of HBV infection? *Hepatology* 2004; 40: 790–2.
16 Mason WS, Halpern MS, England JM *et al.* Experimental transmission of duck hepatitis B virus. *Virology* 1983; 131: 375–84.
17 Tuttleman JS, Pourcel C, Summers J. Formation of the pool of covalently closed circular viral DNA in hepadnavirus-infected cells. *Cell* 1986; 47: 451–60.
18 Moraleda G, Saputelli J, Aldrich CE, Averett D, Condreay L, Mason WS. Lack of effect of antiviral therapy in nondividing hepatocyte cultures on the closed circular DNA of woodchuck hepatitis virus. *J Virol* 1997; 71: 9392–9.
19 Werle-Lapostolle B, Bowden S, Locarnini S *et al.* Persistence of cccDNA during the natural history of chronic hepatitis B and decline during adefovir dipivoxil therapy. *Gastroenterology* 2004; 126: 1750–8.
20 Lok ASF, McMahon BJ. Chronic hepatitis B. *Hepatology* 2007; 45: 507–39.

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Infection of human hepatocyte chimeric mouse with genetically engineered hepatitis C virus and its susceptibility to interferon

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Abstract **We developed a reverse genetics system of hepatitis C virus (HCV) genotypes 1a and 2a using infectious clones and human hepatocyte chimeric mice. We inoculated cell culture-produced genotype 2a (JFH-1) HCV intravenously. We also injected genotype 1a CV-H77C clone RNA intrahepatically. Mice inoculated with HCV by both procedures developed measurable and transmissible viremia. Interferon (IFN) alpha treatment resulted in greater reduction of genotype 2a HCV levels than genotype 1a, as seen in clinical practice. Genetically engineered HCV infection system should be useful for analysis of the mechanisms of resistance of HCV to IFN and other drugs.**

Keywords: Human hepatocyte chimeric mouse; Human serum albumin; HCV RNA; Interferon

1. Introduction

The hepatitis C virus (HCV) infects an estimated 170 million people worldwide [1]. HCV causes persistent infection in adults leading to chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma [2,3]. The most effective therapy for viral clearance is a 48-week combination therapy of pegylated interferon (IFN)-alpha and ribavirin. However, the success rate of this combination therapy is only about 50% [4]. Development of new anti-HCV drug had been severely restricted by the absence of a cell culture system that supports the efficient replication of HCV, as well as the lack of a small animal model. A cell culture system has been developed recently using a unique genotype 2a HCV gnome (JFH-1), which does not require adaptive mutations for efficient replication [5–7]. Chimpanzee was the only useful animal for the study of HCV until recently, although the availability of this model is severely restricted [8]. Recently, HCV-infected mice have been developed by inoculating HCV-infected human serum into chimeric urokinase-type plasminogen activator (uPA)-severe combined immunodeficiency (SCID) mice with engrafted human hepatocytes [9]. This HCV-infected mouse model has been reported to be useful for evaluating anti-HCV drugs such as IFN-alpha and anti-NS3 protease [10]. We have generated a human hepatocyte chimeric mouse where mouse hepatocytes were extensively replaced by human hepatocytes [11], and established a genetically engineered hepatitis B virus (HBV) system [12]. Using this mouse, we show in this paper the development of reverse genetics system of genotypes 1a and 2a after intrahepatic injection of transcribed RNA and intravenous injection of cell culture-produced virus, respectively. We also show here that HCV in these mice can be transmitted to naïve mice. Interferon treatment of these mice resulted in a greater reduction of HCV titer in genotype 2a clone infected mice than in genotype 1a infected mice. As these results are consistent with our clinical experience, we consider this model suitable for the study of resistance of HCV against IFN and other drugs.

2. Materials and methods

2.1. Generation of human hepatocyte chimeric mice and quantification of human serum albumin

Generation of the uPA$^{+/+}$/SCID$^{+/+}$ mice and transplantation of human hepatocytes were performed as described recently by our group [11,12]. All mice used in this study were transplanted with frozen

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Abbreviations: HBV, hepatitis B virus; HCV, hepatitis C virus; HSA, human serum albumin; IFN, interferon; SCID, severe combined immunodeficiency; uPA, urokinase-type plasminogen activator

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human hepatocytes obtained from one donor. Infection, extraction of serum samples, and sacrifice were performed under ether anesthesia. Mouse serum concentrations of human serum albumin (HSA) correlate with the repopulation index [11], and were measured as described previously [12]. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Graduate School of Biomedical Sciences, Hiroshima University.

2.2. HCV RNA transcription and inoculation into chimeric mice

A plasmid containing the full-length genotype 1a HCV cDNA clone, pCV-H77C, was kindly provided by Dr. Robert H. Purcell (National Institutes of Health). Ten micrograms of plasmid DNA, linearized by *Xba*I (Promega, Madison, WI) digestion, was transcribed in a 100-μl reaction volume with T7 RNA polymerase (Promega) at 37 °C for 2 h [13], and analyzed by agarose gel electrophoresis. Each transcription mixture was diluted with 400 μl of phosphate-buffered saline (PBS) and injected into the liver of chimeric mice. Transcripts of plasmid pJFH-1 containing the full-length HCV genotype 2a were transfected into Huh7 cells as described previously [6]. Seventy-two hours after transfection, 200 μl of the culture medium was injected intravenously into the chimeric mice. IFN-treatment was also performed by intramuscular injection of diluted IFN solutions. IFN-alpha was a kind gift from Hayashibara Biochemical Labs, Inc. (Okayama, Japan). Serum samples collected every 2 weeks after inoculation were frozen at −80 °C until further analysis.

2.3. Human serum samples

For control infection experiments, human serum containing a high titer of genotype 1b HCV (2.2×10^6 copies/ml) was obtained from a patient with chronic hepatitis after obtaining a written informed consent. The individual serum samples were divided into small aliquots and separately stored in liquid nitrogen until use.

2.4. RNA extraction and amplification

RNA was extracted from serum samples by Sepa Gene RV-R (Sankojunyaku, Tokyo), dissolved in 8.8 μl RNase-free H_2O, and reverse transcribed by using a random primer (Takara Bio, Inc., Shiga, Japan) and M-MLV reverse transcriptase (ReverTra Ace, TOYOBO Co., Osaka, Japan) in a 20 μl reaction mixture according to the instructions provided by the manufacturer. One microliter of cDNA solution was amplified by Light Cycler (Roche Diagnostic, Japan, Tokyo) for quantitation of HCV. The primers used for amplification were 5′-TTTATCCAAGAAAGGACCC-3′ and 5′-TTCACGCAGAAAGCGTCTAGC-3′. The amplification conditions included initial denaturation at 95 °C for 10 min, followed by 45 cycles of denaturation at 95 °C for 15 s, annealing at 55 °C for 5 s, and extension at 72 °C for 6 s. The lower detection limit of this assay is 10^3 copies/ml. Nested PCR was used with the outer primers NC1 (5′-CAACACTACTCGGCTAGCAGT-3′) and NC2 (5′-CCTGTGAGGAACTACTGTC-3′) and inner primers cc6 (5′-TTTATCCAAGAAAGGACCC-3′) and cc7 (5′-TTCACGCAGAAAGCGTCTAGCttc-3′). The amplification condition included 35 cycles of 94 °C for 30 s, 58 °C for 1 min 30 s, and 72 °C for 1 min after 5 min of initial denaturation at 94 °C followed by 7 min of final extension using Gene Taq (Wako Pure Chemicals, Tokyo) with anti-Taq high according to the instructions provided by the manufacturer (TOYOBO).

2.5. Histochemical analysis of mouse liver

Histopathological analysis and immunohistochemical staining using an antibody against HSA (Bethyl Laboratories Inc.) were performed as described previously [12].

3. Results

3.1. High serum HCV RNA titer in human hepatocyte chimeric mice after inoculation of serum samples obtained from HCV-infected patient

We inoculated 50 μl of genotype 1b serum samples into five chimeric mice intravenously to test their susceptibility to HCV infection. All mice became positive for HCV RNA by nested PCR at 2 weeks after inoculation (Fig. 1). The viremia reached a plateau level at 6–8 weeks after infection, and persisted for more than 12 weeks.

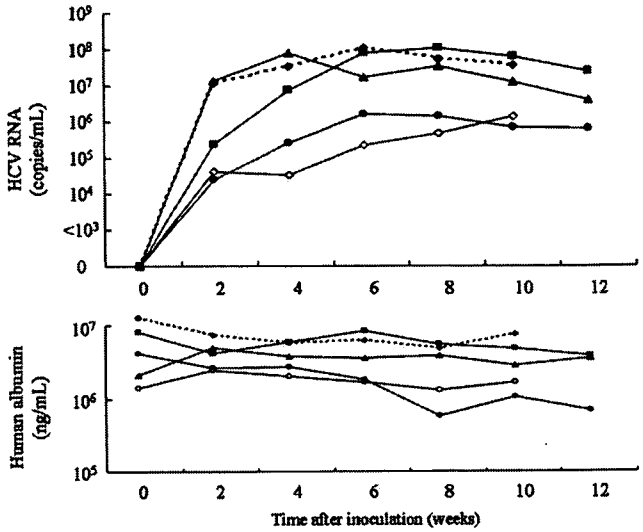

Fig. 1. Serial changes in HCV RNA and human serum albumin in sera of mice inoculated with human serum samples positive for genotype 1b HCV. Fifty microliter serum samples were injected intravenously into each mouse. Mice serum samples were obtained every 2 weeks after injection, and HCV RNA titer was analyzed.

3.2. Infection with in vitro-transcribed genotype 1a HCV RNA and cell culture generated genotype 2a HCV

In the next step, we tried to establish infection of cloned HCV using infectious genotype 1a and genotype 2a clones. In these experiments, we used two different strategies to establish infection using these two clones because genotype 1a has not been confirmed to replicate in cell culture system. We used genotype 1a HCV RNA (CV-H77C), which has been reported to be infectious to chimpanzee [13]. In vitro-transcribed HCV RNA was directly injected intrahepatically in three chimeric mice. We also infected three chimeric mice by intravenous injection of Huh7 cell-produced genotype 2a HCV after transfection of in vitro transcribed RNA from an infectious clone JFH-1. This clone has been shown to be infectious to a chimpanzee [6] and a chimeric mouse [7]. All mice developed measurable viremia 2 weeks after inoculation. At 6 weeks after inoculation, HCV RNA titer was 2.4×10^7 copies/ml (range: 8.8×10^6–2.9×10^7 copies/ml) in genotype 1a HCV-infected mice, and 2.5×10^5 copies/ml (range: 1.4×10^5–3.7×10^5 copies/ml) in genotype 2a HCV-infected mice (Fig. 2).

3.3. Passage experiment of HCV to naïve chimeric mice

We then performed passage experiments using naïve mice. Each of three mice was inoculated intravenously with 10 μl serum samples obtained from the above genotype 1a and genotype 2a HCV-infected mice at week 6. Two weeks after injection, all mice developed measurable viremia, and the titer was 8.5×10^6 copies/ml (range: 1.4×10^6–2.4×10^7 copies/ml) in genotype 1a, and 1.7×10^5 copies/ml (range: 1.5×10^5–2.5×10^5 copies/ml) in genotype 2a HCV-infected mice (Fig. 3).